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α-Aryl-*N*-alkyl Nitrones, as Potential Agents for Stroke Treatment: Synthesis, Theoretical Calculations, Antioxidant, Anti-inflammatory, Neuroprotective, and Brain−Blood Barrier Permeability Properties

Mourad Chioua,[†,▽] David Sucunza,[†,▽] Elena Soriano,[†] Dimitra Hadjipavlou-Litina,*[,‡] Alberto Alcázar,*[,§] Irene Ayuso,[§] María Jesús Oset-Gasque,*[,∥,⊥] María Pilar González,[∥] Leticia Monjas,[#] María Isabel Rodríguez-Franco,[#] José Marco-Contelles,*[,†] and Abdelouahid Samadi[†]

[†]Laboratorio de Radicales Libres y Química Computacional, Instituto de Química Orgánica General (CSIC), Juan de la Cierva, 3, 28006-Madrid, Spain

[‡]Department of Pharmaceutical Chemistry, School of Pharmacy, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece

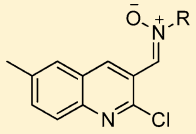
[§]Departamento Investigación, IRYCIS, Hospital Ramón y Cajal, Madrid, Spain

[∥]Departamento de Bioquímica y Biología Molecular II, Facultad de Farmacia, Universidad Complutense de Madrid (UCM), 28040 Madrid, Spain

[⊥]Instituto Universitario de Investigación en Neuroquímica (IUIN), Universidad Complutense de Madrid (UCM), 28040 Madrid, Spain

[#]Instituto de Química Médica (IQM-CSIC), Juan de la Cierva, 3, 28006-Madrid, Spain

ABSTRACT: We report the synthesis, theoretical calculations, the antioxidant, anti-inflammatory, and neuroprotective properties, and the ability to cross the blood−brain barrier (BBB) of (*Z*)-α-aryl and heteroaryl-*N*-alkyl nitrones as potential agents for stroke treatment. The majority of nitrones compete with DMSO for hydroxyl radicals, and most of them are potent lipoxygenase inhibitors. Cell viability-related (MTT assay) studies clearly showed that nitrones **1**−**3** and **10** give rise to significant neuroprotection. When compounds **1**−**11** were tested for necrotic cell death (LDH release test) nitrones **1**−**3**, **6**, **7**, and **9** proved to be neuroprotective agents. In vitro evaluation of the BBB penetration of selected nitrones **1**, **2**, **10**, and **11** using the PAMPA-BBB assay showed that all of them cross the BBB. Permeable quinoline nitrones **2** and **3** show potent combined antioxidant and neuroprotective properties and, therefore, can be considered as new lead compounds for further development in specific tests for potential stroke treatment.



(**2**, **3**)

INTRODUCTION

It is well-established that oxidative stress represents one of the most important molecular events after ischemic damage of membrane lipids, which leads to the death of cerebral tissue.[1] As a consequence, most of current research efforts in this area are focused on blocking and scavenging oxygenated free radicals.[2]

Neuronal membranes are rich in polyunsaturated fatty acids, which are particularly susceptible to free radicals attack at carbons adjacent to double bonds. The produced lipid hydroperoxides are not completely stable in vivo and, in the presence of metals or metal complexes, can be decomposed to reactive radicals, which will trigger the chain reactions. Potential consequences of damage to membrane lipids include changes in fluidity, in permeability, and in the orientation of proteins embedded in the bilayer of the plasma membrane, along with other cellular endomembranes.

