Melatonin Synthetic Analogs as Nitric Oxide Synthase Inhibitors

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Abstract: Nitric oxide (NO), which is produced by oxidation of *L*-arginine to *L*-citrulline in a process catalyzed by different isoforms of nitric oxide synthase (NOS), exhibits diverse roles in several physiological processes, including neurotransmission, blood pressure regulation and immunological defense mechanisms. On the other hand, an overproduction of NO is related with several disorders as Alzheimer's disease, Huntington´s disease and the amyotrophic lateral sclerosis.

Taking melatonin as a model, our research group has designed and synthesized several families of compounds that act as NOS inhibitors, and their effects on the excitability of N-methyl-D-aspartate (NMDA)-dependent neurons in rat striatum, and on the activity on both nNOS and iNOS were evaluated. Structural comparison between the three most representative families of compounds (kynurenines, kynurenamines and 4,5-dihydro-1*H*-pyrazole derivatives) allows the establishment of structure-activity relationships for the inhibition of nNOS, and a pharmacophore model that fulfills all of the observed SARs were developed. This model could serve as a template for the design of other potential nNOS inhibitors.

The last family of compounds, pyrrole derivatives, shows moderate *in vitro* NOS inhibition, but some of these compounds show good iNOS/nNOS selectivity. Two of these compounds, 5-(2-aminophenyl)-1*H*-pyrrole-2-carboxylic acid methylamide and cyclopentylamide, have been tested as regulators of the *in vivo* nNOS and iNOS activity. Both compounds prevented the increment of the inducible NOS activity in both cytosol (iNOS) and mitochondria (i-mtNOS) observed in a MPTP model of Parkinson´s disease.

Keywords*:* Kynurenamine, kynurenine, melatonin, nitric oxide, nitric oxide synthase, pyrazole, pyrrole.

INTRODUCTION

Nitric oxide (NO), an ubiquitous biological messenger involved in a variety of physiological processes, acts as a signal transducer and also exerts a variety of regulatory and citostatic functions [1]. Nitric oxide synthase (NOS) catalyzes the biosynthesis of NO using *L*-arginine (*L*-Arg) as substrate, which is firstly converted in N^{ω}-hydroxy-*L*-arginine, and further oxidized to *L*-citrulline and NO [2].

Three isoforms of NOS have been identified: neuronal NOS (nNOS) [3], endothelial NOS (eNOS) [4], and inducible NOS (iNOS) [5]. These isoforms share approximately 50% sequence identity and have identical overall architecture. Native NOS is a homodimeric enzyme. Each subunit contains a catalytic *N*-terminal oxygenase domain, a *C*-terminal electron-supplying reductase domain, and a calmodulin (CaM)-binding motif linking the two functional domains [6, 7]. The oxygenase domain binds heme, tetrahydrobiopterin (H_4B), and *L*-Arg. The reductase domain binds flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and nicotinamide adenine dinucleotide phosphate (NADPH). The heme domain provides the site for *L*-Arg oxidation, while the FAD and FMN of the reductase domain transfer the electron from NADPH to the heme [8]. Dimerization and H_4B binding are also essential for the catalytic activity [9, 10].

Although similar in their catalytic mechanism, the NOS isoforms are distinguished by their regulation and localization. nNOS and eNOS are constitutively expressed in neurons and endothelial cells, respectively, among other cell types. Their activities are regulated at the posttranslational level, and NO production is completely dependent on Ca^{+2}/CaM binding [11]. However, iNOS is only expressed after induction and is located in macrophages. Its activity is controlled at the transcription level and, once expressed, that isoform will produce NO at a high rate. In addition, iNOS is not regulated by CaM, since CaM is bound with high affinity and functions as a permanent subunit [12]. Every isoform has different physiological functions: blood-vessel dilation (eNOS) [11], neuronal signal transmission (nNOS) [13], immune response such as cytotoxicity against pathogens and tumors (iNOS) [14]. Overproduction of NO has been a factor in numerous disease states. NO overproduction by nNOS has been implicated in strokes [15], migraine headaches [16], and Alzheimer´s disease [17] and with tolerance to and dependence on morphine [18]. Studies with nNOS knockout mice have indicated that NO produced by nNOS during neuronal injury is associated with glutamate toxicity in the brain [19].

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1875-5607/12 $58.00+.00

An overproduction of NO from the inducible isoform (iNOS) has been associated with tissue damage, inflammation, rheumatoid arthritis, and the onset of colitis [20, 21]. Blocking the localized excess production of NO has been identified as a potential means of treating these diseases. However, because of the critical role that eNOS-generated NO plays in vascular regulation, the potential pharmaceutical utility of NOS inhibitors is restricted to the selective inhibition of the neuronal or inducible isoforms [22].

On the other hand, a mitochondrial-localized NOS isoform situated in the internal membrane of mitochondria (mtNOS) was discovered [23-25]. Even there was a controversy among the type(s) of mtNOS in terms of their classification as constitutive or inducible [26, 27], in a recent paper the existence of both constitutive and inducible mitochondrial NOS has been proven (c-mtNOS and i-mtNOS, respectively) [28, 29].

In the last years different selective nNOS and iNOS inhibitors have been described [30-33]. We now focus our interest on melatonin synthetic derivatives.

Melatonin **1** (Fig. **1**) is a hormone synthesized in many organs and tissues of the organisms including the pineal gland [34, 35], that shows inhibitory effects in the rat [36] and human [37, 38] central nervous system (CNS), being this the reason for its anticonvulsant, and neuroprotective properties [39]. Diverse experiments have suggested that melatonin attenuates glutamate-mediated responses in the rat striatum [40], and this inhibitory effect takes place through the inhibition of nNOS [41-43]. nNOS inhibition by melatonin has demonstrated to be dose-dependent and CaM-dependent [44]. Our research group has reported several nNOS inhibitors with a kynurenine structure **2** [44, 45]. A second type of inhibitors are constituted by the kynurenamine derivatives **3** [46], among them the main melatonin brain metabolite N^1-acetyl-5-methoxykynurenamine (AMK: **3**, R^1=OMe, R^2=Me). The third type less flexible nNOS inhibitors bear a 4,5-dihydro-1*H*-pyrazole moiety like in the general formula **4**, [47]. All these compounds inhibit nNOS in a dose-dependent manner and it has been found that AMK rather than melatonin is the active metabolite against nNOS in rat striatum [48]. Finally, 5-phenyl-1*H*-pyrrole-2-carboxamide **5** [49], show moderated both nNOS and iNOS inhibition, and in some cases an interesting iNOS selectivity is observed.

PREPARATION AND BIOLOGICAL ACTIVITY OF SYNTHETIC KYNURENINES (2a-r)

The kynurenine metabolic pathway of *L*-tryptophan in the brain turns out to be of great interest in neuroprotection because kynurenic and quinolinic acids, two intermediate metabolites of this pathway, modulate the N-methyl-D-aspartate (NMDA) receptor in a functionally opposite manner [50-52].

Kynurenic acid is a nonselective antagonist of the $glycine_B$ recognition site at the NMDA receptor and has neuroprotective properties, whereas quinolinic acid, a selective agonist of the NMDA binding site at the NMDA receptor, is a potent neurotoxin and has been involved in the pathogenesis of a variety of neurological disorders [53-56].

In looking for compounds with neuroprotective properties, our research group synthesized a series of kynurenine derivatives, and their effect on both NMDA-

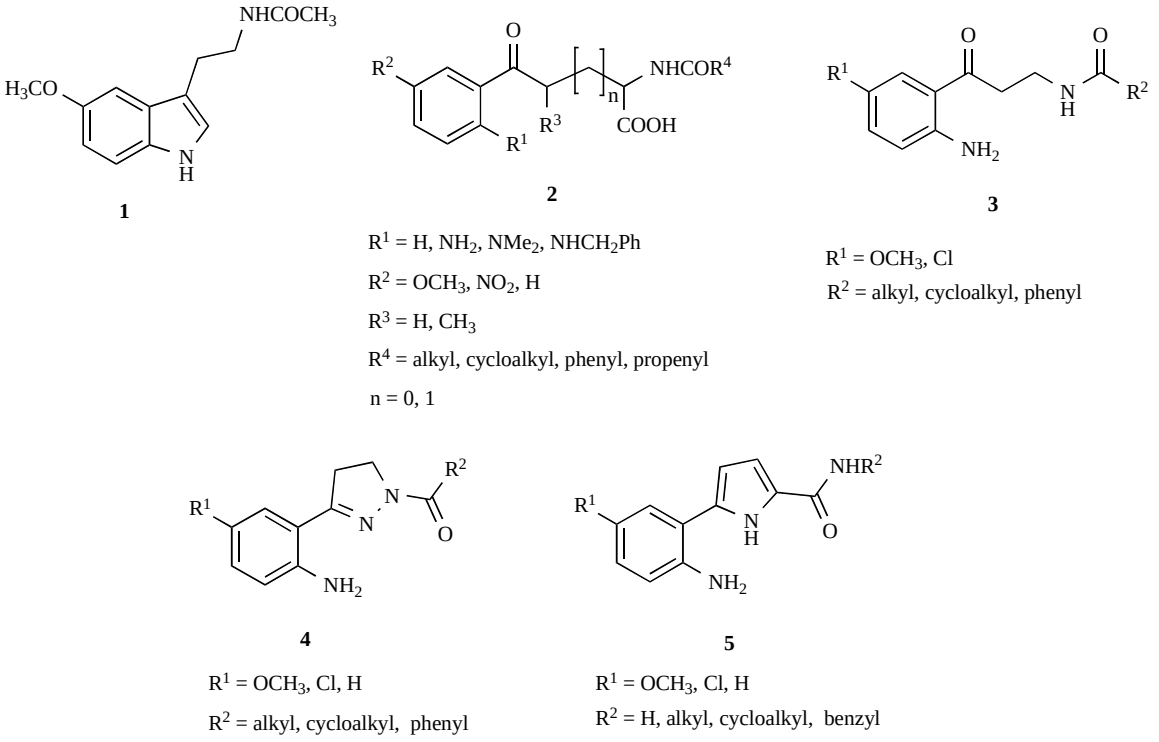
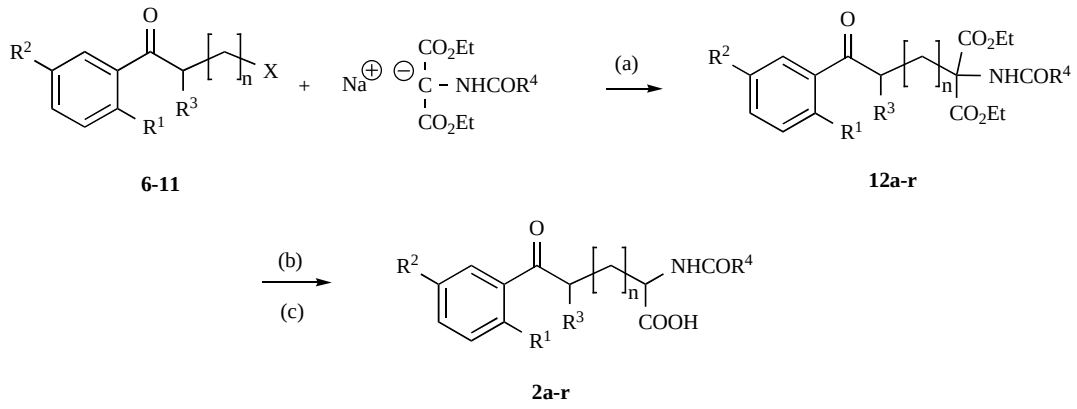


Fig. (1). Melatonin and NOS inhibitors described by our group of research.



