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N,N′-Disubstituted thiourea and urea derivatives: design, synthesis, docking studies and biological evaluation against nitric oxide synthase†

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The synthesis and biological evaluation of new types of *N,N′*-disubstituted thiourea and urea derivatives as inhibitors of both neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) are described. These compounds have been designed by reduction of the carbonyl group in the thiourea and urea kynurenamine derivatives **3** previously synthesized by our research group. The synthetic route performed to this new family also allows us to obtain the molecules **3** with less synthetic steps and higher global yield. Regarding the biological results, in general, the new derivatives **4a–q** inhibit the neuronal NOS isoform better than the inducible one. Furthermore, thioureas exhibit higher inhibition than ureas for both isoenzymes. Among all the tested compounds, **4g** shows significant nNOS (80.6%) and iNOS (76.6%) inhibition values without inhibiting eNOS. This molecule could be an interesting starting point for the design of new inhibitors with application in neurological disorders where both isoenzymes are implicated such as Parkinson's disease.

Introduction

Nitric oxide synthase (NOS) is a family of isoenzymes that convert L-arginine to L-citrulline with nitric oxide (NO) release. There are three human NOS isoforms: endothelial NOS (eNOS) which regulates blood pressure and flow, inducible NOS (iNOS) which is involved in immune response, and neuronal NOS (nNOS) which is essential for neurotransmission.[1] Nonetheless, several studies demonstrated the implication of NOS in many neurodegenerative,[2–7] chronic inflammatory[8,9] and cardiovascular[10,11] diseases. Indeed, NO overproduction, by nNOS or iNOS, leads to cellular and tissue damage through nitrosative and oxidative stress.[12–16] The underproduction of NO by eNOS affects the vascular tone and blood flow controls and causes hypertension. Therefore, the inhibition of nNOS and iNOS but not eNOS is a viable therapeutic strategy to treat and prevent disorders and pathologies as previously mentioned.

A comparison of the NOS structures reveals a huge similarity especially in their substrate binding sites.[17] This fact represents a real challenge in designing selective NOS inhibitors.

In previous efforts to find selective and potent i/nNOS inhibitors, our research group has synthesized and published several families of compounds. Fig. 1 shows some of them with analogous structures: the kynurenine **1**,[18] kynurenamine **2**,[19] and urea and thiourea kynurenamine **3** (ref. 20) derivatives. These last ones have mostly better inhibition results *versus* iNOS. Docking and molecular dynamic studies demonstrated the urea group implication especially in the selectivity process. In addition, both the aromatic ring and the amine group interact with the enzyme through π–cation interaction

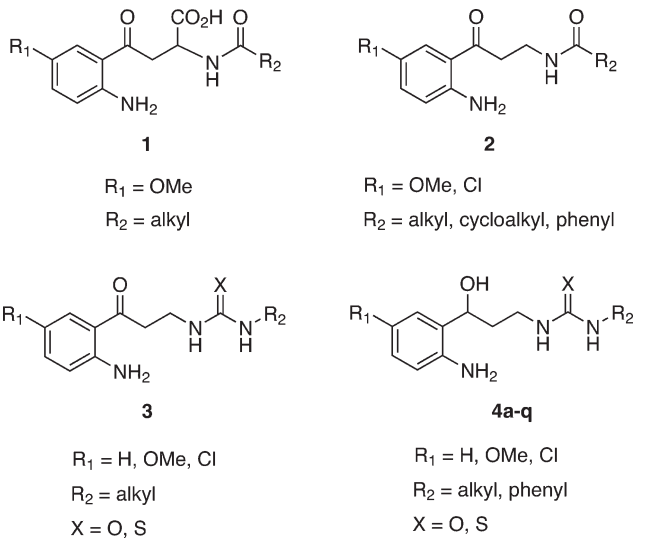

Fig. 1 Some kynurenamine derivatives as NOS inhibitors synthesized by our research group.

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and hydrogen bonds, respectively. However, the carbonyl group does not show a clear role. Therefore, we decided to act at this point, changing this hydrogen bond acceptor residue to another donor such as hydroxyl.

This way, herein we present a new series of compounds **4a–q** where the carbonyl group, present in the three families of the above derivatives, has been replaced by a hydroxyl one, in order to increase the interaction with the enzyme and improve the inhibition. A new synthetic pathway was developed to obtain this series of molecules. Subsequent biological assays and docking studies were performed in this new family of compounds to evaluate the results against NOSs' isoforms.

In addition, compounds **2a–l** previously published and evaluated as nNOS inhibitors[19] were screened against iNOS, and the inhibition data are presented and discussed in this work. *N*,*N*′-Disubstituted thioureas and ureas **4a–q** were designed from the kynurenamines with general skeleton **2**, introducing two structural modifications: replacement of the terminal acyl group by a thiourea or urea one, isosteric to the final guanidine moiety of the NOS substrate (L-Arg) and substitution of the carbonyl group with a hydroxyl residue.

Results and discussion

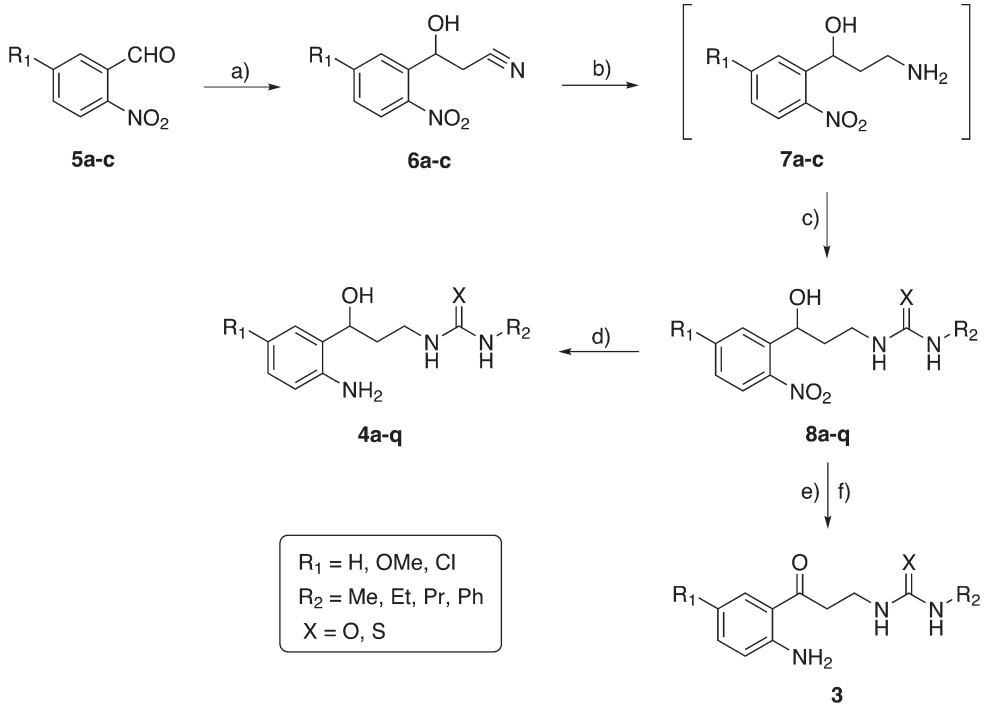
Chemistry

The general synthetic pathway of all the final compounds **4a–q** is represented in Scheme 1. 5-Methoxy-2-nitrobenzaldehyde **5b** (synthesized from 5-hydroxy-2-nitrobenzaldehyde by reaction with MeI in the presence of K_2CO_3/THF),[21] the commercially available 2-nitrobenzaldehyde **5a** and 5-chloro-2-nitrobenzaldehyde **5c** were transformed into the β-hydroxynitriles **6a–c** by treatment with BuLi and acetonitrile in dry THF[22] (70–90% yield). The nitrile group of these last intermediates was reduced selectively with borane in THF[23] to give the 3-amino-1-(2-nitrophenyl)propan-1-ol derivatives **7a–c**. Nucleophilic addition of either alkyl or phenyl isothiocyanate or isocyanate *in situ*, using the microwave (MW) technique, gave the intermediates **8a–q** in good yields (70–85%). The application of the MW technique shortened the reaction time from 18 h (ref. 20 and 24) to 20 min, with respect to the previously described synthesis. Finally, reduction of the phenyl nitro group, with Pd/C in the flow hydrogenator, resulted in the final derivatives **4a–q** (75–91% yield).

On the other hand, this new synthetic route is useful to obtain urea and thiourea kynurenamines **3**. Thereby, as an example, we carried out the oxidation of the hydroxyl group to a carbonyl one in the derivative **8o** (R_1 = Cl, R_2 = Et, X = O), using the Jones reagent followed by the nitro reduction to an amino group[20] (Scheme 1). This alternative synthesis improves the previously described one[20] by shortening the synthetic route (from 8 to 5 steps) and doubling the global yield.

iNOS and nNOS inhibition

Compounds **4a–q** were evaluated *in vitro* as inhibitors of iNOS and nNOS using recombinant isoenzymes. The assays



Scheme 1 Synthesis of *N*,*N*′-disubstituted thiourea and urea derivatives **4a–q**. a) CH_3CN, BuLi, THF, –78 °C, then RT; b) BH_3-THF, 0 °C, then 4 h RT; c) $XCNR_2$, CH_2Cl_2, 20 min (MW); d) 10% Pd/C, MeOH (flow hydrogenation), 60 °C, 60 bar; e) CrO_3, CH_3COCH_3, H_2SO_4, 10 min; f) Fe/$FeSO_4$, H_2O, 95 °C, 3 h.

were made at 1 mM concentration of each compound in order to identify the more active and selective derivatives. Besides, IC_{50} values were measured for the most interesting compounds.

