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Synthesis, structure, theoretical and experimental in vitro antioxidant/pharmacological properties of α-aryl, *N*-alkyl nitrones, as potential agents for the treatment of cerebral ischemia

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ABSTRACT

The synthesis, structure, theoretical and experimental in vitro antioxidant properties using the DPPH, ORAC, and benzoic acid, as well as preliminary in vitro pharmacological activities of (*Z*)-α-aryl and heteroaryl *N*-alkyl-nitrones **6**–**15**, **18**, **19**, **21**, and **23**, is reported. In the in vitro antioxidant activity, for the DPPH radical test, only nitrones bearing free phenol groups gave the best RSA (%) values, nitrones **13** and **14** showing the highest values in this assay. In the ORAC analysis, the most potent radical scavenger was nitrone indole **21**, followed by the *N*-benzyl benzene-type nitrones **10** and **15**. Interestingly enough, the archetypal nitrone **7** (PBN) gave a low RSA value (1.4%) in the DPPH test, or was inactive in the ORAC assay. Concerning the ability to scavenge the hydroxyl radical, all the nitrones studied proved active in this experiment, showing high values in the 94–97% range, the most potent being nitrone **14**. The theoretical calculations for the prediction of the antioxidant power, and the potential of ionization confirm that nitrones **9** and **10** are among the best compounds in electron transfer processes, a result that is also in good agreement with the experimental values in the DPPH assay. The calculated energy values for the reaction of ROS (hydroxyl, peroxyl) with the nitrones predict that the most favourable adduct-spin will take place between nitrones **9**, 10, and **21**, a fact that would be in agreement with their experimentally observed scavenger ability. The in vitro pharmacological analysis showed that the neuroprotective profile of the target molecules was in general low, with values ranging from 0% to 18.7%, in human neuroblastoma cells stressed with a mixture of rotenone/oligomycin-A, being nitrones **18**, and **6**–**8** the most potent, as they show values in the range 24–18.4%.

1. Introduction

For years, the ability of nitrones to act as good radical traps has been in the origin of a number of basic and clinical studies aimed at the investigation of their potential therapeutical application for the treatment of cerebral ischemia, or neurodegenerative diseases where reactive oxygen species (ROS) are implicated.[1] This is the case of (*Z*)-α-phenyl-*N*-*tert*-butylnitrone (PBN),[2] or nitrone NXY-059,[3] a well known free radical scavenger with high neuroprotective profile in rat models of transient and permanent focal ischemia, and stroke model in rodents, that has been launched several times in different programmes in advanced clinical studies, with limited success, but always with renewed interest.[4] However, there is still a large consensus on the convenience of the neuroprotective strategy according to the recently published papers on the area, and the efforts devoted to improving previous results or finding new nitrones for the treatment of ischemic stroke.[5] The case of edaravone, a potent free radical scavenger, recently approved for treatment of patients with acute stroke in Japan,[6] also confirms the viability of this approach. Between the nitrones, PBN, for instance, inhibits the oxidation of lipoproteins,[7] reduces oxidative damage to erythrocytes and peroxidation of lipids due to phenylhydrazine,[8] and protects gerbils from brain stroke and mice against MPTP toxicity.[9] However, the mechanism of action of

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PBN is not clear. In addition, the neuroprotective properties of nitrones seem to be related not to the simple ability to act as ROS traps, but to the suppression of *i*NOS expression, cytokine accumulation, and apoptosis.[10] The formation of nitric oxide (NO) from PBN spin adducts may play a role in the observed effects in the central nervous system.[11]

Based on these grounds, we have recently started a project in the context of a virtual research network ('Retic *RENEVAS*'), *supported by the Spanish government (Instituto de Salud Carlos III, Ministerio de Investigación, Ciencia e Innovación)*, and targeted to the synthesis and biological evaluation of a series of α-aryl, *N*-alkyl (methyl, *tert*-butyl, benzyl) hetero(aryl) or nitrones, as potential drugs for the treatment of cerebral ischemia.

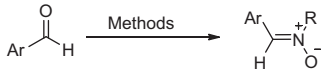
In this work, we report our results in this area, based on the synthesis, structure, and in vitro antioxidant/pharmacological properties of nitrones **6**–**15**, **18**, **19**, **21**, and **23**, shown in Chart 1.

2. Results and discussion

2.1. Synthesis

The selected nitrones **6**–**15**, **18**, **19**, **21**, and **23** in this work have been synthesized using three different methods, as shown in Scheme 1, by reacting the corresponding commercial (**1**, **4**, **17**, and **18**) or easily available aldehydes by the reported procedures, (**2**,[12] **3**,[13] **5**,[14] **20**,[15] and **22**[16]), with the corresponding N-substituted hydroxylamines (method A,[5d] B[17]) or with the appropriate nitro compounds (method C) (Table 1).[18]

Nitrones **6** and **8** (Chart 1) are *N*-alkyl analogues of the well known nitrone **7** (PBN),[2] which has been used here as a reference compound. Nitrones **9–15** (Table 1) have been synthesized and investigated in order to evaluate the effect of different electron donor, protected (OMe) or free (OH), and electron withdrawing (Br) substituents in the aromatic ring, respect to the parent PBN compound. Heteroaryl nitrones **18**, **19**; **21**, and **23** bear a 2-halo(Br, Cl) substituted pyridine, and an indole ring, respectively (Table 1). Known nitrones **6**,[19] **7**,[2,19] **8**[19] showed spectroscopic data in good agreement with those reported in the literature, while new ones (**9**–**15**, **18**, **19**, **21**, and **23**) have analytical and spectral data in accordance with their structure (see Section 4).



Scheme 1. Methods used for the synthesis of the nitrones. Reagents: RNHOH·HCl, Na_2SO_4, Et_3N, THF, MW (method A); RNHOH·HCl, NaOAc, MW (method B); RNO_2, Zn, EtOH (method C).

2.2. Antioxidant evaluation

The in vitro antioxidant activity of these nitrones was initially determined by the use of the DPPH and ORAC tests.

2.2.1. DPPH

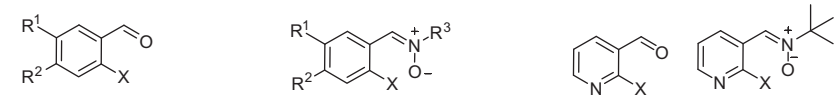
The stable free radical 2,2-diphenyl-1-picylhydrazyl (DPPH) is a useful reagent to investigate the scavenger properties of phenols, catechols and anilines. It is now widely accepted that the reaction between phenols and DPPH proceeds through two different mechanisms: (a) the direct hydrogen atom transfer (HAT) and (b) the sequential proton loss electron transfer (SPLET) (Scheme 2).[20]

A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to colourless/bleached product. In this assay, we measure the DPPH initial absorbance, and the absorbance once the potential antioxidant has been added. The reduction of absorbance is a measure of the free DPPH due to the action of the antioxidant. We have used curcumin as the reference compound.[21] The antioxidant activity was expressed as the RSA% (Radical Scavenging Activity), calculated as follows:

$$\mathrm{RSA}\% = 100[(A_o - A_i)/A_o) \times 100$$

where A_o, and A_i, are the DPPH absorbance in absence and in presence of added nitrone, respectively.