Scheme 1. Schematic pathway followed for the synthesis of kynurenine derivatives **2a-r**. (a) DMF, 5h/RT; (b) NaOH; (c) AcOH/Δ.

Table 1. Structure of kynurenine Derivatives 2a-r

Comp.	R^1	R^2	R^3	R^4	n
2a	NH_2	OCH_3	H	CH_3	0
2b	NH_2	OCH_3	H	C_3H_7	0
2c	$N(CH_3)_2$	OCH_3	H	CH_3	0
2d	$NHCH_2Ph$	OCH_3	H	CH_3	0
2e	H	OCH_3	H	CH_3	0
2f	H	OCH_3	H	C_2H_5	0
2g	H	OCH_3	H	C_3H_7	0
2h	H	OCH_3	H	C_6H_5	0
2i	H	OCH_3	H	C_6H_{11}	0
2j	H	OCH_3	H	C_3H_5[a]	0
2k	H	OCH_3	H	C_3H_5[b]	0
2l	H	NO_2	H	CH_3	0
2m	H	NO_2	H	C_2H_5	0
2n	H	H	H	CH_3	0
2o	H	H	H	C_2H_5	0
2p	H	H	H	C_3H_7	0
2q	H	H	CH_3	C_3H_7	0
2r	H	H	H	C_3H_7	1

[a] 1-Propenyl. [b] Cyclopropyl.

dependent neuronal excitability in rat striatum and on the activity of striatal nNOS were evaluated [44, 45, 57].

Chemistry

Scheme (**1**) represents the general synthetic pathway followed in the preparation of final kynurenine derivatives **2a-r** included in Table (**1**) [41]. The key step is the condensation between the corresponding α - or β - haloacetophenone **6-11** with diethyl sodium acylamidomalonate in dimethylformamide (DMF) at room temperature to yield the amidomalonic acid derivatives **12a-r**. Basic hydrolysis of these intermediates, followed by acidification and heating, yields the final kynurenine analogues **2a-r**.

Biological Results

The effects of the kynurenine derivatives on the excitatory response of striatal neurons to sensorimotor cortex (SMCx) stimulation were evaluated (Fig. **2**) [45]. Electrical stimulation was applied continuously to the deep layers of

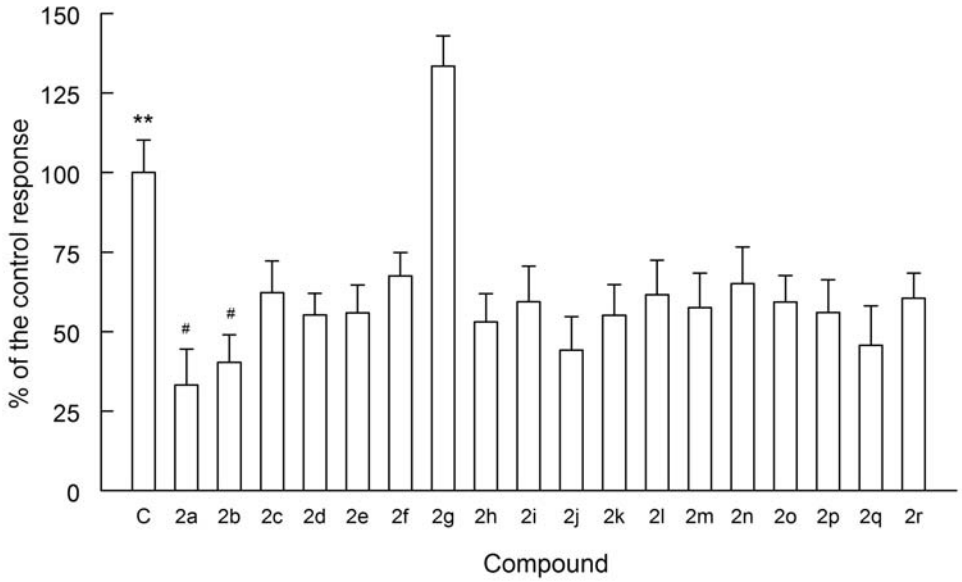


Fig. (2). Effects of microiontophoresis of kynurenine derivatives **2a-r** (1 mM) on the excitatory response of striatal neurons to SMCx stimulation. ***P* < 0,05 vs the others compounds.

motor cortex, and recordings were made from the dorso-medial portion of the striatum. Most of the neurons in this region were quiescent unless excited by cortical stimulation. Only those neurons showing an excitatory response to SMCx stimulation were selected for the study. When a striatal-responding neurone was identified, one kynurenine was microiontophoretically ejected onto this neurone to observe its effect on the excitatory response.

Taking kynurenine **2e** as a reference compounds, it can be observed only one compound **2g** shows an excitatory effect in the SMCx assay. The insertion of an amine group in the benzene ring of compounds **2e** and **2g** to give compounds **2a** and **2b,** respectively**,** produces a significant increment in the inhibitory effect. In fact only these compounds behave as strong inhibitors in the SMCx assay. Substitution of 2'-NH_2 group by a dimethylamine **2c** or benzylamine **2d** produces again a lack of inhibitory effect, giving place to compounds similar to the reference compound **2e**. All other compounds show similar effect than compound **2e**, indicating that substitution of 5'-methoxy group by a 5'-NO_2 or by H, the insertion of an additional methyl group in the side chain, or the lengthening the side chain in one carbon atom produce a similar inhibitory effect than the reference compound.

For further study on the specificity of these compounds on the response of striatal neurons, dose response experiments were carried out (Fig. **3**). In this case, a neuron was continuously stimulated, and different doses of representative compounds were added. It can be observed that for compounds **2a**, **2b**, and **2e** the excitatory response decreased in a dose-dependent manner, whereas for compound **2g** the excitatory response increased also in a dose-dependent manner. Compound **2a** showed significant higher inhibition than the other compounds tested.

The cortico-striatal pathway studied in our experimental paradigm was chosen because this is a glutamate-mediated circuit that mainly uses the NMDA receptor [40, 43, 57-59]. To test if the effects of the synthetic kynurenines described above involved NMDA receptors, a striatal neurone showing excitatory response to SMCx stimulation was chosen for each compound. Once the neurone was found, the electrical stimulation was stopped and the neurone was silenced due to

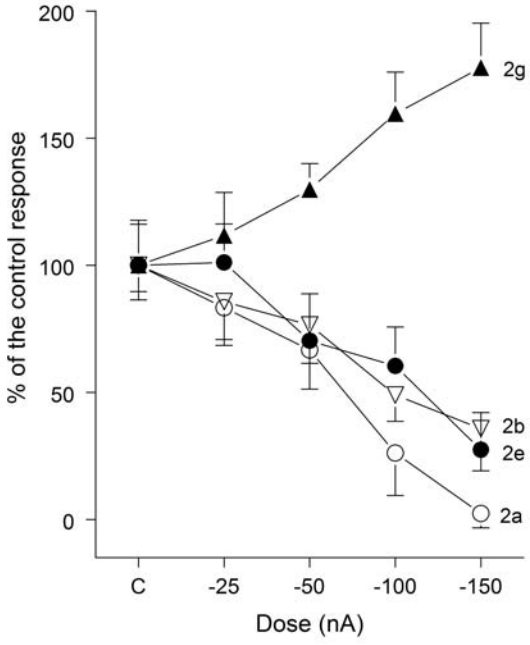


Fig. (3). Dose related effect of kynurenine derivatives iontophoretically applied to striatal neurones. Increasing the ejection current from -25 to -150 nA produces the striatal response to SMCx. Stimulation was attenuated when compounds **2a**, **2b**, and **2e** were iontophoretized. However, the application of compound **2g** increases the excitatory response**.** Data represent the percentage of change in firing rate with respect to control values (mean ± SEM of 10 neurones). **P < 0.05*and ***P < 0.01 vs* to control response.

Table 2. Dose-Dependent Effects of Six Kynurenine Derivatives on Rat Striatal nNOS Inhibition[a]

conc. (M)	comp. 2a	comp. 2b	comp. 2c	comp. 2d	comp. 2e	comp. 2g
0	100.00 ± 2.69	100.00 ± 2.36	100.00 ± 0.84	100.00 ± 1.12	100.00 ± 7.71	100.00 ± 0.56
10^{-11}	21.70 ± 1.65**	14.20 ± 0.67*	-0.18 ± 0.01	-1.25 ± 0.01	7.35 ± 0.31	0.13 ± 0.01
10^{-9}	26.62 ± 0.96**	17.87 ± 0.44**	0.14 ± 0.01	-0.30 ± 0.02	-0.07 ± 0.01	-0.56 ± 0.03
10^{-7}	34.20 ± 2.35**	22.73 ± 1.39**	0.37 ± 0.02	-0.48 ± 0.03	12.76 ± 0.38	-0.91 ± 0.03
10^{-5}	36.00 ± 2.58**	32.64 ± 2.94**	-0.95 ± 0.05	1.84 ± 0.11	-2.90 ± 0.02	0.26 ± 0.01
10^{-3}	68.65 ± 2.72**	45.05 ± 7.02**	1.01 ± 0.06	-0.31 ± 0.02	4.28 ± 0.10	-5.32 ± 0.26

[a] Data represent the mean ± SEM of the percentage of nNOS inhibition of each drug as compared with untreated samples. Each value is the mean of three experiments performed by triplicate in homogenates of four rat striata in each one. *P < 0,05 vs control; **P < 0,01 vs control.

its lack of autoexcitation. Then, NMDA was iontophoretized to induce the excitatory response in this neurone, and, during the NMDA ejection, a series of kynurenines were also iontophoretized. Compounds **2a** and **2b** display stronger inhibitory effects (longer duration of inhibition) than compound **2e**. When the 2'-NH_2 group was blocked with a dimethyl or a benzyl group, the resulting compounds **2c** and **2d**, respectively, behaved as **2e**, thus losing part of their inhibitory effects.