Table 1 illustrates the inhibition percentages *versus* iNOS and nNOS (the kynurenamine derivatives **3a–b**, previously described,[20] were introduced as references).

In general, compounds **4a–q** show better values of inhibition *versus* nNOS than iNOS, since five compounds exhibit good nNOS percentage inhibition (**4g**, 80.6%; **4m**, 70.6%; **4l**, 70.5%; **4a**, 70.2% and **4c**, 51.7%) and only three compounds show good inhibitory activity *versus* iNOS (**4g**, 76.6%; **4m**, 60.4% and **4n**, 55.0%). Regarding the R_1 radical, no clear structure–activity relationship can be concluded. Independently of the substituent nature ((R_1 = H), electron-donating (R_1 = OCH_3) or electron withdrawing (R_1 = Cl)), different inhibition levels can be observed. It seems that the inhibitory activity depends more on X and R_2. Thus, derivatives with a thiourea residue (X = S) show better inhibition data for both isoenzymes than those bearing urea (X = O). In addition, compounds with a methyl substituent as R_2 produce higher inhibition for iNOS such as **4g** and for nNOS such as **4a**, **4g** and **4l**. It is noteworthy that compound **4m** shows good inhibition having R_2 = Et. Finally, **4g** stands out as the best inhibitor of both isoforms.

Furthermore, Table 2 shows the iNOS inhibition values of kynurenamines **2a–l** previously synthesized (not published before), as well as their nNOS values already described[19] in order to compare them with the new *N*,*N*′-disubstituted thioureas and ureas **4a–q**.

The best nNOS inhibitors of derivatives **2a–l** were **2a** (R_1 = OMe, R_2 = Me, 65.4%) and **2b** (R_1 = OMe and R_2 = Et, 50.9%); however, no compound with relevant iNOS inhibition value was found, so they can be considered as selective inhibitors of nNOS *versus* iNOS. Kynurenamine **2a**, the N^1-methyl-5-methoxykynurenamine, is the main brain metabolite of melatonin hormone in mammalians, that mediates some of the actions of this indolamine. Both of them inhibited nNOS activity *in vitro* in a dose-related manner.[25]

If we compare the derivatives **4a–q** with the kynurenamines **2a–l**, we can see that both series inhibit better nNOS than iNOS (compounds **4a–q** having greater potency), unlike the corresponding molecules **3** showing better iNOS inhibition values. Besides, the best inhibitors of the first two families carry a methoxy group as R_1 and a methyl group as R_2 (urea **4g** and kynurenamine **2a**, respectively), while the urea-kynurenamine **3b** which has a chlorine group as R_1 and an ethyl group as R_2 is the best inhibitor of this family and the most selective one. Finally, **4g** has the highest percentage of inhibition of all the tested derivatives with similar structures.

Table 3 includes the IC_{50} data of the most interesting derivatives **4a–q**. Compound **4g** stands out as one with the best values of inhibition, 130 and 180 μM, *versus* nNOS and iNOS, respectively, confirming the potency of this molecule.

eNOS inhibition

We investigated the eNOS inhibitory activity of compound **4g**, using HUVECs incubated with 100 μmol L^{-1} of **4g** or DMSO and measuring the NO production stimulated by the known eNOS activator A23187. This agent increased, in a time dependent manner, NO production. No significant differences ($p > 0.05$) were observed in A23187-stimulated NO production in the presence of **4g**, showing that this compound did not inhibit eNOS (Fig. 2a). Moreover, the endothelium-dependent

Table 1 *In vitro* iNOS and nNOS inhibition (%) observed in the presence of 1 mM concentration of compounds **3a–b** and **4a–q**

Compound	R_1	R_2	X	% iNOS inhibition[a]	% nNOS inhibition[a]
3a[b]	OMe	Me	S	78.20 ± 2.43	46.04 ± 2.85
3b[b]	Cl	Et	O	78.63 ± 1.34	9.86 ± 3.17
4a	H	Me	S	20.74 ± 1.15	70.17 ± 0.77
4b	H	Et	S	19.36 ± 3.33	44.84 ± 0.02
4c	H	Pr	S	31.10 ± 0.55	51.74 ± 1.35
4d	H	Et	O	18.34 ± 0.43	7.81 ± 3.06
4e	H	Pr	O	19.04 ± 1.45	7.00 ± 0.32
4f	H	Ph	O	27.86 ± 1.15	17.69 ± 1.32
4g	OMe	Me	S	76.55 ± 0.33	80.55 ± 2.29
4h	OMe	Pr	S	25.39 ± 1.71	6.02 ± 1.61
4i	OMe	Et	O	26.64 ± 2.05	43.02 ± 0.48
4j	OMe	Pr	O	14.66 ± 0.19	15.81 ± 1.03
4k	OMe	Ph	O	12.33 ± 1.30	12.10 ± 1.08
4l	Cl	Me	S	22.13 ± 0.28	70.53 ± 4.60
4m	Cl	Et	S	60.42 ± 2.31	70.61 ± 3.54
4n	Cl	Pr	S	54.98 ± 3.21	32.40 ± 2.24
4o	Cl	Et	O	33.85 ± 1.86	15.22 ± 2.33
4p	Cl	Pr	O	19.61 ± 3.04	23.81 ± 1.34
4q	Cl	Ph	O	3.11 ± 2.36	38.62 ± 1.63

[a] Values are the mean ± SEM of the percentage of iNOS and nNOS inhibition produced by 1 mM concentration of each compound. Each value is the mean of three experiments performed in triplicate using recombinant iNOS and nNOS enzymes. [b] **3a–b** were used as references.[20]

Table 2 *In vitro* iNOS and nNOS inhibition (%) observed in the presence of 1 mM concentration of compounds **2a–l**

Compound	R_1	R_2	% iNOS inhibition[a]	% nNOS inhibition[b]
2a	OMe	Me	30.74 ± 2.92	65.36 ± 5.6
2b	OMe	Et	0.24 ± 1.95	50.87 ± 1.9
2c	OMe	Pr	7.35 ± 4.48	42.81 ± 1.9
2d	OMe	Bu	14.11 ± 0.89	39.68 ± 1.17
2e	OMe	*c*-C_3H_5	2.66 ± 1.4	40.44 ± 1.84
2f	OMe	*c*-C_4H_7	0.01 ± 1.52	33.75 ± 1.39
2g	OMe	*c*-C_5H_9	0.61 ± 2.49	45.04 ± 1.97
2h	OMe	*c*-C_6H_{11}	9.24 ± 2.1	48.24 ± 2.42
2i	OMe	Ph	10.86 ± 3.73	46.47 ± 2.36
2j	Cl	Me	19.70 ± 0.57	17.70 ± 1.13
2k	Cl	*c*-C_3H_5	5.37 ± 2.59	9.66 ± 4.17
2l	Cl	Ph	26.60 ± 4.52	7.20 ± 1.29

[a] Values are the mean ± SEM of the percentage of iNOS and nNOS inhibition produced by 1 mM concentration of each compound. Each value is the mean of three experiments performed in triplicate using recombinant iNOS and nNOS enzymes. [b] See ref. 19.

Table 3 IC_{50} values (mM) for the inhibition of nNOS and iNOS activity by compounds **4a**, **4c**, **4g**, **4l**, **4m** and **4n**

IC_{50}[a]	4a	4c	4g	4l	4m	4n
nNOS	0.35	0.88	0.13	0.71	0.77	>1
iNOS	>1	>1	0.18	>1	0.80	0.86

[a] The data were obtained by measuring the percentage of inhibition with at least five concentrations of the inhibitor.

relaxation to acetylcholine was not affected by **4g** (Fig. 2b), confirming the absence of eNOS inhibition of this compound. However, L-NAME suppressed this NO-dependent relaxant response.

Cell viability

We investigated the cell viability activity of compound **4g**, using HUVECs. We found that this compound has little toxicity, since only at concentrations >1 mM is cell viability weakly (approximately 20%) reduced (Fig. 3).

Docking studies

Docking studies were performed to propose and understand the binding mode of *N*,*N*′-disubstituted thiourea and urea derivatives **4a–q** inside nNOS and iNOS.

Fig. 4 illustrates the main poses obtained for these compounds in the nNOS (PDB id: 1QW6)[26] binding site. All the ligands interact with Glu592 and a carboxylate moiety of the heme group through the hydroxyl group and both thiourea/urea nitrogens, respectively. Compound **4a** (Fig. 4a) shows additional interactions of the amino group, forming two more hydrogen bonds, one with Trp587 and the other with Glu592. Moreover **4a** has a good orientation in the binding site: the phenyl ring points to the lipophilic region (Phe584 and Pro565) being situated below the heme group establishing a π–cation interaction, the aliphatic chain is directed to Val575 establishing VdW interactions and the thiourea moiety is oriented to a polar pocket formed by three arginines (Arg596, Arg603 and Arg481). In addition, more VdW interactions are established with Gln478.

