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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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Abstract

The synthesis and biological evaluation of a new type of *N,N'*-disubstituted thiourea and urea derivatives as inhibitors of both neuronal and inducible nitric oxide synthase (nNOS and 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 better the neuronal NOS isoform 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 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) involved in immune response, and neuronal NOS (nNOS) 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 NOSs structures reveals a huge similarity especially in their substrate binding sites.[17] This fact represents a real challenge in designing selective NOS inhibitors.

† The authors declare no competing interests.

In previous efforts to find selective and potent i/nNOS inhibitors, our research group has synthesized and published several families of compounds. Figure 1 shows some of them with analogous structures: the kynurenine **1**,[18] kynurenamine **2**,[19] and kynurenamine-urea and thiourea **3**[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. Also both, the aromatic ring and the amine group, interact with the enzyme through π-cation interaction and hydrogen bonds, respectively. However, any clear role was observed for the carbonyl group. Therefore we decided to act at this point, changing this hydrogen bond acceptor residue by 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, waiting increased interaction with the enzyme and improved 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.

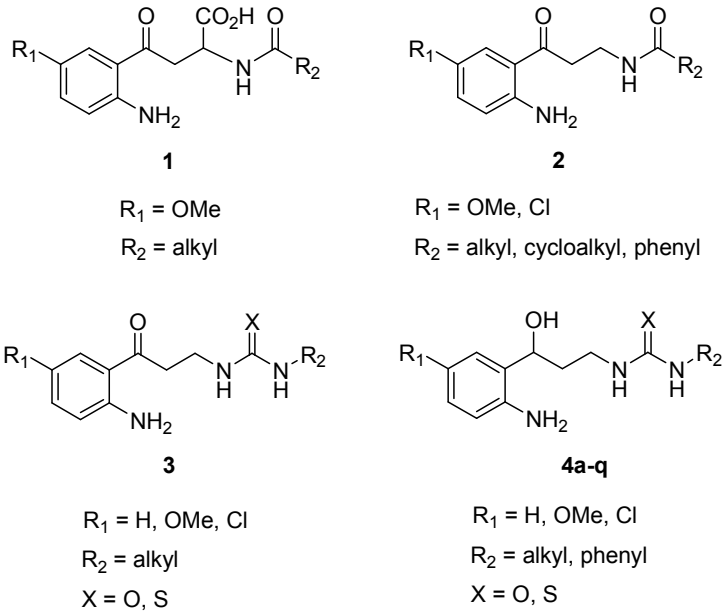

Figure 1. Some kynurenamine derivatives as NOS inhibitors synthesized by our research group.