These results confirm that the assayed kynurenines act on the NMDA receptor in a structure-related manner. Two main possibilities should be kept in mind regarding their mechanism of action: (1) the kynurenines may bind to the NMDA receptor itself in a manner similar to kynurenic acid and some of its derivatives [60-62], or (2) they can modulate nNOS activity, and thus, the intracellular production of NO, the second messenger of the NMDA receptor. Anyway, activation of the NMDA receptor increases Ca^{+2} influx into the cell, resulting in an increase of the nNOS activity by a calcium-calmodulin (CaCaM) complex dependent mechanism [63]. Consequently, intracellular NO concentration increases and diffuses out of the cell, increasing presynaptic glutamate output that further stimulates the NMDA receptor. Thus, the effects of **2a-e** and **2g** on neural NOS activity was assessed, and the results of this assay are summarized in Table (**2**). It can be seen that 10 pM of **2a** or **2b** were able to significantly reduce nNOS activity to 78.3 and 85.8%, respectively. At the maximal concentration tested (1 mM), nNOS was reduced by **2a** and **2b** to 31.5 and 54.9%, respectively. These data suggest that **2a** is more potent than **2b** in the inhibition of striatal nNOS activity.

Interestingly, nNOS was unaffected by the other tested compounds at concentrations ranging from 10 pM to 1 mM. Since compounds **2a** and **2b** share the same structure as **2e** and **2g** except for the presence of an amino group at R^1, these results suggested a relation between this chemical group and the biological activity of these compounds. This suggestion was further supported because nNOS activity was not affected by **2c** and **2d**, two compounds obtained from **2a** by blocking the amino group at R_1 with a dimethyl or a benzyl group, respectively. Finally, compound **2g**, which increases neuronal excitability, did not modify striatal nNOS activity. Compared with melatonin, which has an IC_{50} for nNOS activity above 1 mM [43], we can observe that **2a** is at least 25 times more potent against nNOS. Moreover, the electrophysiological and biochemical effects correlated very well. As can be seen in Table (**3**), the kynurenine with the smaller IC_{50} value for their electrophysiological effects has the stronger inhibitory effect on nNOS activity.

Table 3. IC_{50} Values for Kynurenine Derivatives Obtained from Electrophysiological (nA) and nNOS Activity (M) Experiments

Comp.	IC_{50} Electrophysiological Activity (nA)	IC_{50} nNOS Activity (M)
2a	-69	41×10^{-6}
2b	-99	$> 10^{-3}$
2c	-104	-
2d	-106	-
2e	-111	-
2g	-83	-

PREPARATION AND BIOLOGICAL ACTIVITY OF SYNTHETIC KYNURENAMINES (3a-l)

Kynureamines **3a-l** constituted the second family of NOS inhibitors prepared by our research group [46], since they are quite similar to kynurenines. In fact, the elimination of the COOH group in the active kynurenines yields similar kynurenamines.

In the brain, melatonin is metabolized by the action of the indoleamine 2,3-dioxygenase to afford the N^1-acetyl-N^2-formyl-5-methoxykynurenamine (AFMK). This metabolite is further transformed into AMK, **3a**, and this is one of the more important metabolic pathways of melatonin in mammalians [64, 65]. Furthermore, it has also been proposed that the action of melatonin can be due to one of these metabolites [66]. On the other hand, Inhibition of kynurenine 3-hydroxylase (KYN3OH) has been proposed as a potentially useful strategy for neuroprotection [67, 68], because KYN3OH inhibitors decrease the brain concentration of the neurotoxic quinolinic acid and 3-hydroxykynurenine while increasing the biosynthesis of the

neuroprotective kynurenic acid. Due to these reasons, kynurenamines **3a-l** were synthesized and analyzed as both KYN3OH and NOS inhibitors [42].

Chemistry

Scheme (**2**) represents the general synthetic pathway for all final kynurenamines included in Table (**4**) [42]. 5-Methoxy- and 5-chloro-2-nitrophenyl vinyl ketones **13a** and **13b** were reacted with phthalimide in the presence of NaMeO, and their ketone group was protected by reaction with ethylene glycol and *p*-toluenesulfonic acid to yield the dioxolane derivatives **14a-b** . The reaction of **14a-b** with hydrazine opens the phthalimide moiety, and further acidification (HCl) of the reaction mixture allows the hydrolysis of the dioxolane group to yields the corresponding β -aminoketones **15a-b**, which were not isolated. Acylation in situ of **15a-b** by reaction with acetic anhydride or with the corresponding acyl chloride gives rise to the nitroderivatives **16a-l**. Finally, the reduction of the 2'-NO_2 groups in **16a-l** allows the preparation of the corresponding kynurenamine derivative **3a-l**. This reduction was accomplished by catalytic hydrogenation (H_2, Pd/C) in compounds **3a-i** and by reaction with $Fe/FeSO_4$ in compounds **3j-l** to avoid dechlorination.

Biological Results

Table (**4**) shows the nNOS inhibition in the presence of 1 mM concentration of each kynurenamine **3a-l**. In general, most compounds show good range of inhibition. Regarding the influence of R^2 on the activity, it can be observed that an increment in the volume of R^2 decreases the inhibitory activity. Thus, the change of the Me group by Et, Pr or Bu decreases steadily the percentage of inhibition. The insertion of a cyclopropyl or a phenyl group in R^2 is also detrimental for the activity. In relation to R^1, it can be observed that compounds with R^1 = OMe are about 2 times more active than the corresponding ones with R^1 = Cl, indicating that an electron-withdrawing substituent is detrimental for the activity.

The comparison of the activity of kynurenines **2a-b** with their corresponding kynurenamines derivatives **3a** and **3c** indicates a slightly higher inhibition activity for compounds type **2**. Nevertheless, the differences in activity are not high enough to consider that kynurenines are more potent as nNOS inhibitors.

Table (**4**) also shows the activity of compounds type **3** against KYN3OH, expressed as the percentage of activity of this enzyme in the presence of each final compounds. It can be observed that neither kynurenines **2** nor kynurenamines **3** showed a significant effect on this enzyme, indicating that their potential neuroprotective properties could be due again to the nNOS inhibition.

Among kynurenamines, the melatonin metabolite AMK **3a** is the most potent one, showing a similar inhibition percentage than kynurenine **2a**.

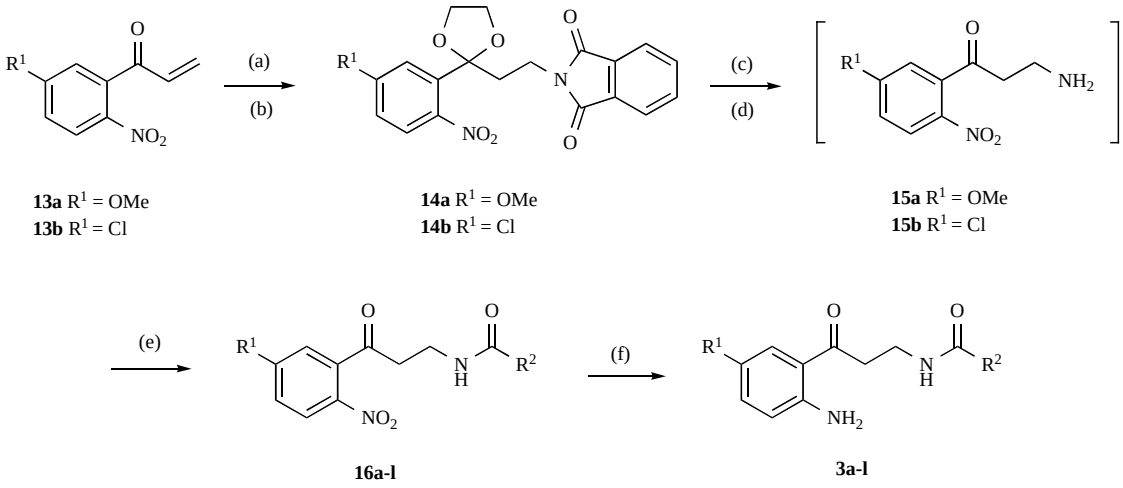
DIGGING INTO THE NOS INHIBITION MECHANISM BY KYNURENINES AND KYNURENAMINES

Activation of the NMDA receptor by glutamate causes a Ca^{2+} influx into the cells and results in the formation of NO from L-Arg [69]. Involved enzymes in this pathway are CaM and nNOS [69].

NO modulates several processes in the CNS such as pain perception, long term potentiation and memory, and cerebral blood flow [70], and has been specifically implicated in the glutamate-dependent excitotoxicity and neuronal death [71].

Melatonin (**1**) exerts neuroprotective properties reflecting both antioxidant [72-74] and inhibitory effects on the CNS [75-77]. Electrophysiological experiments have demonstrated that the excitatory response of striatal neurons to SMCx stimulation in rats is attenuated by the iontophoretic ejection of melatonin [40, 78]. Similarly, some synthetic kynurenines affect the excitatory response of striatal neurons in a structure-related manner [43, 44]. This excitatory response is mainly mediated by glutamate acting on the NMDA receptors [40, 58].

Melatonin **1** and kynurenines **2a** and **2b** inhibit nNOS activity in a dose dependent manner [43, 44], being the calculated IC_{50} values for each compound as follows: **1**, > 1 mM; **2a**, 41 μM; and **2b**, > 1 mM.



13a R^1 = OMe
13b R^1 = Cl

14a R^1 = OMe
14b R^1 = Cl

15a R^1 = OMe
15b R^1 = Cl

16a-l

3a-l

Scheme 2. Synthetic pathway followed in the preparation of kynurenamine derivatives **3a-l**. (a) Phthalimide, NaOMe; (b) $(CH_2OH)_2$, H^+; (c) NH_2NH_2/EtOH; (d) HCl (aq); (e) Ac_2O or R_2COCl;(f) H_2,Pd/C or $Fe/FeSO_4$.