On the other hand, compound **4j** (Fig. 4b) shows no additional hydrogen bonds in the binding site. It presents a

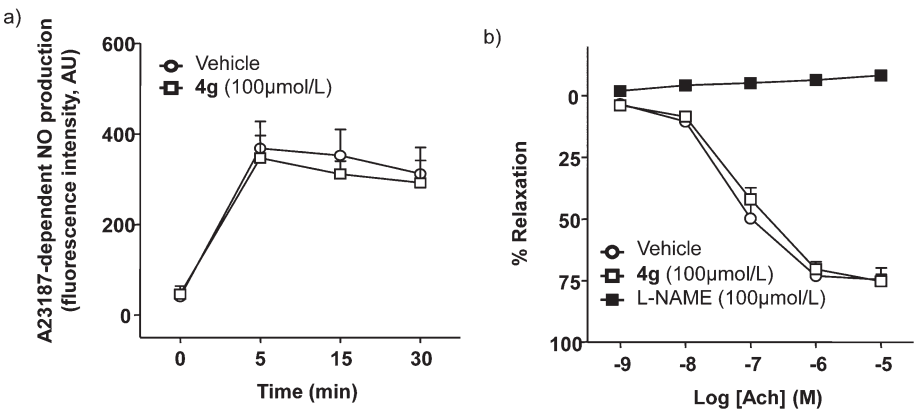


Fig. 2 Effects of **4g** on eNOS activity. a) A23187-dependent NO production in HUVECs incubated with DMSO or **4g** (100 μmol L^{-1}) for 30 min (n = 12). b) Acetylcholine-evoked relaxation in aortic rings with endothelium contracted with 1 μM noradrenaline in the presence of DMSO, **4g** (100 μmol L^{-1}), or L-NAME (100 μmol L^{-1}) for 30 min (n = 5). The data are expressed as the mean ± SEM of n experiments.

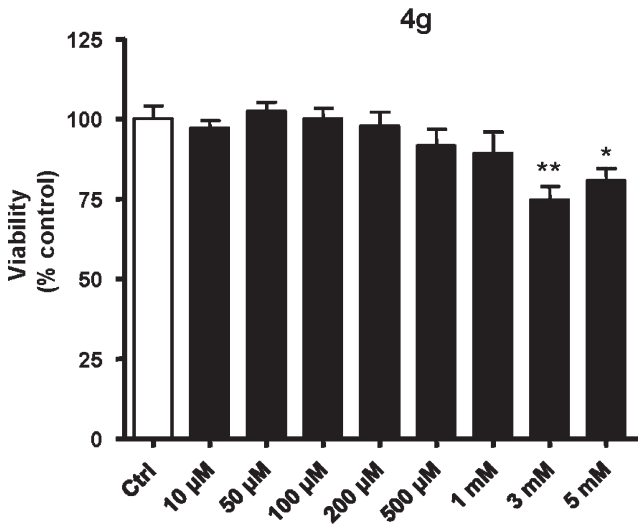


Fig. 3 Study of cell viability of **4g** using MTT assay. *$p < 0.05$ *vs.* control (ctrl); **$p < 0.01$ *vs.* ctrl.

similar orientation to **4a**, but it is longer, consequently it adopts a more constricted conformation which results more unstable and reduces the π–cation interaction, due to the inclination of the aromatic ring with respect to the heme group. This fact can explain the loss of activity of some compounds.

Fig. 5 shows the most common poses obtained for hydroxypropyl derivatives in the iNOS binding site. Compound **4g** (Fig. 5a) forms three hydrogen bonds, one with Glu371 through the hydroxyl group and two with a heme propionate moiety through both thiourea nitrogens. It is noteworthy that, although **4a** has more favourable H-bond contribution inside nNOS, **4g** seems to have higher VdW stabilising contribution inside iNOS. This contribution is performed through the aliphatic chain which interacts with the nearby residues Val346, Pro344 and Gln257, and especially through the thiourea moiety which is oriented to Gln257 and to the arginine pocket.

Other compounds such as **4l** (Fig. 5b) demonstrate weaker interaction with the iNOS (PDB id: 3NW2)[27] binding site. In this case, two hydrogen bonds are established, one between the amino group and a propionate of the heme group and the other between a thiourea nitrogen and Glu371. Moreover, the conformation adopted by **4l** moves away the phenyl ring and the thiourea moiety reducing the π–cation interaction with the heme group and the VdW interactions which decrease the stability and efficiency of the binding process.

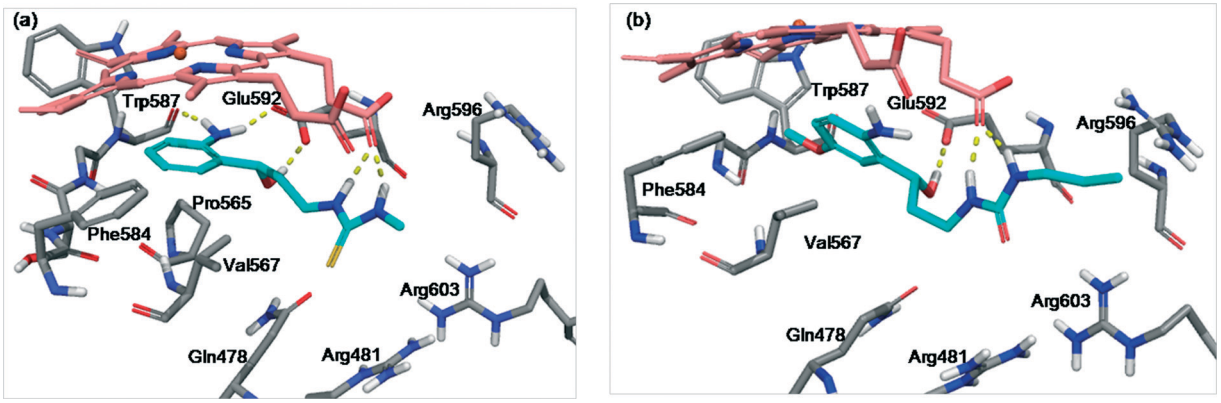


Fig. 4 Detailed view of the main poses obtained for compounds **4a** (a) and **4j** (b) in the nNOS binding site. The dotted lines indicate hydrogen bond interactions between the ligand and the residues of the enzyme.

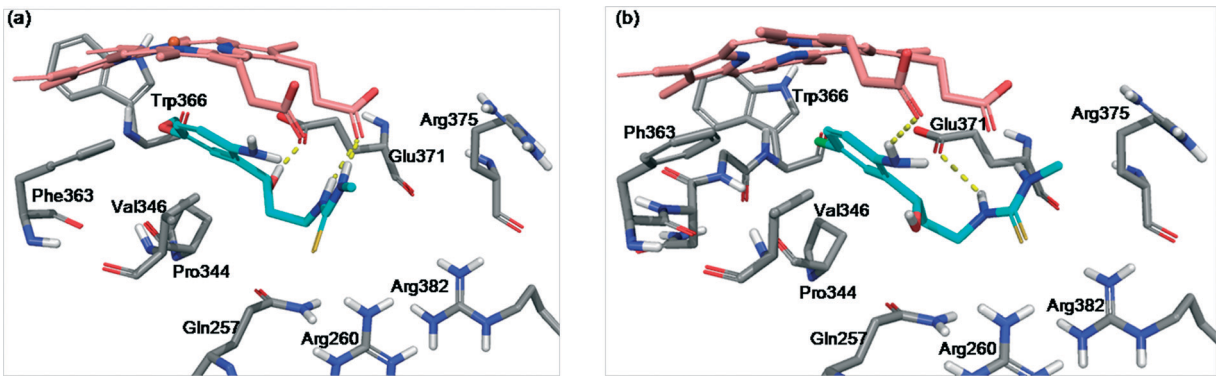


Fig. 5 Detailed view of the main poses obtained for compounds **4g** (a) and **4l** (b) in the iNOS binding site. The dotted lines indicate hydrogen bond interactions between the ligand and the residues of the enzyme.

Conclusions

In summary, we have designed and successfully synthesized a series of novel *N*,*N*′-disubstituted thiourea and urea derivatives. The synthetic pathway of these new compounds can be used to prepare kynurenamine-thioureas and ureas more easily, with less synthetic steps and higher global yield than the previously described route. Their biological evaluation was performed *versus* nNOS and iNOS isoforms for all compounds and *versus* eNOS isoform for **4g**. In general, compounds **4a–q** inhibit the neuronal NOS isoform better than the inducible one, in which the thioureas exhibit higher inhibition than the ureas. The derivatives **4a** and **4g** are the most active compounds with IC_{50} values of 350 and 130 μM, respectively, *versus* nNOS, and 180 μM *versus* iNOS for the second compound. In addition, **4g** does not inhibit eNOS which is necessary to avoid hypertension. Moreover, the cell viability test demonstrated the absence of cell toxicity in compound **4g** at the IC_{50} value. Both *in silico* and *in vitro* studies reveal promising properties for these compounds which could be a reference to design new nNOS and iNOS inhibitors without cytotoxicity and adverse vascular effects, useful in neurodegenerative disorders such as Parkinson's disease, where both isoforms are involved.

Experimental section

Chemistry

Melting points were determined using a capillary melting point apparatus and were uncorrected. Analytical thin layer chromatography was performed using Merck Kieselgel 60F254 aluminum sheets, and the spots were developed with UV light (λ = 254 nm). Flash chromatography was carried out using silica gel 60, 230–240 mesh (Merck), and the eluents used are reported within parentheses. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Inova Unity 300 spectrometer operating at 300.20 for ^{1}H and 75.49 MHz for ^{13}C, on a Varian direct drive 400 spectrometer operating at 400.57 MHz for ^{1}H and 100.73 MHz for ^{13}C, on a Varian Inova Unity 500 spectrometer operating at 499.79 for ^{1}H and 125.68 MHz for ^{13}C and on a Varian direct drive 600 spectrometer operating at 600.25 MHz for ^{1}H and 150.95 MHz for ^{13}C in the deuterated solvents indicated at ambient temperature. Chemical shifts are reported in ppm (δ) and are referenced to the residual solvent peak. IR spectra were recorded on a Perkin Elmer 782 spectrometer. High-resolution mass spectrometry (HRMS) was carried out using a Waters LCT Premier Mass Spectrometer. Elemental analyses were within ±0.4% of the theoretical values. Small scale microwave-assisted synthesis was carried out using an Initiator 2.0 single-mode microwave instrument producing controlled irradiation at 2.450 GHz (Biotage AB, Uppsala). Reaction time refers to holding time at 90 °C, not to total irradiation time. The temperature was measured using an IR sensor outside the reaction vessel. Anhydrous CH_2Cl_2 was used as solvent.