The RSA (%) for nitrones **7** (PBN), **8**–**12**, **18**, **19**, **21**, and **23**, at 0.5 mM, has been incorporated in Table 2. As shown, and as expected, nitrones **9**, **10**, and **13**–**15** bearing free phenol groups provided the best RSA (%) values. Comparing the RSA (%) values for nitrone **10** (25.9 ± 1.8) to compound **15** (40.7 ± 2.5), it is clear that the *para* is preferred to the *meta* for the position of the free

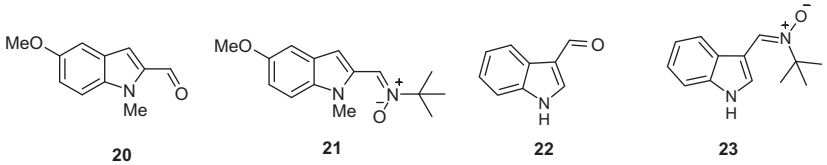


1 R^1, R^2, X= H
2 R^1= OH, R^2= OMe, X= Br
3 R^1, R^2= OMe, X= Br
4 R^1= OMe, R^2= OH, X= H
5 R^1= OMe, R^2= OH, X= Br

6 R^1, R^2, X= H, R^3= Me
7 R^1, R^2, X= H, R^3= *t*-Bu
8 R^1, R^2, X= H, R^3= Bn
9 R^1= OH, R^2= OMe, X= Br, R^3= *t*-Bu
10 R^1= OH, R^2= OMe, X= Br, R^3= Bn
11 R^1, R^2= OMe, X= Br, R^3= *t*-Bu
12 R^1, R^2= OMe, X= Br, R^3= Bn
13 R^1= OMe, R^2= OH, X= H, R^3= Me
14 R^1= OMe, R^2= OH, X= H, R^3= Bn
15 R^1= OMe, R^2= OH, X= Br, R^3= Bn

16 X = Br
17 X = Cl

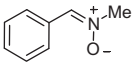
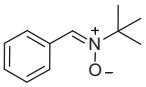
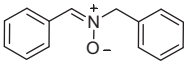
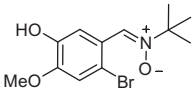
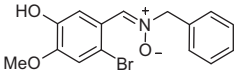
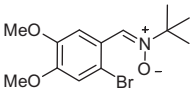
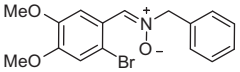
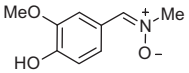
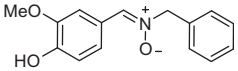
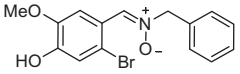
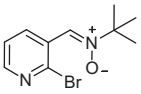
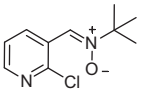
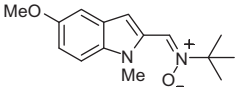
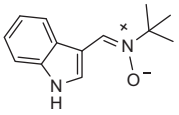
18 X = Br
19 X = Cl



20 **21** **22** **23**

Chart 1.

Table 1
Synthesis of nitrones **6**–**15**, **18**, **19**, **21**, and **23**

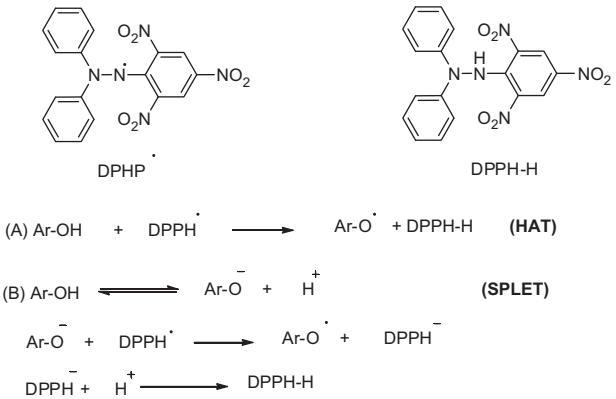
Nitrone	Structure	Time	Yield (method)
6		5 min	75% (B)
7 (PBN)		6 h	48% (C)
8		3 min	84% (B)
9		3 h	65% (A)
10		55 min	91% (A)
11		2 min	42% (B)
12		3 min	64% (B)
13		3 h	60% (A)
14		4 h	55% (A)
15		3 h	70% (A)
18		3 h	76% (A)
19		1 h 45 min	99% (A)
21		6 h	20% (56%)[a] (C)
23		33 h	76% (91%)[a] (A)

[a] Taking into account the recovered unreacted starting product.

hydroxyl group, respect to the C=N(O)Bn moiety, in order to have the best free radical scavenger activity. Similarly, the elimination of the bromine atom at the *ortho* position in nitrone **15** affords a two-fold more potent nitrone **14** (88.2% RSA), the most potent in this series of compounds for this assay. Notice, also, that changing the benzyl (in nitrone **14**) for a methyl (in nitrone **13**) group in the alkyl substituent, has no influence on the RSA(%) values. Finally, the archetypal nitrone **7** (PBN) gave a low RSA(%) value under our experimental conditions, rising to 16% at 10 mM, a result that is in good agreement with the reported 20% at the same concentration.[5c] The other reference molecules used in this assay, such as curcumin and quercetin gave RSA, 35.0% and 94.2%, values, respectively, at 15 μM.

2.2.2. ORAC

The ORAC (Oxygen Radical Absorbance Capacity) assay[22] measures the peroxyl (ROO·) free radical scavenger compounds



Scheme 2. Reaction of phenols with DPPH. Reagents: (A) HAT and (B) SPLET mechanisms.

capability, using fluorescein (FL) as the fluorescent probe. The peroxydation of FL gives nonfluorescent products at 520 nm. Consequently, the remaining fluorescence of FL in presence of the free radical scavenger nitrone must be a measure of the antioxidant activity of the compound under study. There are three ORAC types of assays: hydrophilic ORAC, (ORAC-H), lipophilic ORAC (ORAC-L), and total ORAC. In our work, we used the ORAC-H, using trolox as a reference compound.[23] In Table 2, the values obtained in this experiment, expressed as micromol trolox/micromol compound, are shown. We conclude from these data that most of the nitrones showed a significant antioxidant activity regarding peroxyl radicals, the most potent being nitrone indole **21** (7.98) followed by the phenol bearing *N*-benzyl benzene-type nitrones **10** (7.36), and **15** (6.40). Interestingly, the comparison between nitrone **13** (or **14**) to **10** highlights the importance of a bromine atom in the *ortho* position respect to the nitrone moiety to give nitrones with an important free radical scavenger activity in this assay. This is just the contrary of what we observed in the structure–activity relationship for the bromine motif in the DPPH test (see above). Concerning the type of the *N*-substituent, the benzyl bearing nitrones seem more potent than those bearing a *t*-butyl group (compare nitrones **9** and **10**). Notice, also, that the known nitrone **7** (PBN), was inactive in this assay.

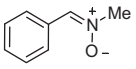
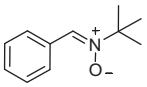
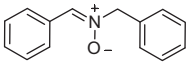
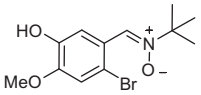
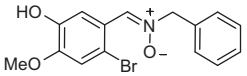
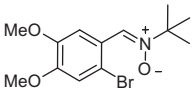
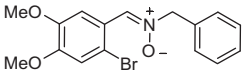
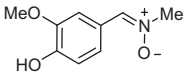
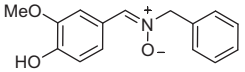
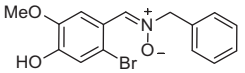
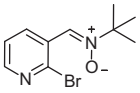
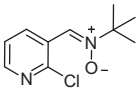
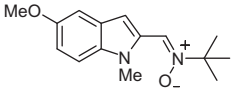
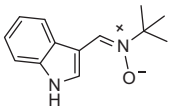
In view of these results, we have investigated next the ability of these nitrones to trap hydroxyl free radicals.

2.2.3. Free radical ·OH scavenger activity

Between the ROS, the ·OH free radical is possibly the most toxic, as it reacts with a number of biological important molecules such as DNA, lipids or carbohydrates. In order to asses the capacity of our molecules to trap this radical species, we have used the benzoate hydroxylation method.[24,25] The hydroxyl radical ·OH generated in a Fenton reaction (Scheme 3) reacts with benzoate to produce the fluorescent hydroxybenzoates, which can be measured spectrofluorimetrically with excitation at 305 nm and emission at 407 nm. However, the hydroxylation of benzoate could be inhibited by ·OH-radical scavengers such as nitrones, and then, the fluorescence generally decrease.