Table 4. Structure and Biological Activities of Kynurenamines 3a-l as nNOS (% Inhibition) and KYN3OH (% Activity) Inhibitors. Biological Activities of Kynurenines 2a and 2b are Also Included for Comparison

Compound	R^1	R^2	% nNOS Inhibition[a]	% KYN3OH Activity[b]
2a	OCH_3	Me	68.49 ± 9.92	100.52 ± 9.7
2b	OCH_3	Pr	45.05 ± 8.56	88.72 ± 10.2
3a	OCH_3	Me	65.36 ± 5.60	101.52
3b	OCH_3	Et	50.87 ± 4.36	99.37
3c	OCH_3	Pr	42.82 ± 4.00	c
3d	OCH_3	Bu	39.65 ± 2.59	97.1
3e	OCH_3	*c*-C_3H_5	40.41 ± 4.27	99.89
3f	OCH_3	*c*-C_4H_7	33.73 ± 2.98	92.22
3g	OCH_3	*c*-C_5H_9	45.04 ± 4.45	96.34
3h	OCH_3	*c*-C_6H_{11}	48.24 ± 4.90	99.64
3i	OCH_3	C_6H_5	46.46 ± 4.46	97.23
3j	Cl	Me	31.69 ± 1.13	c
3k	Cl	*c*-C_3H_5	23.28 ± 3.54	c
3l	Cl	C_6H_5	21.71 ± 2.60	c

[a] Data represent the mean ± SEM of the percentage of nNOS inhibition produced by 1 mM concentration of each compound. [b] Data represent the mean ± SEM of the KYN3OH activity in the presence of 1mM concentration of each compound Each value is the mean of three experiments performed by triplicate in homogenates of four rat striata in each one. [c] Not tested.

On the other hand, it was also proved that melatonin binds CaM with high affinity [79] and the binding is reversible, saturable, and Ca^{2+}-dependent. It was suggested that the inhibitory effect of melatonin on nNOS activity might be produced by removing free cytosolic CaM through a CaM-melatonin interaction [80].

To investigate the mechanism of action of melatonin **1** and synthetic kynurenines **2a** and **2b** on nNOS activity, kinetics studies of the enzyme-substrate reaction were tackled [44] using homogenates of rat striatum and commercially available purified rat brain nNOS. Besides, experiments with urea-polyacrylamide gel electrophoresis (PAGE) were carried out to further assess the possible interaction of these compounds with CaM.

Rat striatal homogenates were incubated with each drug (1 mM) in the presence of increasing concentrations of *L*-Arg (0–10 μM) (Fig. **4**). Striatal nNOS activity was saturable and proportional to the substrate concentration (control). However, the activity of the enzyme was significantly decreased in the presence of 1 mM of melatonin **1** or kynurenines **2a** or **2b**.

Lineweaver-Burk double reciprocal analysis of the data demonstrate that, although K_m values of control, **1**, **2a**, and **2b** were similar, the V_{max} values for these compounds were lower than the control. These results suggest that melatonin **1** and kynurenines **2a** and **2b** behave as noncompetitive inhibitors of nNOS activity.

The existence of a possible interaction between nNOS cofactors and melatonin **1** or kynurenines **2a** and **2b** was also studied using commercially available purified rat nNOS. The effect of melatonin and kynurenines on nNOS activity was investigated in an incubation medium containing 0.0125 U of purified nNOS, 17.5 μM $CaCl_2$, 10 μg/ml CaM, 10 μM FAD, and 30 μM H_4B. In these studies, increasing amounts of CaM, FAD or H_4B were also added to the incubation medium. In the absence of added CaM, these compounds inhibit nNOS in a dose-dependent manner (Fig. **5**). At a 10 μg/ml of CaM, melatonin **1** and kynurenine **2b** were unable to inhibit the nNOS activity, while the inhibition percentage of kynurenine **2a** was significantly decreased. On the other hand, after fixing the CaM concentration at 0.1 μg/ml, different concentrations of FAD (0.1 μM to 10 μM) or H_4B (0.3 μM to 30 μM) in the incubation medium did not modify the enzyme inhibition by melatonin **1** nor by kynurenines **2a** and **2b** on purified nNOS activity (data not shown).

The possible interaction between melatonin **1** or kynurenines **2a** and **2b** and CaM was also studied by electrophoresis, and urea-PAGE of CaM assays were performed. The electrophoretic migration mobility of CaM was determined in the absence or presence of 1 mM **1** (Fig. **6A**), 1 mM **2a** (Fig. **6B**), and 1 mM of **2b** (Fig. **6C**). The presence of these inhibitors produced a similar CaM mobility into the gel that was faster than the CaM mobility obtained in control gels in which these compounds were absent. In the presence of ethylene glycol tetraacetic acid (EGTA) (a Ca^{2+} complexing agent), the migration of CaM was slower than the observed in the absence of this chelating agent, and in these conditions neither melatonin **1**, nor kynurenines **2a** and **2b** were able to modify the migration pattern of CaM. Similar studies were made with other kynurenines, in special

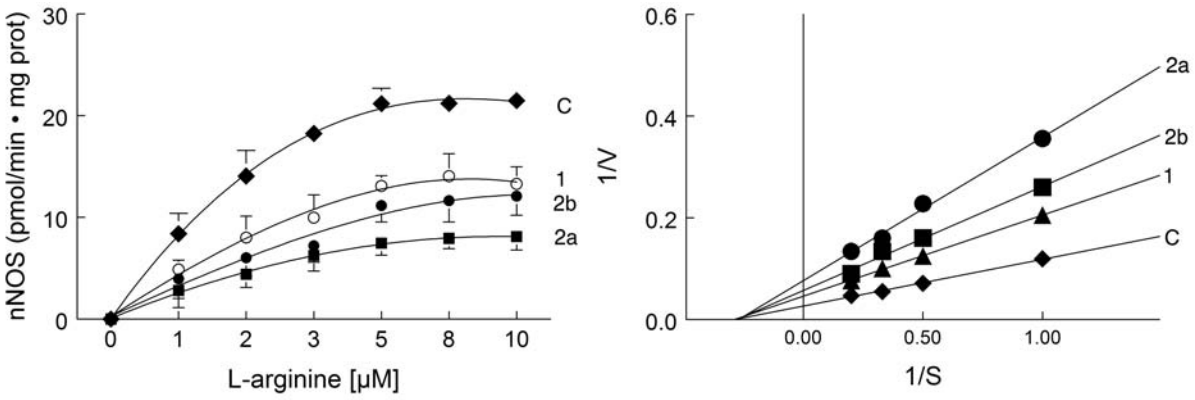


Fig. (4). Experiments with homogenates from rat striatum showing the kinetics of enzyme-substrate reaction in the presence of the indicated drugs. (Left) Effect of **1**, **2a**, and **2b** on rat striatal nNOS activity. Homogenates from rat striatum were incubated for 30 min at 37ºC with increasing concentration of *L*-Arg and in the absence (C) or in the presence of 1mM drug. Each point is the mean ± SEM of three experiments done in triplicate. (Right) Double reciprocal plot of the data showing that the drugs tested modify V_{max} and not K_m values of the enzyme-substrate reaction.

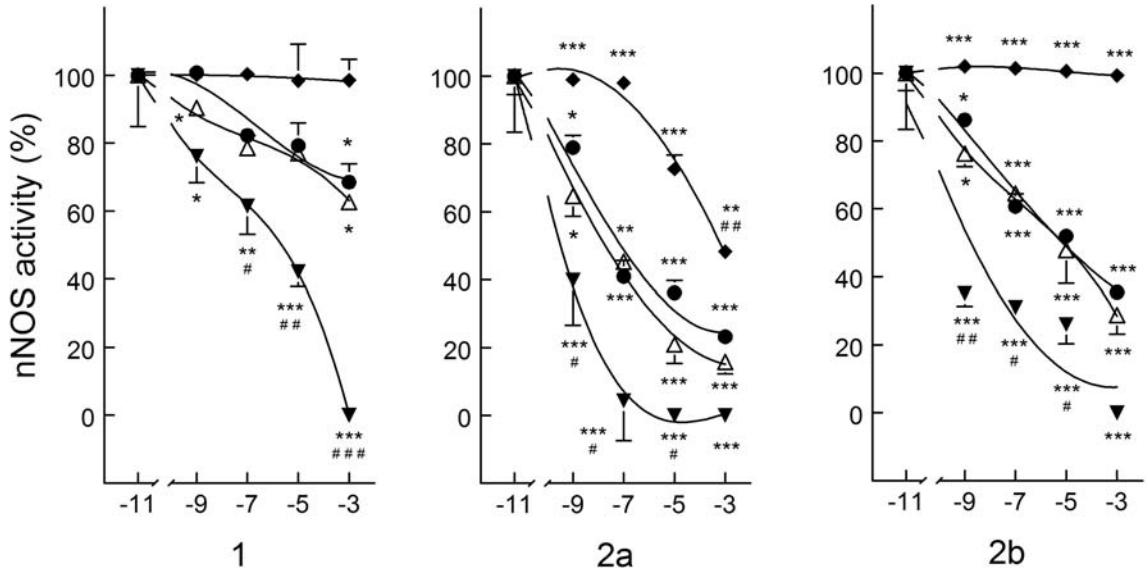


Fig. (5). Effect of melatonin **1** (left) and kynurenines **2a** (center) and **2b** (right) (10^{-9} to 10^{-3} M) on nNOS activity in the presence of increasing concentrations of CaM. (▼, 0 μg/ml; Δ, 0.1 μg/ml; ●, 1 μg/ml; ♦, 10 μg/ml).