General synthetic method of 3-hydroxy-3-(2-nitro-5-substituted phenyl)propanenitrile, 6a–c

BuLi (1.6 M/hexane, 20.3 mL) was added to dry THF (37.5 mL) cooled to −78 °C under argon. Then, a solution of acetonitrile (1.7 mL) in dry THF (4.9 mL) was added dropwise. The mixture was stirred at −78 °C for 1 h. Afterward, a solution of the corresponding benzaldehyde **5a–c** (16.16 mmol) in dry THF (4.9 mL) was added dropwise. The mixture was stirred again at −78 °C for 30 min and then warmed to RT. The reaction was agitated for 15 min at RT and was quenched with cold water (25 mL), diluted with diethyl ether (30 mL) and washed with 2% aqueous HCl (15 mL). The aqueous layer was extracted with diethyl ether (3 × 15 mL). Finally, the organic phase was washed with $NaHCO_3$ saturated solution (10 mL) and brine (15 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The crude mixture was purified by flash chromatography (EtOAc/hexane, 1 : 2).

3-Hydroxy-3-(2-nitrophenyl)propanenitrile, 6a. Yellow solid (70%).[28]

3-Hydroxy-3-(5-methoxy-2-nitrophenyl)propanenitrile, 6b. Yellow solid (90%); mp: 88–90 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 (d, 1H), 7.47 (d, 1H), 6.94 (dd, 1H), 5.81–5.70 (m, 1H), 3.94 (s, 3H), 3.07 (dd, 1H), 2.85 (dd, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.39, 140.30, 139.65, 127.97, 117.03, 114.49, 112.73, 65.50, 56.15, 27.27; HRMS *m*/*z* 245.0540 $[M + Na]^+$, calcd. mass for $C_{10}H_{10}N_2O_4Na$: 245.0538.

3-(5-Chloro-2-nitrophenyl)-3-hydroxypropanenitrile, 6c. Yellow solid (70%); mp: 63–64 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (d, 1H), 8.01 (d, 1H), 7.51 (dd, 1H), 5.69 (dd, 1H), 3.08 (dd, 1H), 2.87 (dd, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.07, 141.37, 138.95, 129.63, 128.61, 126.52, 116.64, 65.18, 27.41; HRMS *m*/*z* 249.0047 $[M + Na]^+$, calcd. mass for $C_9H_7N_2O_3NaCl$: 249.0045.

General synthetic method of 3-amino-1-(2-nitro-5-substituted phenyl)propan-1-ol, 7a–c

3-Hydroxy-3-(2-nitro-5-substituted phenyl)propanenitrile derivatives **6a–c** (1.7 mmol) were treated dropwise with a solution of BH_3 (12.3 mL) in THF at 0 °C under argon. The mixture was stirred for 4 h at RT. Afterward, the reaction mixture was cooled to 0 °C and an ice-cold solution of 6 N HCl (7.5 mL) was added carefully. The THF was evaporated, and the aqueous phase was basified with 4 N NaOH to pH = 10 and extracted with EtOAc (3 × 15 mL). The organic phase was washed with brine, dried over Na_2SO_4 and concentrated. The crude was used for the next step without purification.

General synthetic method of *N*-alkyl or phenyl-*N*′-[3-hydroxy-3-(2-nitro-5-substituted phenyl)propyl]-thioureas and ureas, 8a–q

Thioisocyanate or isocyanate (0.9 mmol) was added, under argon, to a solution of 3-amino-1-(2-nitro-5-substituted phenyl)propan-1-ol derivatives **7a–c** (0.6 mmol) in dry CH_2Cl_2. The reaction mixture was irradiated under microwave

conditions at 90 °C for 20 min. The crude mixture was purified by flash chromatography (EtOAc/hexane, 1 : 1).

***N*-[3-Hydroxy-3-(2-nitrophenyl)propyl]-*N*′-methylthiourea, 8a.** Yellow solid (0.42 mmol, 70%); mp: 40 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.98–7.90 (m, 2H), 7.70–7.63 (m, 1H), 7.44–7.35 (m, 1H), 5.37 (d, 1H), 4.36 (bs, 1H), 3.59–3.42 (m, 1H), 3.03 (s, 3H), 2.89–2.83 (m, 1H), 2.14–1.97 (m, 2H), 1.88–1.73 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 182.69, 146.98, 140.26, 134.13, 128.09, 128.26, 124.61, 66.09, 42.09, 38.92, 27.41; HRMS *m*/*z* 270.0916 $[M + H]^+$, calcd. mass for $C_{11}H_{16}N_3O_3S$: 270.0912.

***N*-Ethyl-*N*′-[3-hydroxy-3-(2-nitrophenyl)propyl]thiourea, 8b.** Yellow oil (0.42 mmol, 70%); ^{1}H NMR (300 MHz, CD_3OD) δ 8.05–7.82 (m, 2H), 7.82–7.61 (m, 1H), 7.59–7.42 (m, 1H), 5.38–5.20 (m, 1H), 3.76–3.59 (m, 1H), 3.52–3.39 (m, 1H), 3.37–3.28 (m, 2H), 2.34–2.08 (m, 1H), 2.04–1.86 (m, 1H), 1.39–1.24 (m, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 185.83, 147.37, 140.47, 133.30, 128.00, 127.72, 123.87, 66.13, 44.33, 38.48, 36.70, 11.78; HRMS *m*/*z* 284.1064 $[M + H]^+$, calcd. mass for $C_{12}H_{18}N_3O_3S$: 284.1069.

***N*-[3-Hydroxy-3-(2-nitrophenyl)propyl]-*N*′-propylthiourea, 8c.** Yellow oil (0.43 mmol, 73%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (dd, 1H), 7.90 (dd, 1H), 7.67–7.63 (m, 1H), 7.42–7.39 (m, 1H), 6.25 (bs, 1H), 6.15 (bs, 1H), 5.35 (d, 1H), 4.35–4.26 (m, 2H), 3.52–3.47 (m, 2H), 3.34–3.31 (m, 2H), 2.09–2.02 (m, 1H), 1.85–1.76 (m, 1H), 1.69–1.62 (m, 2H), 0.99 (t, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 181.91, 147.10, 140.18, 134.07, 128.24, 128.12, 124.60, 66.26, 45.65, 42.16, 38.83, 22.20, 11.55; HRMS *m*/*z* 282.1460 $[M + H]^+$, calcd. mass for $C_{13}H_{20}N_3O_4$: 282.1454.

***N*-Ethyl-*N*′-[3-hydroxy-3-(2-nitrophenyl)propyl]urea, 8d.** Yellow oil (0.48 mmol, 81%); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.93–7.87 (m, 2H), 7.65–7.60 (m, 1H), 7.40–7.35 (m, 1H), 5.30 (dd, 1H), 3.82–3.75 (m, 1H), 3.24–3.14 (m, 3H), 2.01–1.94 (m, 1H), 1.68–1.61 (m, 1H), 1.14 (t, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.61, 147.06, 140.46, 133.70, 128.18, 127.65, 124.18, 65.91, 39.70, 36.96, 35.56, 15.27; HRMS *m*/*z* 268.1302 $[M + H]^+$, calcd. mass for $C_{12}H_{18}N_3O_4$: 268.1297.

***N*-[3-Hydroxy-3-(2-nitrophenyl)propyl]-*N*′-propylurea, 8e.** Yellow oil (0.45 mmol, 76%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93–7.88 (m, 2H), 7.64–7.61 (m, 1H), 7.39–7.36 (m, 1H), 5.30 (dd, 1H), 4.78 (bs, 2H), 3.83–3.77 (m, 1H), 3.19–3.14 (m, 1H), 3.12 (t, 2H), 2.01–1.94 (m, 1H), 1.67–1.61 (m, 1H), 1.55–1.49 (m, 2H), 0.91 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.87, 147.28, 140.61, 133.82, 128.35, 127.78, 124.32, 66.05, 42.67, 39.95, 37.11, 23.43, 11.42; HRMS *m*/*z* 298.1222 $[M + H]^+$, calcd. mass for $C_{13}H_{20}N_3O_3S$: 298.1225.

***N*-[3-Hydroxy-3-(2-nitrophenyl)propyl]-*N*′-phenylurea, 8f.** Yellow oil (0.42 mmol, 70%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93–7.86 (m, 2H), 7.63–7.60 (m, 1H), 7.51–7.44 (m, 1H), 7.39–7.36 (m, 2H), 7.25–7.20 (m, 2H), 7.04–6.99 (m, 1H), 5.34 (dd, 1H), 3.82–3.77 (m, 1H), 3.24–3.21 (m, 1H), 2.02–1.96 (m, 1H), 1.72–1.66 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.62, 147.07, 140.20, 137.84, 133.75, 129.36, 128.42, 127.82, 124.30, 121.81, 121.04, 66.39, 39.07, 37.20; HRMS *m*/*z* 316.1292 $[M + H]^+$, calcd. mass for $C_{16}H_{18}N_3O_4$: 316.1297.