In this regard, free radical scavengers, such as nitrones, have demonstrated to be effective as neuroprotective tools in experimental ischemia studies.[3] However, to date, nitrones have not been successful in human trials. This is the case of nitrone NXY-059,[4] a well-known free radical scavenger with good neuroprotective profile in rat models of transient and permanent focal ischemia and stroke model in rodents, which has been launched several times in different programmes in advanced clinical studies, although with limited success.[5] However, the efforts devoted to improving previous results, or to finding new nitrones for the treatment of ischemic stroke, showed that the neuroprotective strategy is still a choice for the development of new drugs for stroke.[6−12]

The case of edaravone, a potent free radical scavenger recently approved for the treatment of patients with acute stroke,[13] also confirms the viability of this approach. Among the nitrones, (*Z*)-α-phenyl-*N*-*tert*-butylnitrone (PBN), for instance, inhibits the oxidation of lipoproteins,[14] reduces the oxidative damage to erythrocytes, the peroxidation of lipids due to phenylhydrazine,[15] and protects gerbils from brain stroke and mice against MPTP toxicity.[16]

However, PBN mechanism of action is still not clear. It does not seem to be related to its ability to act as ROS trap but to the suppression of inducible nitric oxide synthase expression, cytokine accumulation, and apoptosis.[17] In fact, the formation of nitric oxide (NO) from PBN spin adducts may play a role in

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the observed effects in the central nervous system.[18] On the basis of these grounds, we have recently started a project targeted to the synthesis and biological evaluation of α-aryl, *N*-alkyl nitrones as potential drugs for cerebral ischemia treatment.[19]

In this work, we report the synthesis, theoretical calculations, antioxidant capacity, in vivo antinflammatory properties, and neuroprotective properties as well as the ability of nitrones **1**−**25** to cross the blood−brain barrier (BBB). From these studies, we conclude that the quinoline motif is a simple, readily available privileged heterocyclic ring system, leading to (*Z*)-*N*-((2-chloro-6-methylquinoline-3-yl)methylene)-2-methylpropan-2-amine oxide (nitrone **2**) and (*Z*)-*N*-((2-chloro-6-methylquinoline-3-yl)methylene)-1-phenylmethanamine oxide (nitrone **3**) as potent neuroprotective agents with strong antioxidant capacity, able to cross the BBB, for the potential treatment of stroke.

RESULTS AND DISCUSSION

1. Chemistry. (*Z*)-α-Aryl and heteroaryl *N*-alkyl nitrones **1**−**11** (Table 1) have been synthesized from the corresponding commercial [3-(trifluoromethyl)benzaldehyde, 2-chloro-6-methylquinoline-3-carbaldehyde, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde; 5-bromofuran-2-carbaldehyde] or from the readily available aldehydes [3-(4-formylphenoxy)propyl nitrate[20] and 2-(4-formylphenoxy)ethyl nitrate][20] with *N*-*tert*-butyl- or *N*-benzylhydroxylamine, as shown in Scheme 1. The aldehyde choice was based on the previous literature,[12] our results and experience in the topic,[19] which prompted us to use benzaldehydes substituted with strong electron-withdrawing groups or free phenol highly hindered moieties or with 2(3)-(nitrooxy)alkoxy) chains at position C-4 and heterocyclic (quinoline, furan) carbaldehydes containing halogen substituents in appropriate positions.

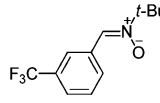
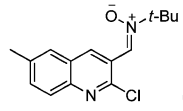
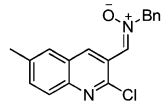
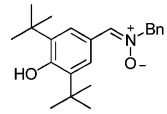
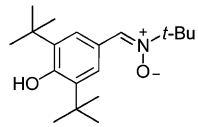
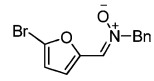
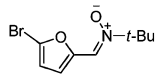
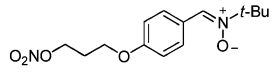
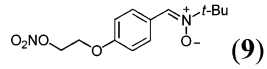
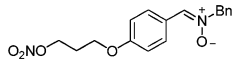
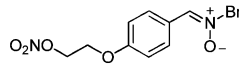
All new or known (**1**,[21] **4**,[22] **5**[22]) nitrones have been characterized by their analytical and spectroscopic data (see Experimental Section). The stereochemistry at the double bond has been assigned as *Z* by comparing NMR data with those analyzed and reported for known nitrones.

With these nitrones in hand, first of all, we carried out some theoretical calculations in order to predict the permeability of these compounds to the BBB, and their neuroprotective capacity.