The fluorescence of the hydroxylated benzoate products was measured after 2 h of incubation at 37 °C, in absence and in presence of the nitrones. The RSA (%) values are shown in Table 2. All the nitrones studied were active in this experiment, showing high values, in the 94–97% range, the most potent corresponding to nitrone **14** that shows a 96.9%. This activity is in the same range as the reference compounds curcumin (96.8%) and querecetin (96.6%).

Table 2
Antioxidant activity of nitrones **6**–**15**, **18**, **19**, **21**, and **23** determined by ORAC, DPPH, and OH· assays[a]

Nitrone	Structure	DPPH [(RSA (%)][b]	ORAC[e]	OH· [(RSA (%)][f]
6		0.8 ± 0.7	0.0 ± 0.0	95.3 ± 0.8
7 (PBN)		1.4 ± 0.9	0.0 ± 0.0	94.9 ± 1.0
8		0.6 ± 0.3	0.01 ± 0.00	95.7 ± 0.5
9		31.1 ± 2.1	5.58 ± 0.33	96.3 ± 0.5
10		25.9 ± 1.8	7.36 ± 0.32	94.6 ± 0.9
11		0.8 ± 0.9	0.03 ± 0.00	96.3 ± 0.8
12		0.2 ± 0.2	0.03 ± 0.002	95.1 ± 1.3
13		87.6 ± 1.2	3.35 ± 0.07	96.5 ± 0.7
14		88.2 ± 1.0	2.93 ± 0.07	96.9 ± 0.7
15		40.7 ± 2.5	6.40 ± 0.35	95.7 ± 1.0
18		3.9 ± 3.8	0.01 ± 0.00	95.1 ± 1.1
19		1.6 ± 1.5	0.01 ± 0.00	94.9 ± 1.3
21		4.8 ± 1.8	7.98 ± 0.21	nd[d]
23		1.2 ± 1.1	3.69 ± 0.15	nd[d]
	Curcumin	35.0 ± 0.3[c]	nd[d]	96.8 ± 0.6
	Quercetin	94.2 ± 0.9[c]	10.49 ± 0.45	96.6 ± 0.5
	Trolox	nd[d]	1.00 ± 0.0	94.3 ± 1.6

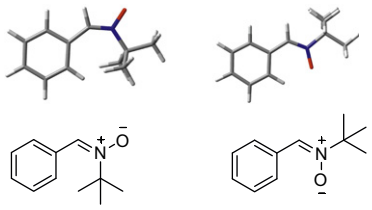
[a] Data are expressed as mean values (±SEM) of at least three different experiments in quadruplicate.
[b] Determined at 0.5 mM.
[c] Determined at 15 μM.
[d] nd, not determined.
[e] micromol trolox/micromol compound.
[f] Benzoic acid methods: determined at 50 μM.

2.3. Computational chemistry. Theoretical calculations

2.3.1. Structural analysis of the nitrones

The most stable geometry for the C=N double bond has been investigated in nitrone **7** ('PBN') (Chart 2). These calculations show

Scheme 3. Fenton's reaction and free radical reaction of radical ·OH with a nitone.



(*E*)-**7** (*Z*)-**7**

Chart 2. Isomers (*E*)- and (*Z*)-**7**.

that the *Z*-isomer is 15.9 kcal/mol more stable than the *E*-isomer, due to a strong steric repulsion between the phenyl and the *t*-Bu or Bn groups in the *E*-isomer. To minimize these repulsions, the C=N bond is elongated from 1.317 Å in the *Z*-isomer to 1.329 Å in isomer *E*-, and the CCN and CNC bond angles open from 126.8° and 121.9° in isomer *Z*- to 138.5°, and to 127.6° in isomer *E*-, respectively. This conclusion is supported by the chemical shifts (δ_H) in the ^{1}H NMR spectra, and the published crystallographic data for *C*-aryl-nitrones.[26]

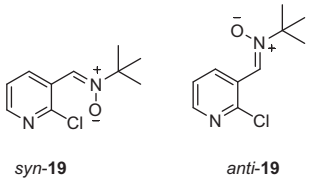
We also evaluated the relative population of *syn*/*anti* rotamers around the C–C bond for nitrone **19** (Chart 3). The repulsion between the aromatic ring and the nitroxyl moiety in the *syn* rotamer induces a distortion of the C–C bond (Table 3) that prevents the aryl-nitrone co-planarity. Obviously, this is a destabilizing effect, absent in the *anti* rotamer, and that contributes to the higher stability of the *anti*-nitrone rotamers.

2.3.2. Antioxidant capacity

First of all, we calculated the potential of ionization of nitrones **7**–**12**, **18**, **19**, **21**, **23**, and curcumin as the reference compound (Table 4).

This parameter detected that nitrones **9** and **10** were the best compounds in order to participate in electron transfer processes, a result that is also in good agreement with the experimental values in the DPPH assay (see Table 2), and the fact that these nitrones are phenol derivatives, well known for their ability to transfer hydrogen atoms.[27] These values also compare very favourably with the potential of ionization obtained in the same experiment for curcumin.

The determination of the physicochemical parameters implicated in the reaction of a radical with a substrate may help in



syn-**19** *anti*-**19**

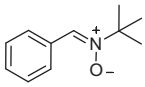
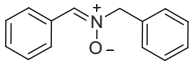
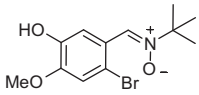
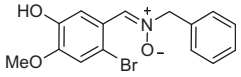
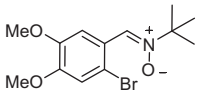
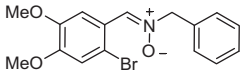
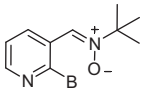
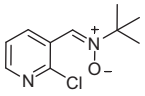
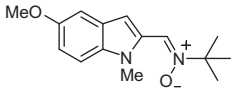
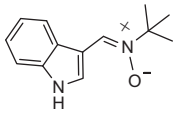
Chart 3.

Table 3
Torsion of the CC bond, and energy differences between the *anti* and *syn* rotamers in nitrones **9**, **11**, **18**, **19**, and **21** (kcal/mol)[a]

	9	11	18	19	21
Dihedral angle NCCC in **A** (°)	35.1	55.5	56.7	54.6	55.3
$\Delta E_{anti\text{-}syn}$ (kcal/mol)	−0.7	−4.2	−6.0	−8.3	−4.5

[a] Geometry optimization using B3LYP/6-31G*.

Table 4
The potential of ionization, Energies for the formation of adducts-spin nitrone-OH· and nitrone-OOH· of nitrones **7–12**, **18**, **19**, **21**, **23**, and curcumin

Nitrone	Structure	Potential of ionization[a] (eV)	ΔE_{rxn} [b] (kcal/mol)	ΔE_{rxn} [c] (kcal/mol)
7 (PBN)		7.10	−54.1	−18.3
8		7.45	−50.7	−19.8
9		6.61	−51.0	−22.4
10		6.70	−49.8	−23.7
11		7.42	−53.4	−20.7
12		7.49	−50.6	−18.3
18		7.10	−52.7	−20.5
19		7.39	−51.8	−16.8
21		7.02	−50.9	−23.7
23		7.12	−48.3	−19.0
	Curcumin	6.38	nd[d]	nd[d]

[a] The potential of ionization of nitrones **7–12**, **18**, **19**, **21**, **23**, and curcumin.
[b] Energies for the formation of adducts-spin nitrone-OH·.
[c] Energies for the formation of adducts-spin nitrone-OOH·.
[d] nd, not determined.

the analysis of the antioxidant activity of our nitrones. In this context, the determination of the reaction free energy in the formation of the adduct-spin gives a good correlation between the values of the potential of ionization and the experimental kinetic constants.[28] Accordingly, this theoretical approach can be useful in order to predict the respective reactivities.

The NPA (Natural Population Analysis) proves that the spin density in the adduct is mainly delocalized over the N–O group.[29] In the case of nitrone **7** (PBN), for instance, the N and O atoms showed values of 355% and 63%, respectively. In Chart 4 we have depicted the calculated spin-density for the nitrone **7** and hydroxyl radical adduct.