Results and discussion

Chemistry

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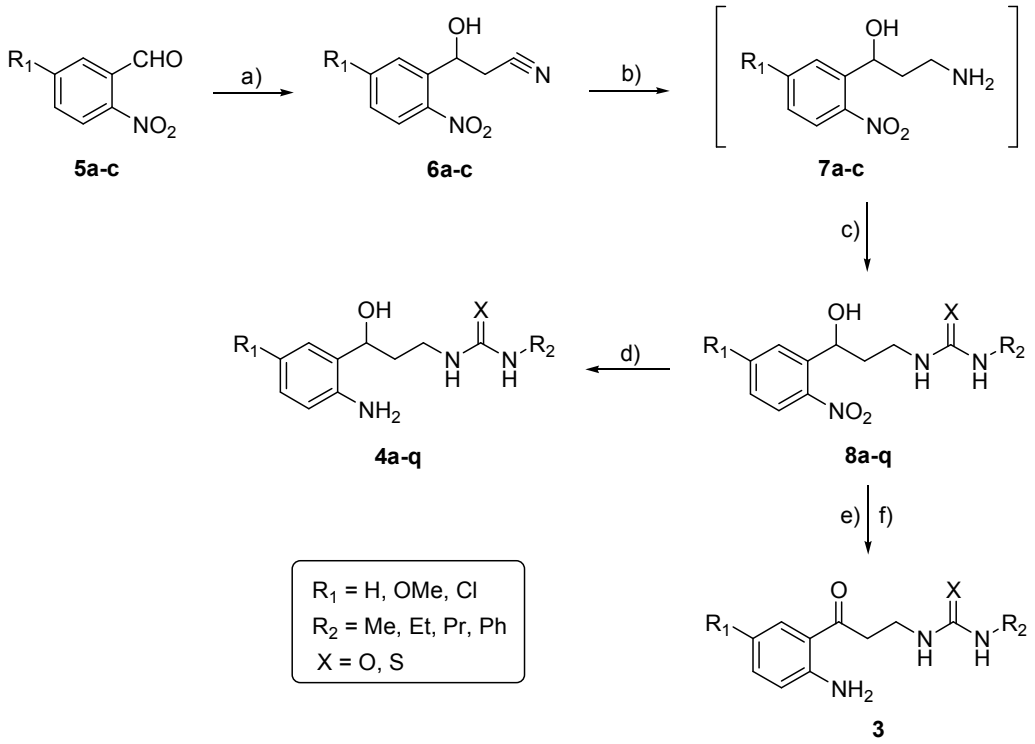
The general synthetic pathway of all the final compounds **4a-q** is represented in Scheme 1. 5-Methoxy-2-nitrobenzaldehyde **5b** (synthesized from 5-hydroxy-2-nitrobenzaldehyde by reaction with MeI in the presence of K_2CO_3/THF),[21] the commercially available 2-nitrobenzaldehyde **5a** and 5-chloro-2-nitrobenzaldehyde **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 microwave (MW) technique, gave the intermediates **8a-q** with good yields (70-85%). The application of the MW technique shortened the reaction time from 18 h[20,24] to 20 min, 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 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.



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

Results and discussion

iNOS and nNOS inhibition

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Compounds **4a-q** were evaluated *in vitro* as inhibitors of iNOS and nNOS using recombinant isoenzymes. The assays were made at 1mM 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 reference).

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 by triplicate using recombinant iNOS and nNOS enzymes. [b]**3a-b** were used as reference.[20]

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 methyl substituent in R_2 produce higher inhibition for iNOS as **4g** and for nNOS such as **4a**, **4g** and **4l**. It is noteworthy that compound **4m** shows good inhibition having a 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**.

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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 intermediates 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 two first families carry a methoxy group in R_1 and a methyl in R_2 (urea **4g** and kynurenamine **2a**, respectively), while the urea-kynurenamine **3b** which has a chlorine in R_1 and an ethyl in 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 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 by triplicate using recombinant iNOS and nNOS enzymes. [b]See Ref.[19]

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

Table 3. IC_{50} values (mM) for the inhibition of nNOS and iNOS activity by the 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]Data were obtained by measuring percentage of inhibition with at least five concentrations of inhibitor.

eNOS inhibition

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We carried out the eNOS inhibitory activity of compound **4g**, using HUVECs incubated with 100 µmol/L of **4g** or vehicle 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 (Figure 2a). Moreover, the endothelium-dependent relaxation to acetylcholine was not affected by **4g** (Figure 2b), confirming the absence of eNOS inhibition of this compound. However, L-NAME suppressed this NO-dependent relaxant response.

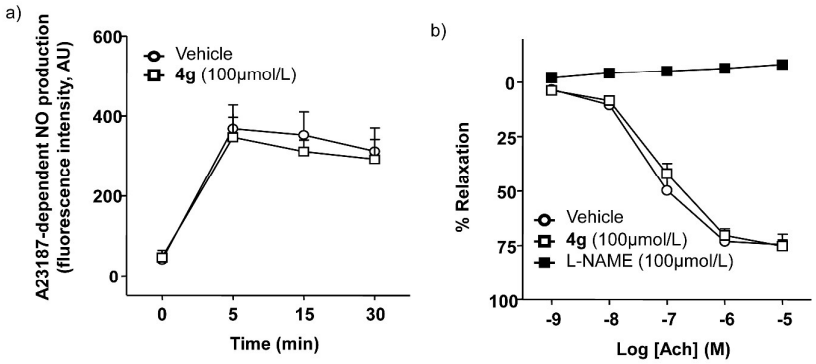

Figure 2. Effects of **4g** on eNOS activity. a) A23187-dependent NO production in HUVECs incubated with DMSO or **4g** (100 µmol/L) for 30 min (n = 12) (c). b) Acetylcholine-evoked relaxation in aortic rings with endothelium contracted with 1 µM noradrenaline in the presence of DMSO, **4g** (100 µmol/L),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