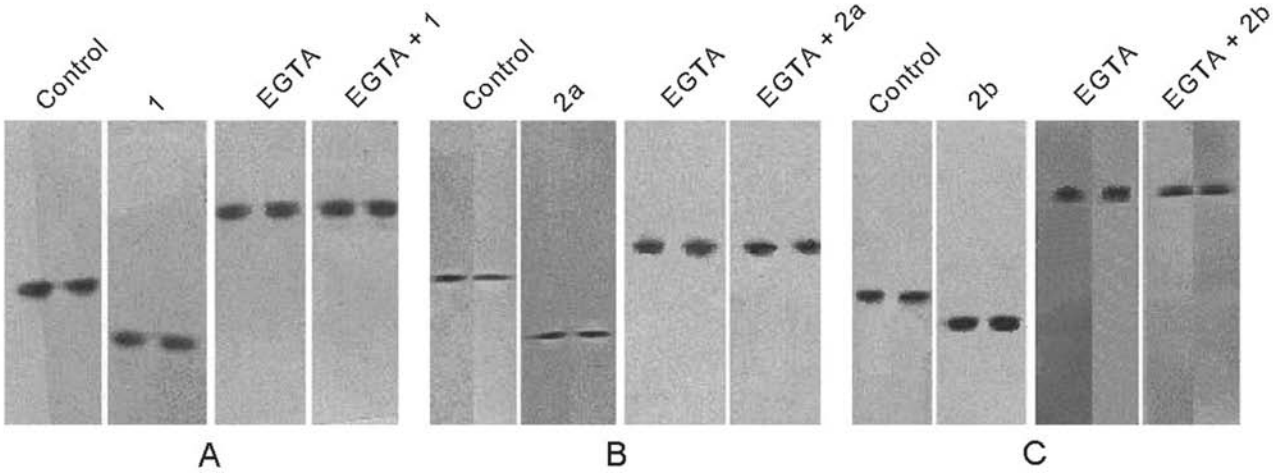


Fig. (6). Effects of melatonin **1** and kynurenines **2a** and **2b** on the electrophoretic mobility of CaM. Urea-PAGE gels were made in the presence of 1mM Ca^{+2} (control) or 2 mM EGTA and in the absence or presence of 1 mM each compound.

those with the amino group blocked **2e** and **2f**, and none of these compounds affected the migration pattern of CaM either in the presence of absence of EGTA.

These data suggest an interaction between melatonin **1** and kynurenines **2a** and **2b** and the CaCaM complex, being the Ca^{+2} ions necessary for this interaction. Dose-dependent experiments with urea-PAGE demonstrate that the kynurenines **2a** and **2b** bind CaCaM with higher affinity than melatonin **1**, thus supporting the results of kinetic experiments [44].

The major findings of these studies are the demonstration that melatonin **1** and kynurenines **2a** and **2b**, inhibit nNOS activity through a mechanism involving a complex formation with CaCaM. The results also show interesting structure-related effects of these kynurenines in their ability to bind CaCaM. Although kynurenines carrying an NH_2 group on their molecule (**2a** and **2b**) bind CaCaM inhibiting nNOS activity, kynurenines lacking the amino group (**2e** and **2g**) are unable to bind CaCaM, losing their inhibitory effect on nNOS. The importance of the NH_2 group in the inhibition of nNOS activity was also demonstrated by blocking it with either a dimethyl or a benzyl group (**2c** and **2d**, respectively); in this case nNOS was not inhibited further.

Finally, similar studies were performed with kynuramine **3a**, the main brain melatonine metabolite. These studies also demonstrated that compound **3a** behaves similarly to melatonin and kynurenines, and inhibits nNOS activity in a dose-dependent manner. Such inhibition is also lost in the presence of increasing amount of CaCaM complex, but not by the addition of other nNOS cofactors. In this case, interaction between kynuramine **3a** and CaM was also studied by means of fluorescence assays, indicating a concentration-dependent inhibition of fluorescence in the presence of compound **3a** [48].

PREPARATION AND BIOLOGICAL ACTIVITY OF SYNTHETIC PHENYLPYRAZOLES (4a-s)

Although NO is not involved in the synaptic transmission under normal conditions, an excessive NO production by some of the NOS-isoenzymes may be detrimental. Thus, it is well-known that an overproduction of NO produces neurotoxicity, and this fact has been associated with several neurological disorders such as Alzheimer's disease [81, 82], amyotrophic lateral sclerosis [83], and Huntington's disease [84]. For this reason, a recent strategy in the development of successful neuroprotective agents is orientated toward the synthesis of new structures that interfere with some step of the complex chemical signaling system involving NOS, including the inhibition of the enzyme itself.

The syntheses of a series of kynurenine **2a-r** and kynuramine **3a-l** derivatives have been described above. Among them, kynurenines **2a** and **2b** show a significant nNOS inhibitory activity, and AMK, **3a**, the main brain metabolite of melatonin is the most potent nNOS inhibitor belonging to the kynuramine family. Compounds **2a-b** and all kynuramine derivatives are characterized by the presence of a 2'-NH_2 group, which restricts the side chain conformational mobility by formation of an intramolecular hydrogen with the carbonyl ketonic groups. This conformational restriction allows kynurenines and kynuramines to mimic the active conformation of melatonin **1** when it interacts with its biological target. Nevertheless, these structures are still quite flexible, and they could adopt several conformations.

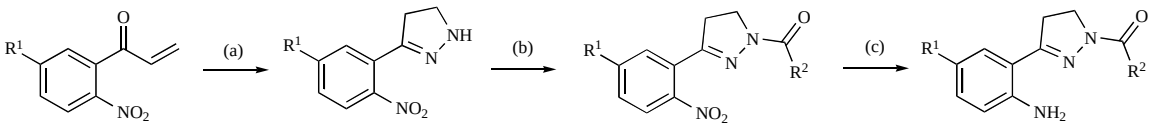
4,5-Dihydro-1*H*-pyrazole derivatives **4a-s** constitute a new type of nNOS inhibitors, showing good inhibitory activity [47]. These compounds are characterized by the presence of a 4,5-dihydro-1*H*-pyrazole fragment as well as the 2'-NH_2 group present in kynurenines and kynuramines. The pyrazole ring restricts the side chain conformational flexibility while the 2'-amine group forms an intramolecular hydrogen bond with the pyrazole N-2 atom, resulting in a less flexible molecule as a whole. To confirm the potential neuroprotective activity of these new compounds, biological assays to test the inhibitory effect against both nNOS and KYN3OH have been performed.

Chemistry

Scheme (**3**) represents the general synthetic pathway for all the final 4,5-dihydro-1*H*-pyrazole derivatives **4a-s** [47]. Reaction of the ketones **13a-c** with hydrazine in ethanol produces the corresponding 4,5-dihydro-1*H*-pyrazoles **17a-c**, which are acylated *in situ* by treatment with acetic anhydride or with the corresponding acyl chloride to obtain the 1-acyl-3-(2-nitro-5-substituted-phenyl)-4,5-dihydro-1*H*-pyrazoles **18a-s**. Finally, reduction of the aromatic nitro group leads to the final compounds **4a-s** in a quantitative manner. The reduction of compounds **4a-j** and **4o-s** was carried out by catalytic hydrogenation (Pd/C), while in compounds **4j-n** reduction was accomplished by reaction with $SnCl_2$ (1:5 molar ratio) in order to avoid dechlorination.

Biological Results

Inhibition of KYN3OH has been proposed as a potentially useful strategy for neuroprotection [67, 68], because KYN3OH inhibitors decrease the brain concentration of the neurotoxic quinolinic acid and 3-hydroxykynurenine while increasing the biosynthesis of the neuroprotective kynurenic acid. For this reason, pyrazoles



13a R^1 = OMe
13b R^1 = Cl
13c R^1 = H

17a R^1 = OMe
17b R^1 = Cl
17c R^1 = H

18a-s

4a-s

Scheme 3. Synthetic pathway followed in the preparation of 4,5-dihydro-1*H*-pyrazole derivatives **4a-s**. (a) NH_2NH_2, EtOH; (b) Ac_2O or R_2COCl, CH_2Cl_2; (c) H_2, Pd/C or $SnCl_2$, EtOH.

(**4**) have been tested as inhibitors of both nNOS and KYN3OH, in order to identify the possible mechanism of action of these molecules.

Table (**5**) illustrates both the nNOS inhibition and KYN3OH activity in the presence of 1mM concentration of each pyrazole. Among pyrazole derivatives, only compounds **4o** and **4b** (with inhibitions of 22.15% and 18.86%, respectively) are very weak inhibitors of KYN3OH, and results obtained for the rest of compounds are not statistically significant.

By contrast, a great number of pyrazoles show good nNOS inhibiton, depending on the substitution on both R^1 and R^2 groups. In general, it can be observed that a cycloalkyl or phenyl substituent in R^2 is better than an alkyl group, being cyclopropyl the better inhibitor in series A and B, and phenyl group in series C. In relation to R^1, it can be observed that compounds belonging to series A or B are more active than those of series C. Only compound **4q** (R^2 = Pr), belonging to series C, shows higher activity than the corresponding one of the other series.

All these biological assays demonstrate that, like kynurenines **2**a and **2b**, 4,5-dihydro-*1H*-pyrazole derivatives **4** could present neuroprotective properties through a nNOS inhibition mechanism. A comparison between both types of compounds could help to define a putative model for the interaction with nNOS.

DEVELOPING OF A PHARMACOPHORE MODEL FOR NOS INHIBITION

A comparison of the conformational behavior of kynurenines **2**, kynurenamines **3** and pyrazoles **4** allowed the development of a pharmacophore model for the NOS inhibition by these classes of compounds. Conformational analyses were performed using Sybyl program [85], running on a HP workstation. Three-dimensional models of all compounds were built from a standard fragment library, and their geometries were subsequently optimized using the Tripos force field [86] including the electrostatic term calculated from Gasteiger and Marsili [87] charges (ε = 1, distance dependent). The method of Powell [88] was used for energy minimization, using the energy gradient as a convergence criteria (0.01 kcal/mol·Å^2). For each compound, a conformational analysis was done by means of the random search procedure implemented in SYBYL (Maximun cycles: 1000, maximun hits = 10, RMS threshold = 0.2 Å, energy cutoff = +20 kcal/mol).