***N*-[3-Hydroxy-3-(5-methoxy-2-nitrophenyl)propyl]-*N*′-methylthiourea, 8g.** Yellow oil (0.43 mmol, 72%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.09 (d, 1H), 7.43 (d, 1H), 6.87 (dd, 1H), 5.51 (d, 1H), 4.39 (bs, 1H), 3.94 (s, 3H), 3.91–3.89 (m, 1H), 3.56–351 (m, 1H), 3.05 (s, 3H), 2.11–2.04 (m, 1H), 1.79–1.74 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.77, 164.22, 144.04, 139.51, 127.70, 113.55, 111.97, 66.22, 55.98, 42.04, 38.62, 30.31; HRMS *m*/*z* 300.1021 $[M + H]^+$, calcd. mass for $C_{12}H_{18}N_3O_4S$: 300.1018.

***N*-[3-Hydroxy-3-(5-methoxy-2-nitrophenyl)propyl]-*N*′-propylthiourea, 8h.** Yellow oil (0.46 mmol, 77%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (d, 1H), 7.40 (d, 1H), 6.84 (dd, 1H), 6.21 (bs, 1H), 6.14 (bs, 1H), 5.48 (d, 1H), 4.42–4.35 (m, 2H), 3.91 (s, 3H), 3.52–3.46 (m, 1H), 3.36–3.30 (m, 2H), 2.07–2.00 (m, 1H), 1.76–1.70 (m, 1H), 1.66 (q, 2H), 0.99 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 182.16, 164.61, 144.37, 139.96, 128.09, 113.93, 112.37, 66.60, 56.36, 45.88, 42.41, 39.08, 22.46, 11.81; HRMS *m*/*z* 326.1159 $[M + H]^+$, calcd. mass for $C_{14}H_{20}N_3O_4S$: 326.1175.

***N*-Ethyl-*N*′-[3-Hydroxy-3-(5-methoxy-2-nitrophenyl)propyl]-urea, 8i.** Yellow oil (0.44 mmol, 74%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.03 (d, 1H), 7.41 (d, 1H), 6.82 (dd, 1H), 5.45 (dd, 1H), 3.90 (s, 3H), 3.87–3.80 (m, 1H), 3.24–3.15 (m, 3H), 2.01–1.94 (m, 1H), 1.61–1.55 (m, 1H), 1.15 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.23, 159.78, 144.63, 139.86, 127.57, 113.59, 112.02, 66.35, 56.06, 39.73, 37.17, 35.77, 15.43; HRMS *m*/*z* 296.1254 $[M + H]^+$, calcd. mass for $C_{13}H_{18}N_3O_5$: 296.1246.

***N*-[3-Hydroxy-3-(5-methoxy-2-nitrophenyl)propyl]-*N*′-propylurea, 8j.** Yellow oil (0.45 mmol, 75%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (d, 1H), 7.41 (d, 1H), 6.85 (dd, 1H), 5.45 (d, 1H), 4.73 (bs, 2H), 3.89 (s, 3H), 3.83–3.72 (m, 1H), 3.26–3.18 (m, 1H), 3.14 (t, 2H), 2.04–1.89 (m, 1H), 1.65–1.48 (m, 3H), 0.92 (t, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.05, 159.77, 144.46, 139.70, 127.39, 113.38, 111.95, 66.39, 55.89, 42.61, 39.28, 37.29, 23.15, 11.23; HRMS *m*/*z* 312.1571 $[M + H]^+$, calcd. mass for $C_{14}H_{22}N_3O_5$: 312.1559.

***N*-[3-Hydroxy-3-(5-methoxy-2-nitrophenyl)propyl]-*N*′-phenylurea, 8k.** Yellow solid (0.48 mmol, 80%); mp: 77–78 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, 1H), 7.38 (d, 1H), 7.34–7.24 (m, 4H), 7.09–7.04 (m, 1H), 6.80 (dd, 1H), 5.49 (dd, 1H), 3.87 (s, 3H), 3.84–3.77 (m, 1H), 3.23–3.17 (m, 1H), 2.01–1.93 (m, 1H), 1.65–1.56 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.10, 157.52, 144.30, 139.62, 138.15, 129.28, 127.52, 124.12, 121.52, 113.42, 111.91, 66.53, 55.89, 39.07, 37.04; HRMS *m*/*z* 368.1214 $[M + H]^+$, calcd. mass for $C_{17}H_{19}N_3O_5Na$: 368.1222.

***N*-[3-(5-Chloro-2-nitrophenyl)-3-hydroxypropyl]-*N*′-methylthiourea, 8l.** Yellow oil (0.45 mmol, 75%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.96–7.90 (m, 2H), 7.36 (dd, 1H), 6.33 (bs, 2H), 5.38 (dd, 1H), 4.36 (bs, 1H), 3.54–3.41 (m, 2H), 3.01 (s, 3H), 2.06–1.95 (m, 1H), 1.78–1.65 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 182.41, 144.83, 142.57, 140.79, 128.44, 128.07, 126.15, 66.67, 41.79, 38.84, 30.29; HRMS *m*/*z* 304.0522 $[M + H]^+$, calcd. mass for $C_{11}H_{15}N_3O_3SCl$: 304.0523.

***N*-[3-(5-Chloro-2-nitrophenyl)-3-hydroxypropyl]-*N*′-ethylthiourea, 8m.** Yellow oil (0.42 mmol, 70%); ^{1}H NMR (300

MHz, $CDCl_3$) δ 7.99–7.82 (m, 2H), 7.35 (dd, 1H), 6.37 (bs, 2H), 5.38 (d, 1H), 4.34 (bs, 1H), 3.75–3.42 (m, 4H), 2.06–1.97 (m, 1H), 1.87–1.56 (m, 1H), 1.32–1.20 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.45, 144.90, 142.53, 140.71, 128.44, 128.02, 126.09, 66.69, 41.75, 38.80, 38.68, 13.99; HRMS *m*/*z* 318.0672 $[M + H]^+$, calcd. mass for $C_{12}H_{17}N_3O_3SCl$: 318.0679.

***N*-[3-(5-Chloro-2-nitrophenyl)-3-hydroxypropyl]-*N*′-propylthiourea, 8n.** Yellow oil (0.45 mmol, %); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.00–7.90 (m, 2H), 7.38 (dd, 1H), 6.36 (bs, 2H), 5.41 (d, 1H), 4.38 (bs,1H), 3.81–3.30 (m, 4H), 2.12–1.96 (m, 1H), 1.80–1.62 (m, 3H), 1.01 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 181.57, 144.88, 142.58, 140.72, 128.46, 128.02, 126.10, 66.65, 45.67, 41.76, 38.89, 22.10, 11.41; HRMS *m*/*z* 332.0833 $[M + H]^+$, calcd. mass for $C_{13}H_{19}N_3O_3SCl$: 332.0836.

***N*-[3-(5-Chloro-2-nitrophenyl)-3-hydroxypropyl]-*N*′-ethylurea, 8o.** Yellow oil (0.51 mmol, 85%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 (d, 1H), 7.90 (d, 1H), 7.34 (dd,1H), 5.33 (dd, 1H), 4.94 (bs, 2H), 4.30 (bs, 1H), 3.85–3.79 (m, 1H), 3.24–3.15 (m, 3H), 1.99–1.92 (m, 1H), 1.61–1.55 (m, 1H), 1.15 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.65, 145.07, 142.88, 140.53, 128.53, 127.80, 125.89, 65.66, 39.76, 36.86, 35.65, 15.25; HRMS *m*/*z* 302.0903 $[M + H]^+$, calcd. mass for $C_{12}H_{17}N_3O_4Cl$: 302.0908.

***N*-[3-(5-Chloro-2-nitrophenyl)-3-hydroxypropyl]-*N*′-propylurea, 8p.** Yellow oil (0.42 mmol, 70%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (d, 1H), 7.90 (d, 1H), 7.38 (dd, 1H), 5.32 (dd, 1H), 3.86–3.80 (m, 1H), 3.20–3.15 (m, 1H), 3.13 (t, 2H), 1.99–1.92 (m, 1H), 1.61–1.55 (m, 1H), 1.56–1.51 (m, 2H), 0.93 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.75, 145.07, 142.85, 140.54, 128.52, 127.79, 125.89, 65.63, 42.60, 39.81, 36.86, 23.25, 11.27; HRMS *m*/*z* 316.1056 $[M + H]^+$, calcd. mass for $C_{13}H_{19}N_3O_4Cl$: 316.1064.

***N*-[3-(5-Chloro-2-nitrophenyl)-3-hydroxypropyl]-*N*′-phenylurea, 8q.** Yellow solid (0.42 mmol, 70%); mp: 53 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92–7.88 (m, 2H), 7.35–7.28 (m, 5H), 7.13–7.08 (m, 1H), 5.37 (dd, 1H), 3.87–3.80 (m, 1H), 3.20–3.15 (m, 1H), 1.98–1.90 (m, 1H), 1.64–1.56 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.65, 144.98, 142.67, 140.61, 137.76, 129.43, 128.44, 127.91, 126.00, 124.64, 122.09, 65.90, 39.32, 36.89; HRMS *m*/*z* 372.0722 $[M + H]^+$, calcd. mass for $C_{16}H_{16}N_3O_4NaCl$: 372.0727.