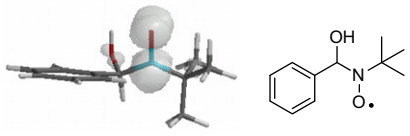


Chart 4. Spin-density in the adduct **7** (PBN)-OH.

In Table 4, we have included the calculated values for all the spin-adducts nitrone-OH·. We did not calculate the correspondence for curcumin, because, in this case, the position of the radical attack is unknown. These data show that the reaction is strongly exothermic, and consequently, this is a very favourable thermodynamic process.[30] The energy differences between the different nitrones were small, taking into account the high exothermicity, a result that is in agreement with the experimental values (see Table 2), showing that all these molecules are good trapping agents, and react similarly to radical ·OH.

The results shown in Table 4 confirm the hypothesis that different mechanisms account for trapping the different ROS, and suggest that the ability to transfer hydrogen atoms has no relation with the stability of the hydroxyl radical.[31]

On the other hand, the computational calculation of the energy reaction in the formation of the adduct-spin between nitrones and the hydroperoxyl ·OOH radical, has previously been used to predict its reactivity.[32] In the case of nitrones **7–12**, **18**, **19**, **21**, and **23**, we have observed that the most stable conformation for the adduct-spin has an intramolecular OO–H···O–N hydrogen bond (for nitrone **21**, see Chart 5) with bond lengths of 1.84 and 2.19 Å. Similarly to the nitrone adducts-spin with radical OH·, the spin density is now transferred and concentrated over the nitroxyl group N–O. In nitrone **7** (PBN), the N and O atoms show values of 39% and 56%, respectively.

The calculated values range from −16.8 to −23.7 kcal/mol (Table 4), showing also an exothermic reaction between OOH· and nitrones, although less strong than in the case of the OH· addition. These values predict that the most favourable adduct-spin will take place between nitrones **9**, **10**, and **21**, a fact that would be in agreement with their experimentally observed scavenger ability (Table 2); conversely, nitrone **23** would not be a good radical trap agent.

The capacity to cross the brain–blood–barrier (BBB) is of paramount importance for a drug in order to reach its biological target and act as an active agent in the CNS. The experimental determination of the BBB parameter is not very easy, and this is the reason why the theoretical prediction, using CSBBB (software ChemSilico)[33] is an alternative path, although the method is not very reliable yet. As shown in Table 5, and taking into account that the higher the log BBB is, the higher the ability to cross the BBB, the predicted capacity in decreasing order would be as follows: **8** ≫ **21** ⩾ **12**, **11** > **10**.

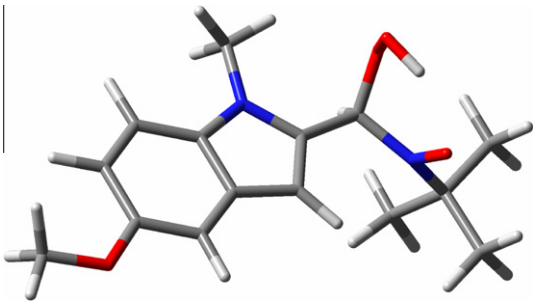
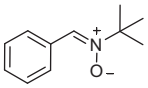
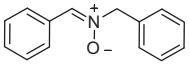
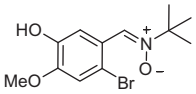
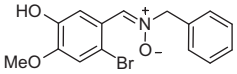
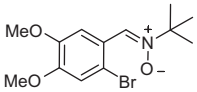
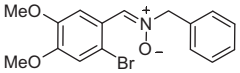
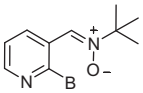
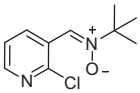
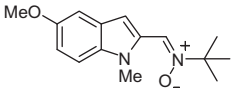
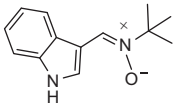


Chart 5. Hydrogen-bond in the adduct-spin nitrone **21**-OOH·.

Table 5
Theoretical determination of log *P*, neuroprotection and log BBB, in nitrones **7–12**, **18**, **19**, **21**, **23**, and curcumin

Nitrone	Structure	log *P*	Neuroprotection[5g] (%)	log BBB[a]
7 (PBN)		1.86	73	0.16 (0.15)
8		2.21	111	0.55 (0.22)
9		2.26	87	0.01 (0.19)
10		2.63	111	0.23 (0.10)
11		2.45	70	0.34 (0.23)
12		2.82	116	0.36 (0.12)
18		1.71	76	0.00 (0.15)
19		1.55	74	0.03 (0.15)
21		2.36	50	0.39 (0.17)
23		2.02	60	−0.05 (0.18)
	Curcumin	3.16	106	−0.95 (0.34)

[a] Standard shifts in parenthesis.

The determination of log *P* (partition coefficient in octanol–water) has been carried out with VCCLAB software.[34] The values in table 5 suggest that these nitrones are soluble in lypophilic media, and, consequently, they should be active in the CNS. According to the Lipinski rules, the log *P* should be less than 5,[35] but others have suggested that the optimal value must be between 1.5 and 2.0.[5g]

Goldstein and colleagues used QSAR for the determination of the neuroprotection of nitrones using the log *P* values and the HOMO orbital energies,[5g] and Eq. 1:

$$\text{Neuroprotection}\ (\%) = 33.2 * \log P - 2.15 * E_{(HOMO)} - 379.43 \quad (1)$$

One of the most interesting results is that nitrones bearing the *N*-benzyl group must show better neuroprotective profile than those with a *N*-*t*-butyl substituent [**12** vs **11**; **8** vs **7**; **10** vs **9**]. In addition, it seems that nitrones bearing the *N*-benzyl group show better capacity to cross the BBB, and the type of the halogen (Br vs Cl in nitrones **18** and **19**) atom has no effect on these properties.

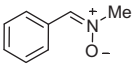
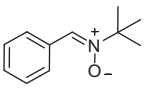
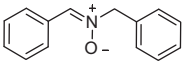
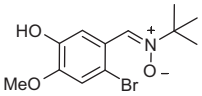
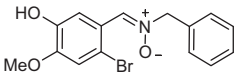
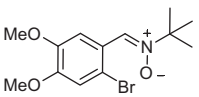
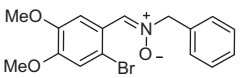
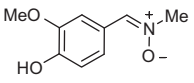
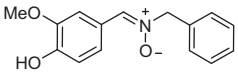
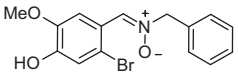
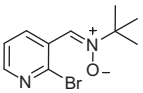
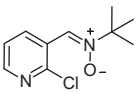
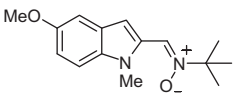
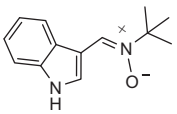
2.4. In vitro neuroprotection assay

Next, the in vitro pharmacological analysis was carried out in order to determine the neuroprotective capacity of these nitrones.

Then, we investigated the neuroprotective effect of nitrones **6–15**, **18**, **19**, **21**, and **23** against rotenone/oligomycin-A-induced death of human neuroblastoma SH-SY5Y cells. The mixture of rotenone plus oligomycin-A blocks mitochondrial electron transport chain complexes I and V, respectively,[36] inducing cell death by oxidative stress.