Cell viability

We carried out the cell viability activity of compound **4g**, using HUVECs. We found that this compound has little toxicity, since only at concentrations >1mM weekly (approximately 20 %) reduced cells viability (Figure 3).

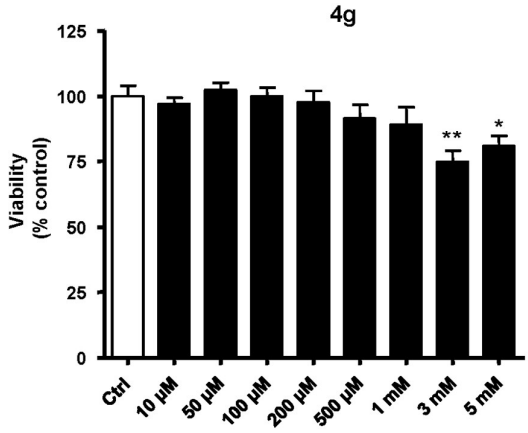

Figure 3. Study of cell viability of **4g** using MTT assay. *p<0.05 vs control (ctrl); **p<0.01 vs ctrl.

Docking studies

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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.

Figure 4 illustrates the main poses obtained for these compounds in the nNOS (PDB id: 1QW6)[26] binding site. All the ligands interact with the Glu592 and a carboxylate moiety of the heme group through the hydroxyl group and both thiourea/urea nitrogens, respectively. Compound **4a** (Figure 4a) shows additional interactions of the amino group, forming two more hydrogen bonds, one with Trp587 and the other with Glu592. Moreover **4a** have 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** (Figure 4b) shows no additional hydrogen bonds in the binding site. Despite it presents a similar orientation to **4a**, being longer adopts a more constricted conformation which results more unstable and reduces the π-cation interaction, due to the inclination of the aromatic ring respect to the heme group. This fact can explain the loss of activity of some compounds.

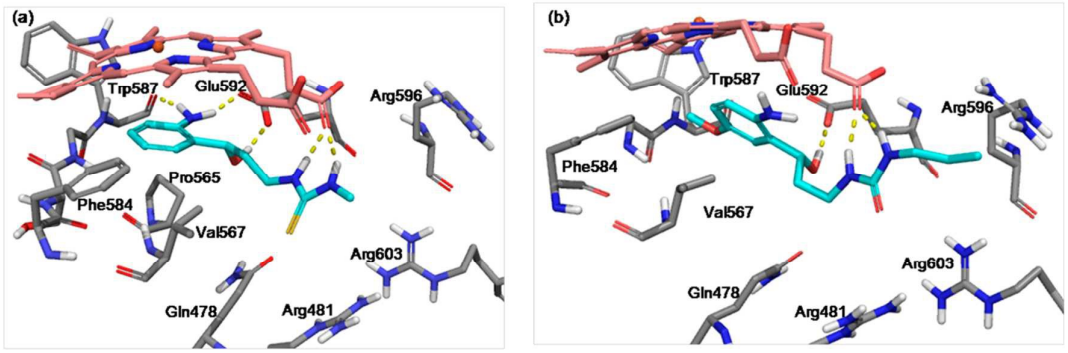

Figure 4. Detailed view of the main poses obtained for compound **4a** (a) and **4j** (b) in the nNOS binding site. Dotted lines indicate hydrogen bond interactions between the ligand and the residues of the enzyme.

Figure 5 shows the most common poses obtained for hydroxypropyl derivatives in the iNOS binding site. Compound **4g** (Figure 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 arginines pocket.