General synthetic method of *N*-alkyl or phenyl-*N*′-[3-(2-amino-5-substituted phenyl)-3-hydroxypropyl]-thioureas and ureas, 4a–q

A solution of each of the nitro precursors **8a–q** (0.5 mmol) in MeOH (10 mL) was passed through the flow hydrogenator under the following conditions: 60 °C, 60 bar, 0.2 mL min^{-1} flow rate and 10% Pd/C as a catalyst. After evaporation of the solvent, the crude mixture was purified by recrystallization (diethyl ether) or by flash chromatography (EtOAc or EtOAc/hexane, 1 : 1).

***N*-[3-(2-Aminophenyl)-3-hydroxypropyl]-*N*′-methylthiourea, 4a.** Yellow oil (0.37 mmol, 75%); ^{1}H NMR (600 MHz, CD_3OD) δ 7.06 (d, 1H), 6.96–6.93 (m, 1H), 6.66 (d, 1H), 6.63–6.60 (m, 1H), 4.72 (dd, 1H), 3.53–3.24 (m, 2H), 2.90 (s, 3H), 2.02–1.96 (m, 2H); ^{13}C NMR (150 MHz, CD_3OD) δ 181.71, 147.42, 130.83, 130.29, 129.13, 120.45, 119.16, 72.55, 42.10, 38.53, 32.02; HRMS *m*/*z* 240.1176 $[M + H]^+$, calcd. mass for $C_{11}H_{18}N_3OS$: 240.1171; anal. calcd for $C_{11}H_{17}N_3OS$: C 55.20, H 7.16, N 17.56, found: C 54.81, H 7.16, N 17.17.

***N*-[3-(2-Aminophenyl)-3-hydroxypropyl]-*N*′-ethylthiourea, 4b.** Yellow oil (0.37 mmol, 75%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.09–7.04 (m, 2H), 6.73–6.70 (m, 1H), 6.64 (dd, 1H), 6.43 (bs, 1H), 6.15 (bs, 1H), 4.78 (dd, 1H), 3.92 (m, 1H), 3.49–3.33 (m, 1H), 3.35–3.30 (m, 2H), 2.21–2.15 (m, 1H), 1.98–1.91 (m, 1H), 1.19 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 181.25, 144.70, 128.67, 127.12, 127.08, 118.54, 116.96, 71.12, 42.09, 38.87, 34.69, 14.18; HRMS *m*/*z* 254.1175 $[M + H]^+$, calcd. mass for $C_{12}H_{20}N_3OS$: 254.1171; anal. calcd for $C_{12}H_{19}N_3OS$: C 56.89, H 7.56, N 16.58, found: C 57.26, H 7.91, N 16.22.

***N*-[3-(2-Aminophenyl)-3-hydroxypropyl]-*N*′-propylthiourea, 4c.** Yellow solid (0.40 mmol, 80%); mp: 50 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.09–7.03 (m, 2H), 6.74–6.69 (m, 1H), 6.64 (d, 1H), 6.38 (bs, 1H), 6.15 (bs, 1H), 4.81–4.75 (m, 1H), 4.07–3.76 (m, 2H), 3.53–3.44 (m, 1H), 3.32–3.17 (m, 2H), 2.22–2.15 (m, 1H), 1.98–1.90 (m, 1H), 1.62–1.55 (m, 2H), 0.94 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 181.61, 144.91, 128.83, 127.22, 127.18, 118.62, 117.06, 71.35, 46.03, 42.13, 34.82, 22.31, 11.55; HRMS *m*/*z* 268.1469 $[M + H]^+$, calcd. mass for $C_{13}H_{22}N_3OS$: 268.1484; anal. calcd for $C_{13}H_{21}N_3OS$: C 58.39, H 7.92, N 15.71, found: C 58.14, H 8.12, N 15.41.

***N*-[3-(2-Aminophenyl)-3-hydroxypropyl]-*N*′-ethylurea, 4d.** White solid (0.41 mmol, 83%); mp: 115 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 7.09 (d, 1H), 7.03–6.98 (m, 1H), 6.71 (d, 1H), 6.69–6.65 (m, 1H), 4.75 (dd, 1H), 3.31 (bs, 1H), 3.29–3.18 (m, 2H), 3.13 (q, 2H), 2.03–1.88 (m, 2H), 1.09 (t, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 159.98, 144.89, 128.19, 127.56, 126.54, 117.67, 116.47, 70.07, 37.00, 35.90, 34.41, 14.34; IR (KBr) ν: 3608.79, 3583.37, 3351.29, 2929.21, 1699.47, 1624.22, 1565.96, 1495.79, 1456.51, 1261.70, 1157.66, 1069.88, 755.76, 665.51 cm^{-1}; HRMS *m*/*z* 238.1475 $[M + H]^+$, calcd. mass for $C_{12}H_{20}N_3O_2$: 238.1477; anal. calcd for $C_{12}H_{19}N_3O_2$: C 60.74, H 8.07, N 17.71, found: C 60.86, H 8.34, N 17.41.

***N*-[3-(2-Aminophenyl)-3-hydroxypropyl]-*N*′-propylurea, 4e.** Yellow solid (0.45 mmol, 90%); mp: 108–109 °C; ^{1}H NMR (600 MHz, CD_3OD) δ 7.11 (d, 1H), 7.03–7.01 (m, 1H), 6.71 (d, 1H), 6.70–6.67 (m, 1H), 4.76 (dd, 1H), 3.31–3.26 (m, 1H), 3.24–3.19 (m, 1H), 3.08 (t, 2H), 2.04–1.98 (m, 1H), 1.97–1.91 (m, 1H), 1.53–1.48 (m, 2H), 0.93 (t, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 161.53, 146.32, 129.61, 128.99, 127.97, 119.09, 117.90, 71.52, 42.88, 38.43, 37.35, 24.47, 11.62; HRMS *m*/*z* 252.1701 $[M + H]^+$, calcd. mass for $C_{13}H_{22}N_3O_2$: 252.1712; anal. calcd for $C_{13}H_{21}N_3O_2$: C 62.13, H 8.42, N 16.72, found: C 62.23, H 8.44, N 16.41.

***N*-[3-(2-Aminophenyl)-3-hydroxypropyl]-*N*′-phenylurea, 4f.** White solid (0.35 mmol, 70%); mp: 95 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 7.36–7.32 (m, 2H), 7.26–7.21 (m, 2H), 7.12 (d, 1H), 7.03–7.01 (m, 1H), 6.99–6.94 (m, 1H), 6.72 (dd, 1H), 6.69–6.66 (m, 1H), 4.80 (dd, 1H), 3.39–3.34 (m, 1H), 3.28–3.26 (m, 1H), 2.09–1.95 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ

160.84, 148.58, 143.26, 132.04, 131.92, 131.30, 130.27, 125.67, 122.55, 121.42, 120.22, 74.02, 40.65, 39.40; IR (KBr) *v*: 3608.66, 3583.14, 3352.13, 2927.61, 1645.83, 1624.22, 1597.47, 1554.11, 1498.40, 1456.94, 1440.69, 1312.68, 1238.51, 1071.53, 754.01, 665.86 cm^{-1}; HRMS *m*/*z* 286.1560 $[M + H]^+$, calcd. mass for $C_{16}H_{20}N_3O_2$: 286.1556; anal. calcd for $C_{16}H_{19}N_3O_2$: C 67.35, H 6.71, N 14.73, found: C 67.49, H 7.09, N 14.63.

***N*-[3-(2-Amino-5-methoxyphenyl)-3-hydroxypropyl]-*N*′-methylthiourea, 4g.** Yellow solid (0.37 mmol, 75%); mp: 50 °C; ^1H NMR (300 MHz, CD_3OD) δ 6.98 (d, 1H), 6.93 (d, 1H), 6.83 (dd, 1H), 4.92 (d, 1H), 3.82 (s, 3H), 3.80–3.78 (m, 2H), 2.98 (s, 3H), 2.12–2.06 (m, 2H); ^{13}C NMR (150 MHz, CD_3OD) δ 182.41, 155.80, 134.34, 131.26, 121.11, 115.91, 114.99, 72.27, 57.35, 38.38, 36.51, 31.05; HRMS *m*/*z* 270.1280 $[M + H]^+$, calcd. mass for $C_{12}H_{20}N_3O_2S$: 270.1276; anal. calcd for $C_{12}H_{19}N_3O_2S$: C 53.51, H 7.11, N 15.60, found: C 53.50, H 7.55, N 15.50.

***N*-[3-(2-Amino-5-methoxyphenyl)-3-hydroxypropyl]-*N*′-propylthiourea, 4h.** Yellow solid (0.38 mmol, 76%); mp: 45 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 6.69 (d, 1H), 6.66 (dd, 1H), 6.62 (d, 1H), 6.50 (bs, 1H), 6.20 (bs, 1H), 4.77 (dd, 1H), 3.72 (s, 3H), 3.67–3.48 (m, 2H), 3.31–3.24 (m, 2H), 2.17–2.08 (m, 1H), 1.98–1.90 (m, 1H), 1.63–1.55 (m, 2H), 0.95 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 181.63, 153.16, 137.56, 129.60, 118.71, 113.95, 113.07, 70.78, 55.92, 45.94, 42.21, 35.18, 22.33, 11.56; HRMS *m*/*z* 298.1580 $(M + H)^+$, calcd. mass for $C_{14}H_{24}N_3O_2S$: 298.1589; anal. calcd for $C_{14}H_{23}N_3O_2S$: C 56.54, H 7.79, N 14.13, found: C 56.50, H 7.65, N 14.50.