The neuroprotective effect of nitrones **6–15**, **18**, **19**, **21**, and **23** against this toxic stimulus was evaluated by the method of MTT metabolic reduction,[37a] at the concentration of 1 μM, in SH-SY5Y neuroblastoma cells exposed to 30 μM rotenone plus 10 μM oligomycin-A for 24 h, as described.[37b] As shown in Table 6, the neuroprotective profile of the target molecules was in general low, with

Table 6
Neuroprotection induced by nitrones **6–15**, **18**, **19**, **21**, and **23** (1 μM) in human neuroblastoma cells stressed with a mixture of rotenone/oligomycin-A[a]

Nitrones	Structure	MTT reduction (% vehicle)	% Protection
6		64.7 ± 5.4	18.7
7 (PBN)		69.6 ± 2.9	18.6
8		70.8 ± 7.1	18.4
9		65 ± 3	11
10		64.2 ± 2.5	8.7
11		63.1 ± 3.3	0
12		65.3 ± 6.9	3
13		60.9 ± 4.7	9.7
14		63.9 ± 6.1	16.9
15		62.8 ± 4.4	13.4
18		60.1 ± 7.3	24
19		61.4 ± 5.5	0
21		62.8 ± 3	4.4
23		64.1 ± 2.1	8
NAC (1 mM)		63.1 ± 3.7	25
Melatonin (10 nM)		59 ± 2.4	17

[a] Data are expressed as the means ± SEM of at least three different cultures in quadruplicate.

nitrones **18**, and **6**–**8**, the most potent, showing values in the range 24–18.4%. Note also that phenol nitrone **14** showed a significant neuroprotective value (16.9%). *N*-Acetylcisteine (NAC) at 1 mM and melatonin at 10 nM were used as positive controls.

3. Conclusions

In the context of our current project targeted to the development of new drugs for the treatment of cerebral ischemia, we have synthesized 11 new nitrones (**9**–**15**, **18**, **19**, **21**, and **23**), and three known nitrones (**6**–**8**) for comparative purposes. The archetypal nitrone **7** (PBN) has been used as internal reference compound. Regarding the in vitro antioxidant activity, for the DPPH radical test, and as expected, nitrones **13** and **14** bearing free phenol groups gave the best RSA (%) values in this assay. In the ORAC analysis, the most potent radical scavenger was nitrone indole **21**, followed by the *N*-benzyl benzene-type nitrones **10**, and **15**. Notice, also, that out of the five nitrones bearing a phenol group, the most potent in the DPPH test was nitrone **14**, bearing an hydroxyl group and no bromine atom in *para* and *ortho* positions, respectively, respect to the nitrone moiety. Conversely, the most potent in the ORAC assay was nitrone **10**, bearing an hydroxyl group and a bromine atom in *meta* and *ortho* positions, respectively, respect to the nitrone group. Very interestingly, the archetypal nitrone **7** (PBN) gave a low RSA value (1.4%) in the DPPH test, or was inactive in the ORAC assay. Concerning the ability to scavenge the hydroxyl radical, we were aware that, since the hydroxyl radical is able to react with everything just limited by its diffusion speed, the hydroxyl scavenging assay cannot make differences between the compounds. Consequently, and not surprisingly, the nitrones studied proved active in this experiment, showing high values in the 94–97% range, the most potent being nitrone **14**.

Regarding the prediction of the antioxidant power using theoretical calculations, the potential of ionization confirms that nitrones **9** and **10** are the best compounds in electron transfer processes, a result that is also in good agreement with the experimental values in the DPPH assay. The calculated energy values for the reaction of ROS (hydroxyl, peroxyl) with the nitrones predict that the most favourable adduct-spin will take place between nitrones **9**, **10** and **21**, a fact that would be in agreement with their experimentally observed scavenging ability. However, nitrone **23** would not be a good radical trap agent.

Finally, the in vitro pharmacological analysis, the neuroprotective profile of the target molecules was in general low, with values ranging from 0% to 18.7%, in human neuroblastoma cells stressed with a mixture of rotenone/oligomycin-A, the most potent nitrones (**6**–**8** and **18**) showing values in the range 24–18.4%.[38]

Overall, the nitrones studied in this work[39] show a good antioxidant and neuroprotective profile, as the first step in our projected investigation of their potential use as drugs for the treatment of cerebral ischemia. Other studies and analyses are now in progress and will be reported in due course.

4. Experimental part

4.1. General methods

Reactions were monitored by TLC using precoated silica gel aluminium plates containing a fluorescent indicator (Merck, 5539). Detection was done by UV (254 nm) followed by charring with sulfuric-acetic acid spray, 1% aqueous potassium permanganate solution or 0.5% phosphomolybdic acid in 95% EtOH. Anhydrous Na_2SO_4 was used to dry organic solutions during work-ups and the removal of solvents was carried out under vacuum with a rotary evaporator. Flash column chromatography was performed using Silica Gel 60 (230–400 mesh, Merck). Melting points were determined on a Kofler block and are uncorrected. IR spectra were obtained on a Perkin–Elmer Spectrum One spectrophotometer. ^{1}H NMR spectra were recorded with a Varian VXR-200S spectrometer, using tetramethylsilane as internal standard and ^{13}C NMR spectra were recorded with a Bruker WP-200-SY. All the assignments for protons and carbons were in agreement with 2D COSY, HSQC, HMBC, and 1D NOESY spectra. Values with (*) can be interchanged. Elemental analyses were conducted on a Carlo Erba EA 1108 apparatus. 1,1-Diphenyl-2-picrylhyrazyl (DPPH) radical, sodium benzoate, $FeSO_4{\cdot}7H_2O$, EDTA, 30% H_2O_2, nitro blue tetrazolium chloride, NADH and phenazine methosulfate were purchased from Sigma–Aldrich. Phosphate buffer (0.1 M and pH 7.4) was prepared mixing an aq KH_2PO_4 solution (50 mL, 0.2 M), and an aq of NaOH solution (78 mL, 0.1 M); the pH (7.4) was adjusted by adding a solution of KH_2PO_4 or NaOH.

4.2. General procedure for the synthesis of nitrones

Method A:[5d] In a 20 mL glass tube equipped with septa, the aldehyde, dry Na_2SO_4 (2.81 equiv) and triethylamine (2 equiv) were suspended in dry THF. Then, the hydroxylamine hydrochloride (2 equiv) was added. The mixture was stirred for 30 s, and then exposed to MWI (250 W) at 80 °C during the time indicated for each compound. When the reaction was over (TLC analysis), the reaction mixture was diluted with water, extracted with CH_2Cl_2, dried over anhydrous sodium sulphate, filtered and the solvent was evaporated. The resultant solid was purified by column chromatography to give pure compounds. *Method B*:[17] In a 20 mL glass tube equipped with septa, the aldehyde, the hydroxylamine hydrochloride (1 equiv) and NaOAc (1.2 equiv) were exposed to MWI (250 W) at 80 °C during the time indicated for each compound. The formed solid was washed with CH_2Cl_2, the solvent was evaporated and the resultant solid was purified by column chromatography to give pure compounds. *Method C*:[18] To a solution of the aldehyde in EtOH (0.14 M) was added methyl-2-nitropropane (2 equiv) and zinc (3 equiv). The reaction mixture was cooled to 0 °C, and glacial acetic acid (6 equiv) was added dropwise. The reaction mixture was stirred at room temperature for 6 h, and then was kept at 0 °C for 12 h. The precipitate was filtered, washed with EtOH. The solvent was evaporated and the resultant solid was purified by column chromatography to give pure compounds

4.2.1. (*Z*)-α-Phenyl-*N*-methylnitrone (6)[19]

Following the general procedure (method B), reaction of benzaldehyde (**1**) (0.38 mL, 3.76 mmol), *N*-methylhydroxylamine hydrochloride (0.25 g, 3.13 mmol) and NaOAc (0.308 g, 3.76 mmol), after 5 min and column chromatography (hexane/EtOAc, 1:1, v/v), gave compound **6** (0.317 g, 75%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.26–8.18 (m, 2H), 7.46–7.40 (m, 3H), 7.38 (s, 1H), 3.85 (s, 3H); MS (EI) *m/z* (%): 135 $[M]^+$ (66), 134 $[M-H]^+$ (66). Anal. Calcd for C_8H_9NO: C, 71.09; H, 6.71; N, 10.36. Found: C, 70.87; H, 6.82; N, 10.24.