Conformational analysis of kynurenines **2** indicates the existence of two main conformational families depending on the orientation of the carbonyl group. Because of the

Table 5. Structure and Biological Activities of 4,5-Dihydro-1*H*-Pyrazole Derivatives 4a-s. R^1 Defines the Series A (R^1 = OMe), B (R^1 = Cl) and C (R^1 = H)

Compound	R^1	R^2	% nNOS Inhibition[a]	% KYN3OH Activity[b]
4a	OCH_3	Me	38.04 ± 1.53	98.84 ± 11.3
4b	OCH_3	Et	53.27 ± 2.83	81.14 ± 8.6
4c	OCH_3	Pr	34.70 ± 1.32	96.43 ± 10.6
4d	OCH_3	Bu	49.76 ± 1.53	120.50 ± 12.3
4e	OCH_3	*c*-C_3H_5	62.24 ± 4.68	123.35 ± 11.1
4f	OCH_3	*c*-C_4H_7	38.30 ± 3.33	96.54 ± 9.07
4g	OCH_3	*c*-C_5H_9	49.87 ± 4.13	105.19 ± 10.1
4h	OCH_3	*c*-C_6H_{11}	62.20 ± 1.91	87.36 ± 7.9
4i	OCH_3	Ph	58.92 ± 3.55	c
4j	Cl	Me	47.58 ± 4.01	110.59 ± 10.5
4k	Cl	Et	46.56 ± 5.66	97.74 ± 11.1
4l	Cl	Pr	34.43 ± 3.70	97.74 ± 10.2
4m	Cl	*c*-C_3H_5	70.24 ± 5.60	98.12 ± 10.1
4n	Cl	Ph	61.12 ± 3.11	105.71 ± 9.5
4o	H	Me	33.69 ± 3.62	77.85 ± 8.6
4p	H	Et	36.47 ± 4.52	85.25 ± 7.8
4q	H	Pr	52.39 ± 2.24	84.95 ± 7.9
4r	H	*c*-C_3H_5	38.79 ± 3.18	86.93 ± 9.4
4s	H	Ph	57.07 ± 3.13	82.46 ± 9.9

[a] Data represent the mean ± SEM of the percentage of nNOS inhibition produced by 1 mM concentration of each compound. Each value is the mean of three experiments performed by triplicate in homogenates of four rat striata in each one. [b] Data represent the mean ± SEM of the KYN3OH activity in the presence of 1mM concentration of each compound. [c] not tested.

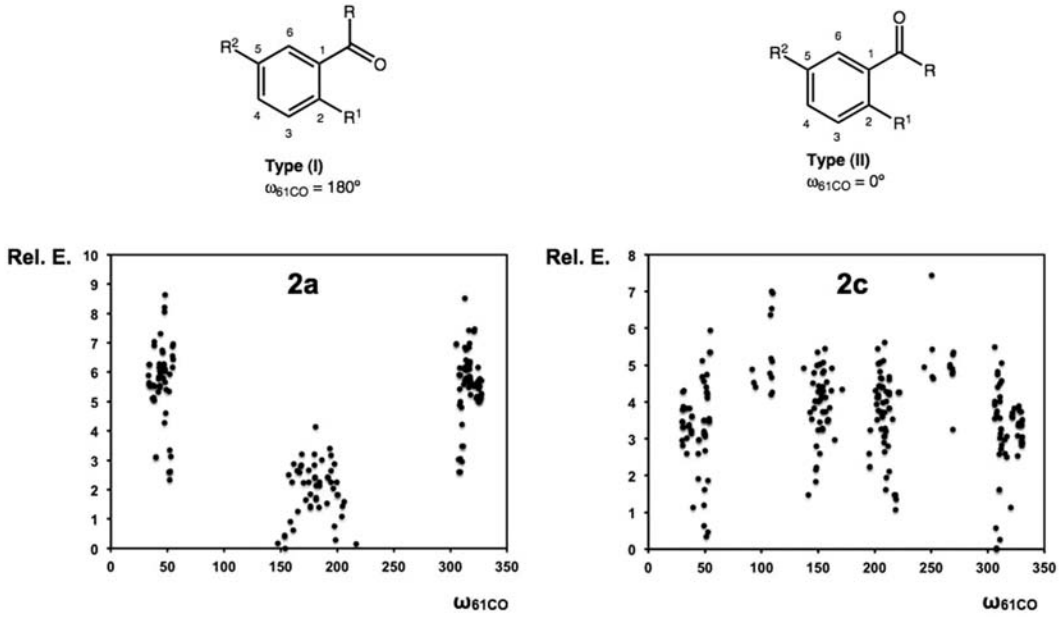


Fig. (7). (up) The two main conformational families found for compounds **2a-r**. (down) Representation of the relative energy (Rel. E., kcal/mol) versus ω_{61CO} (ω, degree) for all the conformations found in compounds **2a** and **2c**. Conformations type (**I**) are situated near 180º, and type (**II**) between 0-50º or 300-350º.

conjugation with benzene, the carbonyl group prefers to be coplanar (or almost coplanar) with the aromatic ring and can adopt two possible conformations: in one of them the oxygen atom points toward the R^1 substituent, and in the other it is orientated in the opposite direction conformation type **I** and type **II**, respectively (Fig. **7**).

Fig. (**7**) also shows, as an example, the energy of the different conformations found for compounds **2a** and **2c** as a function of the torsional angle C_6-C_1-C=O (ω_{61CO}). Zone near 180º corresponds to conformations type **I**, and zones between 0-50º or 300-350º include conformations type **II**. In compound **2a** ($R^1 = NH_2$), the intramolecular hydrogen bond formed between both the 2-amine and the CO groups stabilizes conformations type **I**, and consequently the zone near 180º is more populated and includes the more stable conformers. On the contrary, compound **2c** ($R^1 = N(CH_3)_2$) shows a wide distribution of the conformations over all the values of ω_{61CO}, being more populated conformations type **II**, which are also the most stable ones. This is a consequence of the high volume of the dimethylamine group that prevents the conjugation with the aromatic ring of either the carbonyl or the amine groups. In other compounds, the behavior is intermediate between both compounds **2a** and **2c**, depending on the formation or not of the intramolecular hydrogen bond.

In this sense, in compound **2e** ($R^1 = H$), the populations of both families are almost equal and both have similar energies, and in compound **2d** ($R^1 = NHCH_2Ph$), the 2-benzylamine substituent still allows the formation of the intramolecular hydrogen bond, and the conformational behavior of this compound is similar to that of **2a**, showing a more populated region near 180º that includes more stable conformations (type **I**). These facts confirm the importance of this intramolecular hydrogen bond in the conformational behavior of these compounds.

The conformational analysis of kynurenines shows that conformations type **I** are favored by about 3 kcal/mol in compounds **2a** and **2d** and by 0.5 kcal/mol in **2e**. Only in **2c** the most stable conformer belongs to type **II** conformational family. The presence of the 2-amine groups in **2a** and **2b** restricts the flexibility of these compounds and allows the formation of an additional hydrogen bond between them and their potential biological target.

Conformational analysis of kynurenamines **3** indicates a behavior similar to that of kynurenines **2**. Two main conformational families similar to that of kynurenines **2** (families **I** and **II**) were identified. Fig. (**8**) shows as an example the most stable conformer of compound **3a**, the main brain metabolite of melatonin. For comparison, the most stable conformer found for compounds **2a** have also been included. In general, conformations of family **I** are stabilized by the formation of an intramolecular hydrogen bond between the CO and the NH_2 moieties, whereas conformations belonging to family **II** are more energetic.

Finally, conformational analysis of 4,5-dihydro-1*H*-pyrazoles **4** indicates the existence of four different conformers. Fig. (**8**) shows the most stable conformer of compound **4j**, as an example. The greater stability of this comformer is due to three structural aspects: (i) The existence of a hydrogen bond between the 2'-NH_2 group and the N-2 nitrogen of the pyrazole ring that stabilizes this conformation. (ii) The stabilization due to the conjugation between the C=N double bond and the benzene ring. (iii) The s-cis conformation adopted by the amide bond, which

minimizes the steric interactions between the methyl group and the C-5 atom of the pyrazole ring.

In all cases, the intramolecular hydrogen bond makes that the side-chain of these compounds adopts a similar orientation of the melatonin sidechain, like is also shown in the most stable conformer found for melatonin in the conformational analysis (Fig. **8**).

To find the similarities between the conformational behavior of compounds **1**, **2**, **3** and **4**, we have compared all the conformations of melatonin, kynurenine and/or kinurenamine derivatives, with the four conformers found for each pyrazole compound.

The importance of the 2-NH_2 group in the pharmacophore of kynurenines have been mentioned previously. Since the nitrogen atom of the amide group acts as hydrogen bond donor in kynurenines or kynurenamines but not in pyrazole derivatives, this nitrogen is not a common pharmacophoric feature. Instead of that, the terminal carbonyl group could acts as a hydrogen bond acceptor, and probably behaves in a similar way in the three types of molecules on interacting with the enzyme. Finally, the benzene ring is also a common structural template for both families of compounds.

For these reasons, in the matching process the benzene ring, the N atom of the 2'-NH_2 group and the carbon atom directly joined to the benzene ring were chosen in order to superimpose all compounds. In the fitting process, the benzene ring, the 2'-NH_2 group and the amide oxygen atom have been selected as potential pharmacophoric groups.

Using this procedure, one low energy conformer of melatonin, kynurenine and kynurenamine that fitted very well over the most stable conformer of pyrazole derivatives was found. In all cases, the most stable conformer is stabilized by the presence of the intramolecular hydrogen bond. Fig. (**9**) shows as an example the superimposition of melatonin and compounds **2a** and **3a** over the most stable conformer of compound **4j**. It can be seen that the relative energy of the conformation of melatonin and compound **2a** and **3a** are small (2.82, 1.40 and 2.75 kcal/mol, respectively).

In the kynurenine family, substitution of the methyl group **2a** by a propyl **2b** decreases the nNOS inhibitory effect from 68.5 % to 45.1 %. In kynurenamines, a similar behavior was observed since an increment in the volume R^2 substituyent decreases the inhibitory activity. Nevertheless, in the pyrazole derivatives, substitution of the methyl group by a more bulky one increases the nNOS inhibitory effect.