***N*-[3-(2-Amino-5-methoxyphenyl)-3-hydroxypropyl]-*N*′-ethylurea, 4i.** Yellow solid (0.39 mmol, 78%); mp: 128–129 °C; ^1H NMR (600 MHz, CD_3OD) δ 6.79 (d, 1H), 6.71 (d, 1H), 6.67 (dd, 1H), 4.76 (dd, 1H), 3.73 (s, 3H), 3.32–3.27 (m, 1H), 3.25–3.20 (m, 1H), 3.15 (q, 2H), 1.98–1.91 (m, 2H), 1.11 (t, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 161.40, 154.32, 139.07, 131.79, 119.39, 114.52, 113.66, 70.94, 56.09, 38.39, 37.67, 35.85, 15.76; HRMS *m*/*z* 268.1647 $(M + H)^+$, calcd. mass for $C_{13}H_{22}N_3O_3$: 268.1661; anal. calcd for $C_{13}H_{21}N_3O_3$: C 58.41, H 7.92, N 15.72, found: C 58.50, H 7.63, N 15.75.

***N*-[3-(2-Amino-5-methoxyphenyl)-3-hydroxypropyl]-*N*′-propylurea, 4j.** White solid (0.37 mmol, 75%); mp: 130 °C; ^1H NMR (300 MHz, CD_3OD) δ 6.83 (d, 1H), 6.76 (d, 1H), 6.71 (dd, 1H), 4.81 (dd, 1H), 3.78 (s, 3H), 3.36–3.25 (m, 2H), 3.13 (t, 2H), 2.04–1.95 (m, 2H), 1.61–1.50 (m, 2H), 0.98 (t, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 160.09, 152.88, 137.70, 130.33, 117.95, 113.13, 112.27, 69.55, 54.70, 41.47, 36.97, 36.26, 23.04, 10.19; IR (KBr) *v*: 3608.56, 3583.07, 3351.03, 2931.27, 2352.07, 1613.25, 1568.33, 1503.31, 1431.20, 1258.55, 1157.68, 1069.54, 1041.27, 840.60, 757.42, 665.87 cm^{-1}; HRMS *m*/*z* 282.1813 $(M + H)^+$, calcd. mass for $C_{14}H_{24}N_3O_3$: 282.1818; anal. calcd for $C_{14}H_{23}N_3O_3$: C 59.77, H 8.24, N 14.94, found: C 59.79, H 8.40, N 14.75.

***N*-[3-(2-Amino-5-methoxyphenyl)-3-hydroxypropyl]-*N*′-phenylurea, 4k.** White solid (0.37 mmol, 75%); mp: 157 °C; ^1H NMR (500 MHz, CD_3OD) δ 7.36–7.31 (m, 2H), 7.27–7.21 (m, 2H), 6.98–6.95 (m, 1H), 6.79 (d, 1H), 6.70 (d, 1H), 6.65 (dd, 1H), 4.79 (dd, 1H), 3.41–3.35 (m, 1H), 3.40–3.27 (m, 1H), 3.31 (s, 3H), 2.03–1.94 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 157.13, 152.92, 139.55, 137.64, 130.37, 128.35, 121.99, 118.87, 118.02, 113.14, 112.25, 69.77, 54.67, 36.92, 36.01; IR (KBr) *v*: 3608.39, 3583.00, 3292.54, 2922.27, 1621.06, 1556.13, 1502.84, 1451.39, 1253.58, 1042.40, 757.87, 665.28 cm^{-1}; HRMS *m*/*z* 316.1667 $[M + H]^+$, calcd. mass for $C_{17}H_{22}N_3O_3$: 316.1661; anal. calcd for $C_{17}H_{21}N_3O_3$: C 64.74, H 6.71, N 13.32, found: C 64.72, H 6.57, N 13.52.

***N*-[3-(2-Amino-5-chlorophenyl)-3-hydroxypropyl]-*N*′-methylthiourea, 4l.** Yellow solid (0.39 mmol, 79%); mp: 55 °C; ^1H NMR (400 MHz, CD_3OD) δ 7.18 (d, 1H), 7.02 (dd, 1H), 6.72 (d, 1H), 4.80 (dd, 1H), 3.37–3.35 (m, 2H), 2.97 (s, 3H), 2.08–2.03 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 181.67, 143.62, 129.94, 127.15, 126.02, 121.99, 117.36, 69.08, 41.16, 34.81, 29.30; HRMS *m*/*z* 274.0775 $[M + H]^+$, calcd. mass for $C_{11}H_{17}N_3OSCl$: 274.0781; anal. calcd for $C_{11}H_{16}N_3OSCl$: C 48.26, H 5.89, N 15.35, found: C 48.60, H 5.99, N 15.73.

***N*-[3-(2-Amino-5-chlorophenyl)-3-hydroxypropyl]-*N*′-ethylthiourea, 4m.** Yellow solid (0.36 mmol, 72%); mp: 48 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 7.05 (d, 1H), 7.02–6.98 (m, 1H), 6.56 (d, 1H), 4.76 (dd, 1H), 4.55–4.35 (m, 1H), 4.34–4.25 (m, 1H), 4.24–4.17 (m, 2H), 2.15–2.05 (m, 1H), 1.96–1.87 (m, 1H), 1.28 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 182.70, 142.94, 128.98, 128.17, 126.75, 123.14, 118.03, 69.25, 46.39, 40.64, 34.73, 14.10; HRMS *m*/*z* 288.0941 $[M + H]^+$, calcd. mass for $C_{12}H_{19}N_3OSCl$: 288.0937; anal. calcd for $C_{12}H_{18}N_3OSCl$: C 50.08, H 6.30, N 14.60, found: C 50.44, H 6.47, N 14.22.

***N*-[3-(2-Amino-5-chlorophenyl)-3-hydroxypropyl]-*N*′-propylthiourea, 4n.** White solid (0.40 mmol, 80%); mp: 130 °C; ^1H NMR (400 MHz, CD_3OD) δ 7.13 (d, 1H), 6.96 (dd, 1H), 6.67 (d, 1H), 4.74 (dd, 1H), 3.71–3.60 (m, 1H), 3.56–3.46 (m, 1H), 3.45–3.42 (m, 2H), 2.03–1.97 (m, 2H), 1.62–1.55 (m, 2H), 0.94 (t, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 181.17, 143.63, 129.93, 127.14, 126.02, 121.98, 117.35, 69.15, 45.56, 41.24, 34.85, 21.98, 10.20; IR (KBr) *v*: 3608.26, 3583.16, 3326.85, 2930.44, 1627.13, 1488.78, 1261.21, 1101.68, 808.25, 756.63, 665.93 cm^{-1}; HRMS *m*/*z* 302.1096 $[M + H]^+$, calcd. mass for $C_{13}H_{21}N_3OSCl$: 302.1094; anal. calcd for $C_{13}H_{20}N_3OSCl$: C 51.73, H 6.68, N 13.92, found: C 51.51, H 7.16, N 13.54.

***N*-[3-(2-Amino-5-chlorophenyl)-3-hydroxypropyl]-*N*′-ethylurea, 4o.** Yellow solid (0.45 mmol, 91%); mp: 48 °C; ^1H NMR (300 MHz, CD_3OD) δ 7.10 (d, 1H), 6.97 (dd, 1H), 6.67 (d, 1H), 4.71 (dd, 1H), 3.33–3.24 (m, 2H), 3.23–3.17 (m, 2H), 1.95–1.87 (m, 2H), 1.09 (t, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 159.97, 143.45, 130.15, 127.10, 126.11, 122.11, 117.48, 69.22, 36.91, 35.94, 34.43, 14.33; IR (KBr) *v*: 3608.63, 3583.26, 3337.67, 2923.67, 1623.12, 1564.07, 1488.68, 1260.36, 1061.42, 755.82, 665.63 cm^{-1}; HRMS *m*/*z* 294.0982 $[M + Na]^+$, calcd. mass for $C_{12}H_{18}N_3O_2NaCl$: 294.0985; anal. calcd for $C_{12}H_{18}N_3O_2NaCl$: C 53.04, H 6.68, N 15.46, found: C 53.38, H 7.02, N 15.52.

***N*-[3-(2-Amino-5-chlorophenyl)-3-hydroxypropyl]-*N*′-propylurea, 4p.** Yellow solid (0.41 mmol, 82%); mp: 137 °C; ^1H NMR (300 MHz, CD_3OD) δ 7.03 (d, 1H), 6.96 (dd, 1H), 6.59 (d, 1H), 4.70 (dd, 1H), 3.39–3.03 (m, 4H), 1.93–1.71 (m, 2H), 1.53–1.46 (m, 2H), 0.92 (t, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 159.99, 143.54, 130.23, 127.15, 126.11, 122.15, 117.58, 69.32,

42.83, 36.92, 35.93, 22.58, 10.46; IR (KBr) ν: 3608.76, 3583.12, 3352.21, 2929.75, 1625.87, 1489.82, 1456.67, 1261.04, 1071.09, 756.29, 665.60 cm^{-1}; HRMS m/z 286.1318 $[M + H]^+$, calcd. mass for $C_{13}H_{21}N_3O_2Cl$: 286.1322; anal. calcd for $C_{13}H_{20}N_3O_2Cl$: C 54.64, H 7.05, N 14.70, found: C 54.34, H 7.33, N 14.84.