4.2.2. (*Z*)-α-Phenyl-*N*-*tert*-butylnitrone (7)[2,19]

Following the general procedure (method C), reaction of benzaldehyde (**1**) (0.48 mL, 4.71 mmol), 2-methyl-2-nitropropane (1.02 mL, 9.42 mmol), Zn (924 mg, 14.13 mmol) and acetic acid (1.61 mL, 28.26 mmol) in EtOH (35 mL), and column chromatography (0.5% of MeOH in DCM), gave compound **7** (400 mg, 48%) as a solid: mp 72–4 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40–8.12 (m, 2H), 7.53 (s, 1H), 7.44–7.32 (m, 3H), 1.60 (s, 9H); MS (EI) *m/z* (%): 177 [M] (33), 121 (25), 57 (100). Anal. Calcd for $C_{11}H_{15}NO$: C, 74.54; H, 8.53; N, 7.90. Found: C, 74.29; H, 8.71; N, 8.02.

4.2.3. (*Z*)-α-Phenyl-*N*-benzylnitrone (8)[19]

Following the general procedure (method B), reaction of benzaldehyde (**1**) (0.38 mL, 3.76 mmol), *N*-benzylhydroxylamine hydrochloride (0.5 g, 3.13 mmol) and NaOAc (0.308 g, 3.76 mmol), after 3 min, and column chromatography (hexane/EtOAc from 5:1 to 2:1, v/v), gave compound **8** (668 mg, 84%) as a solid: mp 84–6 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.25–8.12 (m, J = 9.8 Hz, 2H), 7.54–7.30 (m, 9H), 5.01 (s, 2H); MS (EI) *m/z* (%): 211 [M] (31), 91 (100). Anal. Calcd for $C_{14}H_{13}NO$: C, 79.59; H, 6.20; N, 6.63. Found: C, 79.31; H, 6.42; N, 6.72.

4.2.4. (*Z*)-α-(2′-Bromo-5′-hydroxy-4′-methoxyphenyl)-*N*-*tert*-butylnitrone (9)

Following the general procedure (method A), reaction of 2-bromo-5-hydroxy-4-methoxybenzaldehyde (**2**)[12] (44 mg, 0.192 mmol), Na_2SO_4 (77 mg, 0.54 mmol), Et_3N (50 μL, 0.38 mmol) and *N*-*tert*-butylhydroxylamine hydrochloride (48 mg, 0.384 mmol) in THF (1.3 mL), after 3 h, and column chromatography (hexane/EtOAc from 2:1, v/v), nitrone **9** (38 mg, 65%) as a solid: mp 186–8 °C; IR (KBr) 2975, 2937, 2762, 1598, 1498, 1428, 1392, 1362, 1274, 1098, 1028 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.18 (s, 1H), 8.77 (s, 1H), 8.00 (s, 1H), 7.01 (s, 1H), 3.85 (s, 3H), 1.62 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.7, 145.6, 131.8, 121.9, 116.2, 115.6, 114.6, 71.3, 56.0, 28.6; MS (EI) *m/z* (%): 302 [M] (5), 222 (19), 166 (100), 57 (40). Anal. Calcd for $C_{12}H_{16}BrNO_3$: C, 47.70; H, 5.34; N, 4.64; Br, 26.44. Found: C, 47.54; H, 5.21; N, 4.72; Br, 26.45.

4.2.5. (*Z*)-α-(2′-Bromo-5′-hydroxy-4′-methoxyphenyl)-*N*-benzylnitrone (10)

Following the general procedure (method A), reaction of 2-bromo-5-hydroxy-4-methoxybenzaldehyde (**2**)[12] (200 mg, 0.866 mmol), Na_2SO_4 (344 mg, 2.42 mmol), Et_3N (0.24 mL, 1.73 mmol) and *N*-benzylhydroxylamine hydrochloride (276 mg, 1.73 mmol) in THF (5.8 mL), after 55 min, and column chromatography (hexane/AcOEt 1:1, v/v), gave nitrone **10** (266 mg, 91%) as a solid: mp >230 °C; IR (KBr) 3082, 2836, 1592, 1501, 1454, 1439, 1413, 1277, 1207, 1174, 1144, 1026 cm^{-1}; ^{1}H NMR (300 MHz, DMSO) δ 9.50 (s, 1H), 8.79 (s, 1H), 7.94 (s, 1H), 7.60–7.27 (m, 5H), 7.20 (s, 1H), 5.13 (s, 2H), 3.80 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 149.3, 145.20, 134.6, 131.4, 129.0, 128.4, 128.3, 122.1, 116.1, 114.9, 111.8, 70.3, 55.9; MS (EI) *m/z* (%): 336 [M] (1), 256 (54), 91 (100). Anal. Calcd for $C_{15}H_{14}BrNO_3$: C, 53.59; H, 4.20; N, 4.17; Br, 23.77. Found: C, 53.38; H, 4.43; N, 4.33; Br, 23.66.

4.2.6. (*Z*)-α-(2-Bromo-4,5-dimethoxyphenyl)-*N*-*tert*-butylnitrone (11)

Following the general procedure (method B), reaction of 2-bromo-4,5-dimethoxybenzaldehyde (**3**)[13] (0.47 g, 1.92 mmol) with *N*-*tert*-butylhydroxylamine hydrochloride (0.2 mg, 1.6 mmol) and NaOAc (157 mg, 1.92 mmol), after 2 min, and column chromatography [hexane/AcOEt, from 5:1 to 2:1, v/v], gave compound **11** (213 mg, 42%) as a solid: mp 123–5 °C; IR (KBr) 2971, 1502, 1285, 1217, 1168, 1023 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.18 (s, 1H), 7.95 (s, 1H), 7.04 (s, 1H), 3.91 (s, 3H), 3.86 (s, 3H), 1.58 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 150.3, 147.8, 129.1, 122.9, 115.6, 115.4, 111.7, 71.6, 56.2, 56.1, 28.4; MS (EI) *m/z* (%): 315 [M] (4), 236 (13), 180 (100), 57 (34). Anal. Calcd for $C_{13}H_{18}BrNO_3$: C, 49.38; H, 5.74; N, 4.43. Found: C, 49.61; H, 5.92; N, 4.48.

4.2.7. (*Z*)-α-(2′-Bromo-4′,5′-dimethoxyphenyl)-*N*-benzylnitrone (12)

Following the general procedure (method B), reaction of 2-bromo-4,5-dimethoxybenzaldehyde (**3**)[13] (1.1 g, 4.5 mmol), with *N*-benzylhydroxylamine hydrochloride (0.6 g, 3.8 mmol) and NaOAc (0.369 g, 4.5 mmol), after 3 min, and column chromatography (hexane/EtOAc from 5:1 to 2:1, v/v), gave nitrone **12** (841 mg, 64%): mp 164–6 °C; IR (KBr) 3068, 3010, 2951, 1592, 1497, 1443, 1387, 1277, 1218, 1171 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.08 (s, 1H), 7.78 (s, 1H), 7.57–7.26 (m, 5H), 6.98 (s, 1H), 5.00 (s, 2H), 3.84 (s, 3H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 150.5, 147.6, 133.1, 133.07, 129.3, 129.0 (2 C), 122.3, 115.3, 115.0, 111.5, 71.7, 56.1, 56.0; MS (EI) *m/z* (%): 349 [M−1] (4), 270 (54), 91 (100). Anal. Calcd for $C_{16}H_{16}BrNO_3$: C, 54.87; H, 4.61; N, 4.00; Br, 22.82. Found: C, 54.87; H, 4.55; N, 4.02; Br, 22.68.