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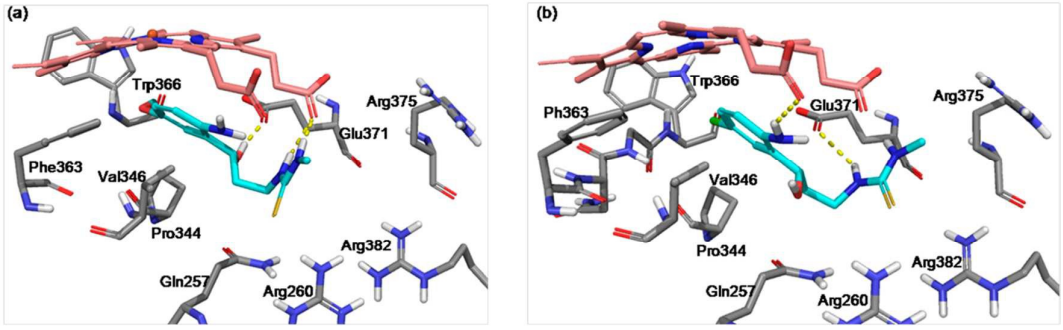

Figure 5. Detailed view of the main poses obtained for compound **4g** (a) and **4l** (b) in the iNOS binding site. Dotted lines indicate hydrogen bond interactions between the ligand and the residues of the enzyme.

Other compounds such as **4l** (Figure 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.

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 easier, 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 better the neuronal NOS isoform than the inducible one, exhibiting the thioureas 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 the hypertension. Moreover cell viability test demonstrated the absence of cell toxicity in compound **4g** at 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 citotoxicity and adverse vascular effect, useful in neurodegenerative disorders such as Parkinson disease, where both isoforms are involved.

Experimental section

Chemistry

Melting points were determined using a capillary melting point apparatus and are uncorrected. Analytical thin layer chromatography was performed using Merck Kieselgel 60 F254 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

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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 on a Waters LCT Premier Mass Spectrometer. Elemental analyses were within ±0.4% of the theoretical values. Small scale microwave-assisted synthesis was carried out in an Initiator 2.0 single-mode microwave instrument producing controlled irradiation at 2.450 GHz (Biotage AB, Uppsala). Reaction time refers to hold time at 90ºC, not to total irradiation time. The temperature was measured with 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-substitutedphenyl)propanenitrile, 6a-c.

BuLi (1.6 M/hexane, 20.3 mL) was added to dry THF (37.5 mL) cooled to -78 ºC 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 ºC 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 ºC 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 x 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 ºC; ^{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 ºC; ^{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-substitutedphenyl)propan-1-ol, 7a-c.

3-Hydroxy-3-(2-nitro-5-substitutedphenyl)propanenitrile derivatives **6a-c** (1.7 mmol) were treated dropwise with a solution of BH_3 (12.3 mL) in THF at 0 ºC under argon. The mixture was stirred during 4h at RT. Afterward the reaction mixture was cooled to 0 ºC 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 x 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.

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Thioisocianate or isocianate (0.9 mmol) was added, under argon, to a solution of 3-amino-1-(2-nitro-5-substitutedphenyl)propan-1-ol derivatives **7a-c** (0.6 mmol) in dry CH_2Cl_2. The reaction mixture was irradiated under microwave conditions at 90 ºC 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 ºC; ^{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

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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 ºC; ^{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.

† The authors declare no competing interests.

***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: 53ºC; ^{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-substitutedphenyl)-3-hydroxypropyl]-thioureas and ureas, 4a-q.

A solution of each nitro precursor **8a-q** (0.5 mmol) in MeOH (10 mL) was passed through the Flow-hydrogenator under the following conditions: 60ºC, 60 bar, 0.2 mL/min flow rate and 10% Pd/C as 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 ºC; ^{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

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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 ºC; ^{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 ºC; ^{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: 95ºC; ^{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) ν: 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 ºC; ^{1}H 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 ºC; ^{1}H 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'*-ehylurea, 4i.** Yellow solid (0.39 mmol, 78%); mp: 128 - 129 ºC; ^{1}H 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,

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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 ºC; ^{1}H 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) ν: 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 ºC; ^{1}H 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) ν: 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 ºC; ^{1}H 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 ºC; ^{1}H 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 ºC; ^{1}H 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) ν: 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.