***N*-[3-(2-Amino-5-chlorophenyl)-3-hydroxypropyl]-*N*′-phenylurea, 4q.** White solid (0.35 mmol, 71%); mp: 105 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 7.36–7.32 (m, 2H), 7.26–7.22 (m, 2H), 7.13 (d, 1H), 6.99–6.94 (m, 2H), 6.67 (d, 1H), 4.76 (dd, 1H), 3.41–3.35 (m, 1H), 3.31–3.27 (m, 1H), 2.01–1.92 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 157.14, 143.71, 139.52, 130.01, 128.36, 127.13, 126.10, 122.02, 121.94, 118.90, 117.38, 69.44, 36.89, 35.65; HRMS m/z 320.1168 $[M + H]^+$, calcd. mass for $C_{16}H_{19}N_3O_2Cl$: 320.1166; anal. calcd for $C_{16}H_{18}N_3O_2Cl$: C 60.09, H 5.67, N 13.14, found: C 60.21, H 5.96, N 13.38.

General synthetic method of *N*-[3-(2-amino-5-chlorophenyl)-3-oxopropyl]-*N*′-ethylurea 3b from *N*-[3-(5-chloro-2-nitrophenyl)-3-hydroxypropyl]-*N*′-ethylurea 8o

The Jones reagent (0.418 mL), freshly prepared (a mixture of 2.67 g of chromic anhydride and 2.3 mL of H_2SO_4 dissolved in 10 mL of water), was added to a solution of **8o** (0.742 mmol) in acetone (3 mL). After 10 min stirring, the reaction was quenched with ice-water (25 mL) and saturated $NaHSO_4$ solution (5 mL). The resulting mixture was extracted with ethyl acetate, filtered, dried (Na_2SO_4) and concentrated under vacuum. The crude mixture was purified by flash chromatography (EtOAc/hexane, 3 : 1). The next step was the nitro group reduction to amino one using Fe/$FeSO_4$ in water as previously described.[20]

***N*-[3-(5-Chloro-2-nitrophenyl)-3-oxopropyl]-*N*′-ethylurea.** Following the procedure described in this section, 0.67 mmol of a white solid was obtained (90%) (spectroscopic data).[20]

***N*-[3-(2-Amino-5-chlorophenyl)-3-oxopropyl]-*N*′-ethylurea 3b.** Yellow solid (75%).[20]

Biological procedures

In vitro nNOS and iNOS activity determination

L-Arginine, L-citrulline, *N*-(2-hydroxymethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), DL-dithiothreitol (DTT), hypoxantine-9-β-D-ribofuranosid (inosine), ethylene glycol-bis-(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), bovine serum albumin (BSA), Dowex-50 W (50 × 8-200), FAD, NADPH and 5,6,7,8-tetrahydro-L-biopterin dihydrochloride (H_4-biopterin), tris-(hydroxymethyl)-aminomethane (Tris-HCl) and calcium chloride were obtained from Sigma-Aldrich Química (Spain). L-[^{3}H]-arginine (47.4 Ci $mmol^{-1}$) was obtained from Perkin Elmer (Spain). Calmodulin from bovine brain and recombinant iNOS and nNOS were obtained from Enzo Life Sciences (Spain).

The i/nNOS activity was measured by the Bredt and Snyder method,[29] monitoring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline. The final incubation volume was 100 μL and consisted of 10 μL of an aliquot of recombinant i/nNOS added to a buffer with a final concentration of 25 mM Tris-HCl, 1 mM DTT, 4 μM H_4-biopterin, 10 μM FAD, 0.5 mM inosine, 0.5 mg mL^{-1} BSA, 0.1 mM $CaCl_2$, 10 μM L-arginine, 10 μg mL^{-1} calmodulin (only for nNOS) and 50 nM L-[^{3}H]-arginine, at pH 7.6. The reaction was started by the addition of 10 μL of 7.5 mM NADPH and 10 μL of each of the derivatives **4a–q** in ethanol (10%) to give a final concentration of 1 mM. The tubes were vortexed and incubated at 37 °C for 30 min. Control incubations were performed by the omission of NADPH. The reaction was halted by the addition of 400 μL of cold 0.1 M HEPES, 10 mM EGTA, and 0.175 mg mL^{-1} L-citrulline, pH 5.5. The reaction mixture was decanted into a 2 mL column packet with Dowex-50 W ion-exchange resin (Na^+ form) and eluted with 1.2 mL of water. L-[^{3}H]-citrulline was quantified by liquid scintillation spectroscopy. The retention of L-[^{3}H]-arginine in this process was greater than 98%. Specific enzyme activity was determined by subtracting the control value, which usually amounted to less than 1% of the radioactivity added. The nNOS activity was expressed as picomoles of L-[^{3}H]-citrulline produced (per mg of protein per min).

eNOS inhibition

Quantification of NO in human umbilical vein endothelial cells (HUVECs)

The investigation has been conducted according to the principles expressed in the Declaration of Helsinki and has been approved by the Ethics Committee for the Human Investigation, at the University of Granada, Granada, Spain (Approval no. 201302400001491). Subjects have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Endothelial cells were isolated from human umbilical cord veins using a previously reported method with several modifications.[30] The cells were cultured (Medium 199 + 20% fetal bovine serum + penicillin/streptomycin 2 mmol L^{-1} + amphotericin B 2 mmol L^{-1} + glutamine 2 mmol L^{-1} + HEPES 10 mmol L^{-1} + endothelial cell growth supplement 30 μg mL^{-1} + heparin 100 mg mL^{-1}) under 5% CO_2 at 37 °C. HUVECs were then used to measure NO production by diaminofluorescein-2 (DAF-2) fluorescence, as described previously.[30] Briefly, cells were incubated for 30 min in the presence of **4g** at a concentration of 100 μmol L^{-1}. After this period, cells were washed with PBS and then were pre-incubated with L-arginine (100 μmol L^{-1} in PBS, 5 min, 37 °C). Subsequently, DAF-2 (0.1 μmol L^{-1}) was incubated for 2 min, and then the calcium ionophore calimycin (A23187, 1 μmol L^{-1}) was added for 30 min and cells were incubated in the dark at 37 °C. Then the fluorescence (arbitrary units) was measured using a spectrofluorimeter (Fluorostart, BMG Labtechnologies, Offenburg, Germany). The autofluorescence was subtracted from each value. In some experiments, N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol L^{-1}) was added 15 min before the addition of

L-arginine. The difference between fluorescence signal without and with L-NAME was considered NO production.

Tissue preparation and measurement of tension

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with the principles outlined in the Declaration of Helsinki and approved by the Ethics Committee for the welfare of experimental animals, at the University of Granada, Granada, Spain (Approval no. 459-bis-CEEA-2012). Male Wistar rats (250–300 g), obtained from Harlam Laboratories SA (Barcelona, Spain), were euthanized by a quick blow on the head followed by exsanguination. The descending thoracic aortic rings were dissected, and the rings were then mounted in organ chambers filled with Krebs solution (composition in mmol L^{-1}: NaCl, 118; KCl, 4.75; $NaHCO_3$, 25; $MgSO_4$, 1.2; $CaCl_2$, 2; KH_2PO_4, 1.2; and glucose, 11) and were stretched to 2 g of resting tension by means of two L-shaped stainless-steel wires inserted into the lumen and attached to the chamber and to an isometric force-displacement transducer (UF-1, Cibertec, Madrid, Spain), and the data were recorded using a recording and analysis system (MacLab AD Instruments), as described previously.[30] After equilibration, aortic rings with a functional endothelium were incubated with DMSO, **4g** (100 μmol L^{-1}), or L-NAME (100 μmol L^{-1}) for 30 min and contracted with phenylephrine (1 μmol L^{-1}). Once a plateau contraction was reached, a concentration–response curve was constructed by cumulative addition of acetylcholine. The results are expressed as percentage of phenylephrine-evoked contraction. The data are expressed as the mean ± SEM, and *n* reflects the number of aortic rings from different rats.

Cell viability tests

HUVECs in suspension were seeded at 1×10^4 cells per well in a 96-well microtiter plate, and these cells were incubated at 37 °C for up to 24 hours. The cell supernatants were removed and replaced by fresh medium before MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis. Then the cells were exposed to serial dilutions of **4g** (10 μM–5 mM) for 30 minutes. At the chosen time, 20 μL of 5 mg mL^{-1} MTT in PBS was added to the cells and further incubated at 37 °C. After washing, 100 μL of DMSO were added into each well, and the absorbance at 570 nm was measured using a multi-well plate reader (Model 680XR, BIO-RAD) with subtraction of the blank value at 630 nm and was compared with control, untreated cells.

Statistical analysis

The data are expressed as the mean ± SEM. Statistically significant differences between groups were calculated by Student's *t* test for unpaired observations or for multiple comparisons by an ANOVA followed by a Newman Keuls test. $p < 0.05$ was considered statistically significant.

Docking studies

The suite of programs Maestro (Schrödinger, LCC[31]) was used for the docking studies. The Cartesian coordinates for the two proteins iNOS and nNOS were obtained from the available X-ray structures with PDB id. 3NW2 and 1QW6, respectively (Protein Data Bank). These receptors were prepared using the Protein Preparation Wizard module[32] implemented in Maestro. The charge and coordination sphere of the Fe(II) ion within the heme prosthetic group were manually redefined before replacing and minimizing all hydrogens in the receptor. LigPrep[33] program was used to generate the 3D structures of a set of conformers of **4a–q**. Geometries of this set were optimized using the MacroModel module. A rigid docking protocol was followed using Glide program in Standard Precision (SP) mode and taking into account the best 5 solutions. Figures were built using PyMOL (v1.3, Schrödinger, LLC).[34]

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