4.2.8. (*Z*)-α-(4′-Hydroxy-3′-methoxyphenyl)-*N*-methylnitrone (13)

Following the general procedure (method A), reaction of commercial 3-hydroxy-4-methoxybenzaldehyde (**4**) (0.15 g, 1 mmol), dry Na_2SO_4 (0.39 g, 2.8 mmol), *N*-methylhydroxylamine hydrochloride (0.16 g, 2 mmol) and triethylamine (0.28 mL, 2 mmol) in THF (1.35 mL), after 3 h, and column chormatography (2% of MeOH in DCM), gave nitrone **13** (0.18 mg, 60%): mp 177–9 °C; IR (KBr) 2992, 2963, 2938, 2744, 2585, 1594, 1528, 1423, 1403, 1289, 1261, 1153, 1124 cm^{-1}; ^{1}H NMR (400 MHz, DMSO) δ 9.63 (br s, 1H, OH), 8.11 (d, J = 1.6 Hz, 1H, CH-2), 7.64 (s, 1H), 7.54 (dd, J = 8.4 and 1.6 Hz, 1H, CH-6), 8.21 (d, J = 8.4 Hz, 1H, CH-5), 3.74 (s, 3H, OCH_3), 3.68 (s, 3H, NCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.3 (C-3), 146.8 (C-4), 133.8 (CH=N), 122.9 (C-1), 122.3 (C-6), 115.2 (C-5), 111.4 (C-2), 55.4 (OCH_3), 53.5 (NCH_3); MS (ES) *m/z* (%): 204 [M+Na]$^+$ (30), 182.2 [M+H]$^+$ (100). Anal. Calcd for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.45; H, 6.31; N, 7.84.

4.2.9. (*Z*)-α-(4′-Hydroxy-3′-methoxyphenyl)-*N*-benzylnitrone (14)

Following the general procedure (method A), reaction of commercial 4-hydroxy-3-methoxybenzaldehyde (**4**) (0.15 g, 1 mmol), dry Na_2SO_4 (0.39 g, 2.8 mmol), *N*-benzylhydroxylamine hydrochloride (0.32 g, 2 mmol) and triethylamine (0.28 mL, 2 mmol) in THF (1.35 mL), after 4 h, and column chromatography (1% of MeOH in DCM), gave compound **14** (0.14 g, 55%): mp 160–2 °C; IR (KBr) *ν* 3226, 2956, 2934, 2556, 1625, 1580, 1464, 1288 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 (d, J = 2 Hz, 1H, CH-2), 7.48–7.42 (m, 2H), 7.41–7.35 (m, 3H), 7.31 (s, 1H, CH=N), 7.19 (dd, J = 8.4 and 2 Hz, 1H, CH-6), 6.90 (d, J = 8.8 Hz, 1H, CH-5), 6.07 (br s, 1H, OH), 5.01 (s, 2H, CH_2), 3.84 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.4 (C-4), 146.3 (C-3), 135.2 (CH=N), 133.0 (C-Ph); 129.1 (2 × CH), 128.8 (2 × CH), 124.1 (C-6), 122.6 (C-1), 114.4 (C-5), 110.7 (C-2), 70.3 (CH_2), 55.8 (OCH_3); MS (ES) *m/z* (%): 258.2 [M+H]$^+$ (100), 280.2 [M+Na]$^+$ (30). Anal. Calcd for $C_{15}H_{15}NO_3$: C, 70.02; H, 5.88; N, 5.44. Found: C, 69.88; H, 5.75; N, 5.31.

4.2.10. (*Z*)-α-(2′-Bromo-4′-hydroxy-5-methoxyphenyl)-*N*-benzylnitrone (15)

Following the general procedure (method A), reaction of of 2-bromo-4-hydroxy-5-methoxybenzaldehyde (**5**)[14] (0.044 g, 0.19 mmol), dry Na_2SO_4 (0.077 g, 0.54 mmol), triethylamine (0.050 mL, 0.38 mmol) and *N*-benzylhydroxylamine hydrochloride (0.12 g, 1 mmol) in THF (1 mL), after 3 h, and column chromatography (3% of MeOH in DCM), gave nitrone **15** (0.12 g, 70%). mp 206–208 °C; IR (KBr) *ν* 3114, 3002, 2961, 2936, 2906, 2694, 2518, 1579, 1505, 1478, 1415, 1391, 1295, 1209, 1169, 1113, 875, 704 and 508 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.15 (s, 1H, CH-6), 7.85 (s, 1H, CH=N), 7.53–7.37 (m, 5H), 7.16 (s, 1H, CH-3), 6.09 (br s, 1H, OH), 5.06 (s, 2H, CH_2), 3.91 (s, 3H, OCH_3); ^{13}C NMR (300 MHz, $CDCl_3$) δ 148.0, 145.3, 133.8, 132.9, 129.3, 129.1, 129.0, 121.5, 118.7, 115.7, 111.3, 71.5, 56.1; EM (ES) *m/z* (%): 336.0 [M+H]$^+$ (100), 360.0 [M+Na+H]$^+$ (17), 695.3 [2M+Na]$^+$ (1);. Anal. Calcd for

$C_{15}H_{14}BrNO_3$: C, 53.59; H, 4.20; N, 4.17; Br, 23.77. Found: C, 53.37; H, 4.08; N, 4.23; Br, 23.49.

4.2.11. (*Z*)-α-(2′-Bromo-3-pyridyl)-*N*-*tert*-butylnitrone (18)

Following the general procedure (method A), reaction of commercial 2-bromonicotinaldehyde (**16**) (100 mg, 0.54 mmol), Na_2SO_4 (216 mg, 1.52 mmol), Et_3N (0.15 mL, 1.08 mmol) and *N*-*t*-butylhydroxylamine hydrochloride (135 mg, 1.08 mmol) in THF (4 mL), after 3 h, and column chromatography (hexane/EtOAc from 4:1, v/v), gave compound **18** (106 mg, 76%) as yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.56 (dd, J = 8.0, 2.0 Hz, 1H), 8.24 (dd, J = 4.7, 2.0 Hz, 1H), 8.02 (s, 1H), 7.27 (dd, J = 7.9, 4.7 Hz, 1H), 1.57 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.6, 143.0, 136.7, 128.1, 127.2, 123.0, 72.6, 28.3; MS (EI) *m/z* (%): 257 [M] (23), 201 (11), 177 (16), 121 (75), 57 (100). Anal. Calcd for $C_{10}H_{13}BrN_2O$: C, 46.71; H, 5.10; N, 10.89; Br, 31.08. Found: C, 47.00; H, 5.39; N, 10.73; Br, 30.87.

4.2.12. (*Z*)-α-(2′-Chloro-3-pyridyl)-*N*-*t*-butylnitrone (19)

Following the general procedure (method A), reaction of commercial 2-chloronicotinaldehyde (**17**) (100 mg, 0.71 mmol), Na_2SO_4 (283 mg, 1.99 mmol), Et_3N (0.19 mL, 1.4 mmol) and *N*-*t*-butylhydroxylamine hydrochloride (177 mg, 1.4 mmol) in THF (5 mL), after 105 min and column chromatography (hexane/EtOAc from 4:1, v/v), gave compound **19** (154 mg, 99%): mp 36–8 °C; IR (KBr) 2975, 1556, 1377, 1140 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.67 (d, J = 7.9 Hz, 1H), 8.30 (d, J = 4.2 Hz, 1H), 8.05 (s, 1H), 7.29 (dd, J = 7.9, 4.2 Hz, 1H), 1.61 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.7, 149.2, 136.9, 125.9, 124.8, 122.8, 72.5, 28.3; MS (ES) *m/z* (%): 213 [M$^+$+1], 235 [M+Na]$^+$, 447 [2M+Na]$^+$. Anal. Calcd for $C_{10}H_{13}ClN_2O$: C, 56.47; H, 6.16; N, 13.17; Cl, 16.67. Found: C, 56.16; H, 6.40; N, 12.99; Cl, 16.35.