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***N*-[3-(2-Amino-5-chlorophenyl)-3-hydroxypropyl]-*N'*-ethylurea, 4o.** Yellow solid (0.45 mmol, 91%); mp: 48 ºC; ^{1}H 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) ν: 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 ºC; ^{1}H 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 ºC; ^{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 chromic anhydride and 2.3 mL H_2SO_4 dissolved to 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 activities 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-50W (50 x 8-200), FAD, NADPH and 5,6,7,8-tetrahydro-L-

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biopterin dihydrocloride (H_4-biopterin), tris-(hydroxymethyl)-aminometane (Tris-HCl) and calcium chloride were obtained from Sigma-Aldrich Química (Spain). L-[^{3}H]-arginine (47.4 Ci/mmol) 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 BSA, 0.1 mM $CaCl_2$, 10 µM L-arginine, 10 µg/mL 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 derivative **4a-q** in ethanol (10%) to give a final concentration of 1 mM. The tubes were vortex and incubated at 37 ºC 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 L-citrulline, pH 5.5. The reaction mixture was decanted into a 2 mL column packet with Dowex-50W 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 (/mg of protein/min).

eNOS inhibition

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

The investigation have been conducted according to the principles expressed in the Declaration of Helsinki, and have been approved by the Ethics Committee for the Human Investigation, at 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 + Amphotericin B 2 mmol/L + Glutamine 2 mmol/L + HEPES 10 mmol/L + endothelial cell growth supplement 30 µg/mL + Heparin 100 mg/mL) 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 during 30 min in the presence of the **4g** at the concentration of 100 µmol/L. After this period, cells were washed with PBS and then were pre-incubated with L-arginine (100 µmol/L in PBS, 5min, 37ºC). Subsequently, DAF-2 (0.1 µmol/L) was incubated for 2 min and then the calcium ionophore calimycin (A23187, 1 µmol/L) was added for 30 min and cells were incubated in the dark at 37ºC. Then the fluorescence (arbitrary units) was measured using a spectrofluorimeter (Fluorostart, BMG Labtechnologies, Offenburg, Germany). The auto-fluorescence was subtracted from each value. In some experiments, N^G-nitro-L-arginine methyl ester (L-NAME, 100 µmol/L) 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

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the Ethics Committee for the welfare of experimental animals, at 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 rings were then mounted in organ chambers filled with Krebs solution (composition in mmol/L: 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 recorded in a recording and analysis system (MacLab AD Instruments), as described previously.[30] After equilibration, aortic rings with a functional endothelium were incubated with vehicle (DMSO), **4g** (100 µmol/L), or L-NAME (100 µmol/L) for 30 min, and contracted with phenylephrine (1µmol/L). Once a plateau contraction was reached, a concentration-response curve was constructed by cumulative addition of acetylcholine. Results are expressed as percentage of phenylephrine-evoked contraction. 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 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 MTT in PBS was added to the cells and further incubated at 37 °C. After washing, 100 µL of DMSO were added in each well and absorbance at 570 nm was measured on a multi-well Plate Reader (Model 680XR, BIO-RAD) with subtraction of blank value at 630 nm, and compared with control, untreated cells.

Statistical analysis

Data are expressed as the mean ± SEM. Statistically significant differences between groups were calculated by Students´ 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. 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 with Glide program using a 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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Keywords

N,*N'*-disubstituted thiourea, *N*,*N'*-disubstituted urea, Inducible nitric oxide synthase (iNOS), Neuronal nitric oxide synthase (nNOS), Oxide nitric synthase (NOS) inhibitors.

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† The authors declare no competing interests.

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N,*N'*-disubstituted thioureas and ureas as nNOS and iNOS inhibitors were synthesized. Thiourea 4g was the best inhibitor without eNOS inhibition.