If we accept these findings as correct, a scheme for the interaction could be drawn (Fig. **9**), and summarized as follow:

(a) A pocket in the enzyme that accommodates the benzene rings present in kynurenines, kinurenamine and pyrazoles (black solid arc). Substitution of 5'H (series C) by OMe (series A) or Cl (series B) increases the inhibitory activity in practically all compounds, and the electronic nature of the 5'-substituent (MeO, Cl, H) seems to be important for the biological activity. Nevertheless, the influence of the substituent is not absolutely clear and it cannot be decided what is the main type of interaction (hydrophobic or electronic)

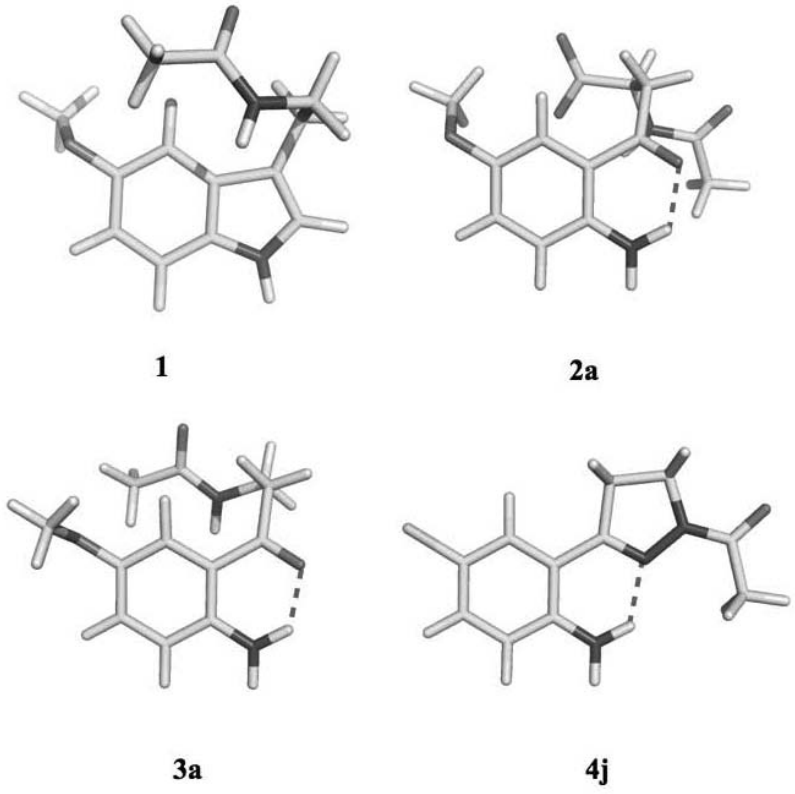


Fig. (8). The most stable conformers found for compounds of **1**, **2a**, **3a** and **4j**.

with this part of the enzyme. Probably both types should be important.

(b) A hydrogen acceptor residue for the interaction with the free NH bond of the 2'-NH_2 group or the indole NH bond of melatonin. Formation of this hydrogen bond has proven to be necessary since in the kynurenine family, elimination, mono- or bialkylation of the 2'-NH_2 group arise in a lack of nNOS inhibitory activity.

(c) A hydrogen bond donor residue that can interacts with the amide oxygen atom of melatonin, kynurenine, kynurenamine and pyrazole derivatives. Both oxygen atoms match very well in the superimposed structures and have the same character as hydrogen bond acceptors.

(d) The R^2 substituent is accommodated in two different and near zones of the binding pocket. Since an increment in the R^2 volume in kynurenines and kynurenamines provokes a decreasing in the activity, these molecules must orientate its R^2 substituent to a region with a small steric tolerance (black points arc). The methyl group of the acetamide moiety of melatonin can probably be inserted in this hindered region. On the contrary, R^2 substituent in pyrazoles must be orientated to a zone sterically allowed (black broken arc).

PREPARATION AND BIOLOGICAL ACTIVITY OF PYRROLE DERIVATIVES (5a-u)

A new family of NOS inhibitors has been obtained from the 3-phenyl-4,5-dihydro-1*H*-pyrazole **4** by substituting the of pyrazole moiety by a pyrrole ring [49].

Chemistry

These compounds are 5-(5-substituted-2-aminophenyl)-1*H*-pyrrole-2-carboxilic acid alkylamides, and the synthesis of these new molecules is described in Scheme (**4**) [49]. The synthesis pathway begins with the reaction of 2-nitrocinnamaldehyde derivatives **20a-c** with ethyl azidoacetate to yield the corresponding 2-azido-5-(2-nitro-5-substituted-phenyl)-penta-2,4-dienoic acid ethyl ester **21-c**. While 2-nitrocinnamaldehyde **20c** is commercially available, compounds **20a** and **20b** have been prepared from the corresponding 2-nitro-5-substituted-benzaldehyde **19a-b,** by Wittig reaction with Ph_3P=CHCHO. Azides derivatives **21a-c** cyclizise and yield the corresponding nitrophenylpyrroles **22a-c** when heated in *p*-xylene. These derivatives were hydrolyzed to yield the carboxylic acid derivatives, which in turn were transformed into the acyl chloride and treated with the appropriated amine to yield the corresponding N-substituted carboxamide **23a-u.** Finally, compounds **5a-u** were obtained by reduction of the nitro group in the corresponding derivative **23a-u**, performed by treatment with $Fe/FeSO_4$.

Biological results

Table (**6**) shows the inhibition percentage of nNOS and iNOS isoforms *in vitro* produced by a 1mM concentration of each compound **5a-u**, compared with the control assays.

Results are quite variable. Among compounds **5a-f** (R^1= OMe), **5a** and **5b** do not inhibit nNOS. An increment in the volume of *N*-carboxamide substituent increases the inhibition percentage, and compounds **5c** and **5d** are the best inhibitors in this series of compounds. In compounds **5e** and **5f** (R^2 = c-C_5H_9, CH_2Ph), a decreasing of the nNOS inhibition can be observed again.

Compounds **5g-k** (R^1 = Cl) show higher nNOS inhibition percentage, indicating that this substituent is the better one for the inhibition of this enzyme. Compound **5k** with a *N*-cyclopropyl substituent is the best inhibitor in this series. Compounds **5i** and **5j** also show good inhibition percentage, indicating that a R^2 must be a group with a moderate volume.

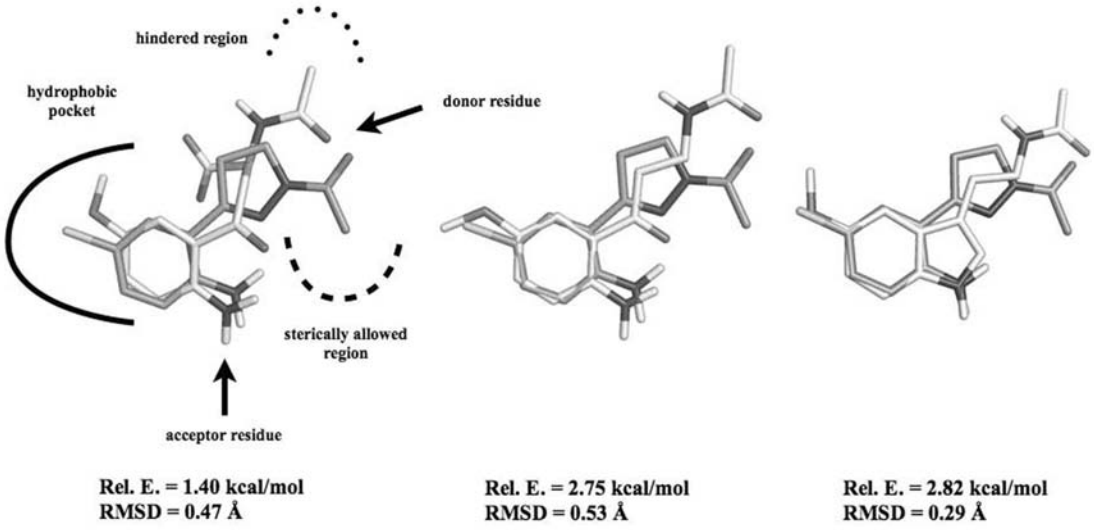
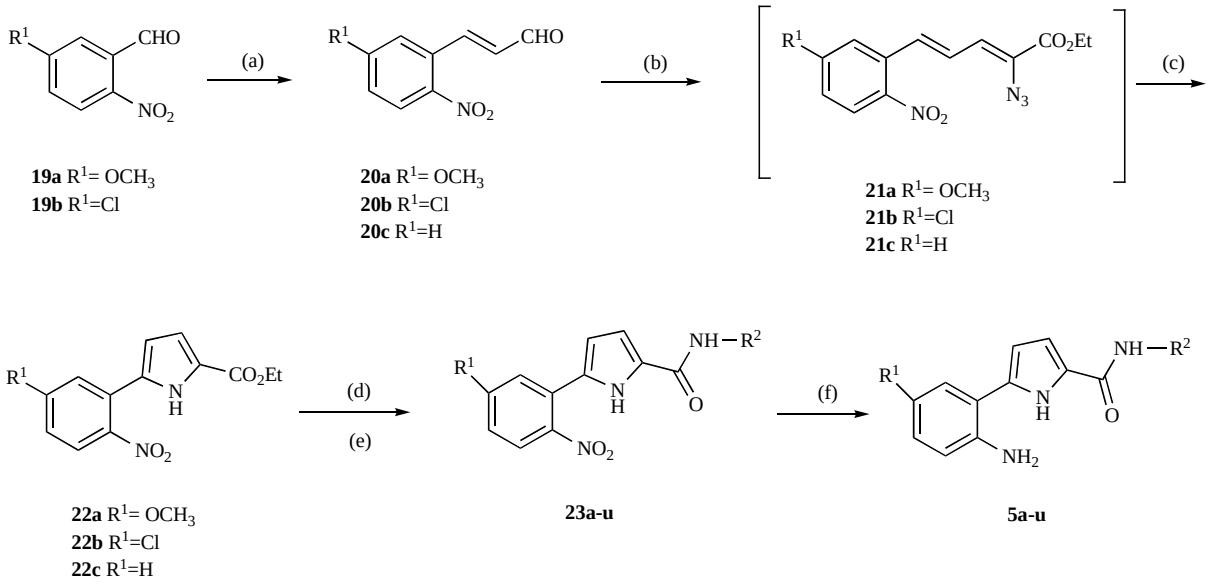


Fig. (9). Superimposition for the corresponding conformations of compound **4j** (dark color) and compounds **2a** (clear color) (left), compound **3a** (clear color) (center), and **1** (clear color) (right), Relative energies correspond to the conformations of **2a**, **3a** and **1**, respectively**.** RMSD indicates the goodness of the superimposition considering the aromatic ring, the NH_2 group and the amide oxygen atom as fitting atoms.



Scheme 4. Synthetic pathway followed in the preparation of pyrrole derivatives **5a-u**. (a) Ph_3P=CHCHO;(b) $N_3CH_2CO_2Et$; (c) thermolysis, p-xylene; (d) NaOH, then AcOH; (e) $SOCl_2$, then R^2NH_2/TEA; (f) Fe/$FeSO_4$.