4.2.13. (*Z*)-α-*N*-(5-Methoxy-1-methyl-1*H*-2-indolyl)-*N*-*tert*-butylnitrone (21)

Following the general procedure (method C), reaction of 5-methoxy-1-methyl-1*H*-indol-2-carbaldehyde (**20**)[15] (303 mg, 1.6 mmol), 2-methyl-2-nitropropane (0.34 mL, 3.2 mmol), Zn (314 mg, 4.8 mmol) and acetic acid (0.55 mL, 9.6 mmol) in EtOH (12 mL), after column chromatography (hexane/EtOAc, from 4:1 to 1:1, v/v), gave unreacted starting material **20** (189 mg), and nitrone **21** (87 mg, 56%): mp 182–4 °C; IR (KBr) 2953, 1561, 1510, 1484, 1339, 1212, 1144, 1028 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.73 (s, 1H), 7.15 (d, J = 8.9 Hz, 1H), 7.06 (d, J = 2.3 Hz, 1H), 6.92 (dd, J = 8.9, 2.3 Hz, 1H), 3.83 (s, 3H), 3.70 (s, 3H), 1.62 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 154.4, 133.9, 130.8, 128.0, 120.0, 115.2, 109.8, 107.6, 102.8, 70.6, 55.9, 30.0, 28.5; MS (EI) *m/z* (%): 260 [M] (100), 229 (9), 204 (35), 187 (94). Anal. Calcd for $C_{15}H_{20}N_2O_2$: C, 69.20; H, 7.74; N, 10.76. Found: C, 69.31; H, 7.83; N, 10.60.

4.2.14. (*Z*)-α-(1*H*-3-Indolyl)-*N*-*tert*-butylnitrone (23)

Following the general procedure (method A) reaction of 1-*H*-indol-3-carbaldehyde (**22**)[16] (100 mg, 0.69 mmol), Na_2SO_4 (172 mg, 1.38 mmol), Et_3N (0.20 mL, 1.38 mmol) and *N*-*t*-butylhydroxylamine hydrochloride (172 mg, 1.38 mmol) in THF (5 mL), after 33 h, and column chromatography (11% of MeOH in EtOAc) gave unreacted **22** (16 mg) and compound **23** (114 mg, 91%): mp 209–211 °C; IR (KBr) 3066, 2873, 1602, 1505, 1442, 1362, 1233, 1097 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.99 (s, 1H), 9.01 (d, J = 2.7 Hz, 1H), 7.98 (s, 1H), 7.67 (d, J = 8.8 Hz, 1H), 7.39 (d, J = 8.8 Hz, 1H), 7.28–7.06 (m, 2H), 1.64 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 135.9, 129.6, 126.7, 124.8, 123.0, 120.7, 117.7, 112.2, 107.9, 68.6, 28.5; MS (EI) *m/z* (%): 216 [M] (100), 160 (98), 117 (93), 57 (48). Anal. Calcd for $C_{13}H_{16}N_2O$: C, 72.19; H, 7.46; N, 12.95. Found: C, 72.01; H, 7.62; N, 12.77.

4.3. Determination of antioxidant activity

4.3.1. DPPH radical

The assay was carried out in 96-well microplates and the final reaction mixture was 200 μL per well. A methanol DPPH solution (1.35×10^{-4} M, final concentration) (2.7 mL) was mixed with different concentrations of the nitrones (0, 100, 200, 300, 400, and 500 μM, final concentration) (0.3 mL) prepared freshly from a methanolic stock solution (10^{-3} M). The resulting mixture was incubated for 2 h at rt in the dark. After this time, 200 μL was placed in a 96-well microplate and the absorbance of DPPH radical was measured at 517 nm in a spectrophotometric plate reader (FluoStar OPTIMA, BMG Labtech). All reaction mixtures were prepared in quadruplicate and at least three independent runs were performed for each sample. The antioxidant activity was determined as the RSA% (sadical scavenging activity), calculated as follows: $RSA\% = 100[(A_o - A_i)/A_o) \times 100$; where A_o and A_i is the DPPH absorbance in absence and in presence of added nitrone concentration *i*, respectively. Data are expressed as means ± SEM.

4.3.2. ORAC assay

The radical scavenging activity of the nitrone was determined by the ORAC method using fluorescein as a fluorescence probe.[23] Briefly, the reaction was carried out at 37 °C in 75 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) contained fluorescein (70 nM), 2,2′-azobis(2-methyl-propionamidine)-dihydrochloride (12 mM), and antioxidant [Trolox (1–8 μM) or nitrone (at different concentrations)]. A Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters was used. The equipment was controlled by the Fluostar Galaxy software version (4.11–0) for fluorescence measurement. Black 96-well untreated microplates (Nunc, Denmark) were used. The plate was automatically shaken before the first reading and the fluorescence was recorded every minute for 98 min. 2,2′-Azobis(2-methyl-propionamidine)-dihydrochloride and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM phosphate buffer (pH 7.4). All reaction mixtures were prepared in duplicate and at least three independent assays were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$AUC = 1 + \sum_{i=1}^{i=98} f_i/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time *i*. The net AUC corresponding to a sample was calculated as follows:

$$\text{net AUC} = AUC_{antioxidant} - AUC_{blank}$$

The net AUC was plotted against the antioxidant concentration and the regression equation of the curve was calculated. The ORAC value was obtained by dividing the slope of the latter curve between the slope of the Trolox curve obtained in the same assay. Final ORAC values were expressed as μmol of Trolox equivalents/μmol of nitrone. Data are expressed as means ± SEM.

4.3.3. The benzoic acid method for the hydroxyl radical scavenging activity

In a screw-cap test tube, sodium benzoate (10 mmol), $FeSO_4 \cdot 7H_2O$ (10 mmol), and EDTA (10 mmol) were added. Then, different concentrations of nitrone (0, 10, 25, 50, 75, 100, 200, 300, 400,

and 500 μM) prepared freshly from a methanolic stock solution (10^{-3} M) and a phosphate buffer (pH 7.4) (0.1 mol) were mixed to give a total volume of 1.8 mL. Finally, H_2O_2 (10 mmol) was added, and the whole incubated at 37 °C for 2 h. After incubation, the fluorescence was measured at 407 nm emission with excitation at 305. All reaction mixtures were prepared in quadruplicate and at least three independent runs were performed for each sample. Data are expressed as means ± SEM.

4.3.4. Computational methods

Calculations have been carried out with GAUSSIAN03 (revision B.03).[40] The geometry optimization has been undertaken with the hybrid method DFT B3LYP,[41] using 6-31G(d) as bases of calculation. In the case of radical adducts, the final spin contamination values are very small ($0.75 < S^2 < 0.76$). Both estimated spin and charge densities have been obtained by NPA analysis[42] over the electronic wave functions.

4.3.5. Neuroprotection assay: quantification of SH-SY5Y cell viability by the MTT test

Cell death and neuroprotection were studied in the human neuroblastoma cell line SH-SY5Y, a kind gift from Dr. F. Valdivieso (Centro de Biología Molecular, CSIC, Madrid, Spain). SH-SY5Y cells were maintained in a 1:1 mixture of F-12 Nutrient Mixture (Ham12) and Eagle's minimum essential medium (EMEM) (Sigma–Aldrich, Madrid, Spain) supplemented with 15 non-essential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (reagents from Invitrogen, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For assays, SH-SY5Y cells were subcultured in 48-well plates at a seeding density of 1×10^5 cells per well. Cells were treated with the drugs before confluence in EMEM with 1% FBS. Cells were used at a low passage number (<13). Cell viability, virtually the mitochondrial activity of living cells, was measured by quantitative colorimetric assay with the mitochondrial probe MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (Sigma–Aldrich, Madrid, Spain). Briefly, SH SY5Y cells were seeded into 48-well culture plates and allowed to attach. MTT (5 mg/mL) was added, and incubation was carried out in the dark, at 37 °C for 2 h, followed by cell lysis, and spectrophotometric measurements at 540 nm. The tetrazolium ring of MTT can be cleaved by active reductases in order to produce a precipitated formazan derivative. The formazan produced was dissolved by adding 200 μL DMSO, resulting in a coloured compound whose optical density was measured in an ELISA reader at 540 nm. All MTT assays were performed in triplicate in cells of different batches.

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38. It is worth to point out that all the nitrones studied in this work are stable in the solvents used. We thank one of referees for comments on this regard.
39. It is well known that the low stability was in the origin of the failure of the clinical assays on PBN. Thus, in our case, it is important to highlight that in the experimental conditions of the antioxidant assays (see Section 4), the new nitrones were stable in the solvents used. We thank one the reviewers for attracting our attention to this point.
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