Table 6. Structure and *In Vitro* nNOS and iNOS Inhibition (%) Observed in Presence of 1mM Concentration of Compounds 5a-u

Comp.	R^1	R^2	% nNOS inhibition[a]	% iNOS inhibition[a]
5a	OCH_3	H	0.00 ± 2.01	8.95 ± 0.50
5b	OCH_3	Me	0.00 ± 2.6	26.17 ± 6.85
5c	OCH_3	Pr	43.45 ± 3.38	6.78 ± 3.93
5d	OCH_3	c-C_3H_5	32.54 ± 2.63	21.36 ± 4.68
5e	OCH_3	c-C_5H_9	15.33 ± 2.42	26.25 ± 3.22
5f	OCH_3	CH_2Ph	8.89 ± 1.13	22.41±1.94
5g	Cl	H	34.48 ± 0.99	5.34 ± 2.34
5h	Cl	Me	17.11 ± 0.74	1.27 ± 3.39
5i	Cl	Et	33.40 ± 2.46	2.8 ± 1.75
5j	Cl	Bu	36.46 ± 4.13	3.33 ± 1.27
5k	Cl	c-C_3H_5	48.07 ± 1.30	7.2 ± 0.31
5l	H	H	15.01 ± 2.66	3.07 ± 1.79
5m	H	Me	0.00 ± 0.21	32.68 ± 2.78
5n	H	Et	4.15 ± 0.97	20.49 ± 5.19
5o	H	Pr	0.00 ± 2.17	13.17 ± 5.2
5p	H	Bu	0.00 ± 1.80	7.53 ± 2.76
5q	H	c-C_3H_5	2.92 ± 0.87	0.00 ± 1.75
5r	H	c-C_4H_7	8.44 ± 1.87	20.1 ± 4.78
5s	H	c-C_5H_9	5.36 ± 3.19	52.79 ± 1.7
5t	H	c-C_6H_{11}	13.40 ± 0.46	17.2 ± 7.54
5u	H	CH_2Ph	7.52 ± 2.50	28.11 ± 2.39

[a]Data represent the mean ± SEM of the percentage of nNOS and iNOS inhibition produced by 1mM concentration of each compound. Each value is the mean of three experiments performed by triplicate in homogenates of four rat striata in each one.

Finally, compounds **5l-u** show a low inhibition percentage. This fact indicates that a non-substituted benzene moiety is detrimental for the nNOS inhibition activity.

Table (**6**) also shows the iNOS inhibition observed in the presence of 1mM concentration of compounds **5**. Since compounds **5g-k** do not inhibit iNOS, it seems that a 5'-Cl substituent is detrimental for the activity and consequently a 5'-MeO substituent or an unsubstituted benzene ring are preferable. Regarding the influence of the *N*-substituent over the activity, the available information is also confused: compounds **5b** and **5m** (R^2 = Me) or **5n** (R^2 = Et) show moderate inhibition percentages, compound **5d** (R^1 = OMe, R^2 = c-C_3H_5) also show a moderate inhibition, but compound **5q** (R^1 = Cl, R^2 = c-C_3H_5) does not inhibit. On the other hand, a further increase in the R^2 volume gives place to an increment in the inhibition activity. Compounds **5e**, **5f**, and **5u** show moderate inhibitions and compound **5s** (R^1 = H, R^3 = c-C_5H_9) is the best inhibitor in all the series.

Melatonin **1** reduces the iNOS activity and expression in several inflammatory models [89], the mitochondrial i-mtNOS activity and expression [90, 91] and the nNOS activity. It has also found that melatonin shows neuroprotection properties in different models, including MPTP-induced Parkinson's disease [92]. For these reasons, the most active iNOS inhibitors **5m** and **5s** have been selected to test its ability to reduce the *in vivo* NOS activity in cytosol and mitochondria in the substantia nigra (SN) of the MPTP model of Parkinson's disease. Fig. (**10)** shows the NOS relative activities in each cell fraction considering the NOS activities in control animals as 100%.

MPTP administration slightly decreases the nNOS and c-mtNOS activities in cytosol and mitochondria, respectively. Melatonin administration significantly reduces nNOS and c-mtNOS activities in MPTP-treated mice. Administration of compounds **5m** and **5s** reduce the nNOS activity in a lesser extent than melatonin, but they provoked a stronger reduction in c-mtNOS activity than that produced by melatonin.

MPTP administration increases 10 times the cytosol iNOS activity in relation to that of control animals. Melatonin administration partially counteracts the effect of MPTP on iNOS activity, while administration of compounds **5m** and **5s** significantly reduces the MPTP-induced iNOS activity to control values. On the other hand, MPTP increases 2 times the i-mtNOS activity whereas melatonin absolutely prevented this effect of MPTP. Interestingly, compounds **5m** and **5e** were much more efficient than melatonin in reducing i-mtNOS activity in MPTP-treated animals.

More in deep studies are needed in order to clarify the biological mechanism by which these molecules diminish the NOS activities *in vivo*. At present we are testing two possibilities: i) compounds **5m** and **5s** can be metabolized so that their *in vivo* activity can be due to a common metabolite that interacts with iNOS, and ii) these molecules, instead of a direct blockade of the NOS activity could modify the genomic expression of iNOS (or i-mtNOS), diminishing by this indirect route the activity of these enzymes. The last possibility seems to be more probable. In fact, melatonin exerts some of its functions through its interaction with ROR/RZR nuclear receptors [93] and compounds **5a-u** could behave in a similar way. Even if the molecular mechanism is still unknown, compounds **5m** and **5s** selectively decrease the NOS activity due to the inducible isoforms of this enzyme in both cytosol and mitochondria. Since i-NOS and i-mtNOS are those that suffer higher alteration in several

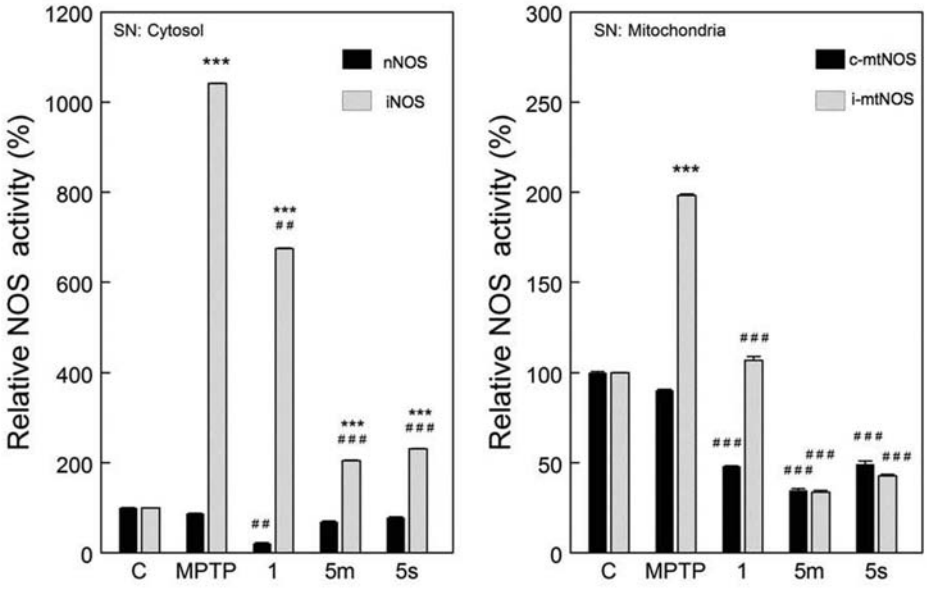


Fig. (10). Relative NOS activities (%) measured in both the cytosol and mitochondria cell fractions isolated from the SN in mice treated with MPTP, MPTP/**1**, MPTP/**5m** and MPTP/**5s**. C represents control animals treated with the vehicle (ethanol/saline). Data represents means ± SEM of seven experiments performed by triplicate in homogenates of four SN in each one. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control.

physiological disorders, the potentiality of these molecules in the development of compounds with interesting pharmacological properties is clear.

CONCLUSIONS

In this work a series of melatonin synthetic analogs with kynurenine, kynurenamine and phenyl-pyrazole structures and interesting properties as nNOS inhibitors are presented. Structural comparison between the three families of compounds, allows establishing a pharmacophore model that fulfills all the observed SARs. This model could serve as a template for the design of other potential nNOS inhibitors. Substitution of the dihydro-pyrazole ring by a pyrrole one gives place to the last family of compounds, which show a significant smaller nNOS inhibitory activity, but some derivatives show interesting results to prevent the increase of the inducible NOS activity. Compounds more effective than melatonin to reduce the i-mtNOS activity in animals treated with MPTP were obtained from these assays, indicating a clear potential of these molecules in the development of compounds with useful pharmacological properties.

At the present, our research is centered in the preparation and biological evaluation of new compounds with a more lipophilic heterocycle rings (thiadiazole, oxadiazole or quinazoline) in order to obtain selectivity towards the iNOS/i-mtNOS isoforms.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

This study was partially supported by the Junta de Andalucía through project no. P06-CTS-01914.

ABREVIATIONS

AFMK	=	N^1-acetyl-N^2-formyl-5-methoxykynurenamine
AMK	=	N^1-acetyl-5-methoxykynurenamine
L-Arg	=	*L*-arginine
CaCaM	=	calcium-calmodulin
CaM	=	calmodulin
c-mtNOS	=	constitutive mitochondrial nitric oxide synthase
CNS	=	central nervous system
DMF	=	dimethylformamide
EGTA	=	ethylene glycol tetraacetic acid
eNOS	=	endothelial nitric oxide synthase
FAD	=	flavin adenine dinucleotide
FMN	=	flavin mononucleotide
H_4B	=	tetrahydrobiopterin
i-mtNOS	=	inducible mitochondrial nitric oxide synthase
iNOS	=	inducible nitric oxide synthase
KYN3OH	=	kynurenine 3-hydroxylase
mt-NOS	=	mitochondrial nitric oxide synthase
MPTP	=	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADPH	=	nicotinamide adenine dinucleotide phosphate
NMDA	=	N-methyl-D-aspartate
nNOS	=	neuronal nitric oxide synthase
NO	=	nitric oxide
NOS	=	nitric oxide synthase
PAGE	=	polyacrylamide gel electrophoresis
SMCx	=	sensorimotor cortex
SN	=	substantia nigra

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Received: September 23, 2011 Revised: February 17, 2012 Accepted: February 19, 2012