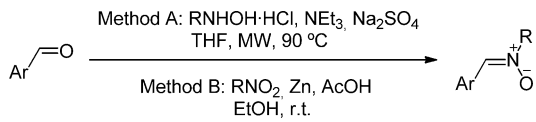
2. Theoretical Calculations. Because of the high attrition rate of central nervous system drug candidates during clinical trials, the assessment of BBB penetration in early research is particularly important. The BBB is the most important barrier between systemic circulation and the central nervous system (CNS) due to its substantially larger surface area. It constitutes a major challenge to the treatment of most brain disorders.[23] In the majority of the cases, the access of medicines to the brain is restricted at the level of the brain capillary endothelial wall, which forms the BBB. Therefore, potential CNS drugs frequently do not achieve sufficient drug concentration in the brain to show pharmacological activity.

One of the most commonly used parameters to quantify brain penetration is the ratio of concentration measured in the brain to that in the blood at steady state, expressed as logBB (log [brain]/[blood]). Although the extent of brain exposure is affected by various factors including influx and efflux transporters at the BBB, most compounds do cross the BBB via passive diffusion.

Table 1. Structure of the Nitrones 1−11, Synthetic Method (Scheme 1), and Chemical Yields

Nitrone	Method	Yield (%)
 (**1**)[21]	B	39
 (**2**)	B	28
 (**3**)	A	64
 (**4**)[22]	A	95
 (**5**)[22]	A	70
 (**6**)	A	81
 (**7**)	A	71
 (**8**)	A	63
 (**9**)	A	45
 (**10**)	A	87
 (**11**)	A	90

Scheme 1. Methods for the Synthesis of Nitrones 1−11



Because of our insufficient understanding of the molecular recognition processes involved in various active transport systems, the time-consuming, expensive, and difficult experimental in vivo determination of logBB, the in silico prediction of BBB permeation, is a research area of enormous interest. Most of the developed computational approaches are based on physicochemical properties, such as molecular weight, *n*-octanol−water partition coefficient, molecular surface area, and Gibbs free energy. Herein, we have applied the prediction software CSBBB[24] based on topological structure descriptors and developed by the use of artificial neural networks.

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The results, which are summarized in Table 2, suggest that all the nitrones, except for those bearing a 3-(nitrooxy) alkoxy) benzylidene substituent (nitrones **8**–**11**), should present a good brain penetration profile with logBB values in the range 0.11–0.64, the most permeable being nitrones **2** (logBB = 0.62) and **5** (logBB = 0.64).

Table 2. Estimated logBB and Percentage of Neuroprotection for Nitrones 1–11

nitrone	logBB (sd)[a]	neuroprotection (%)[b]
1	0.51 (0.12)	12
2	0.62 (0.17)	70
3	0.52 (0.14)	72
4	0.55 (0.16)	82
5	0.64 (0.23)	80
6	0.29 (0.14)	42
7	0.11 (0.18)	37
8	−0.26 (0.23)	30
9	−0.25 (0.23)	18
10	−0.28 (0.18)	31
11	−0.15 (0.19)	22

[a]Standard deviation shown in parentheses. [b]See ref 25 for details.

On other hand, it is well-established that organic molecules incorporating a nitrone moiety can act as free radical trapping agents (spin traps) and are capable of opposing oxidative challenges, although the operational mechanism of nitrones might not exclusively reside in their capacity to directly scavenge free radicals. Thus, the addition of free radicals to the carbon–nitrogen double bond of such molecules occurs very easily, yielding nitroxide radical species, which are generally much more stable and biochemically less harmful than the original free radicals. Because free radicals react via their SOMO (single occupied molecular orbital), improved spin trapping properties are expected for heteroatom radicals formed from nitrones (SOMO–HOMO control). The presence of heterocyclic ring and extended conjugation with the nitrone function would improve the bioavailability and reduce the toxicity characteristics of the nitrones in comparison to compounds described in the literature. As a matter of fact, high-level theoretical calculations indicate that these molecules possess a higher energy HOMO (highest occupied molecular orbital) compared to the nitrones with reduced conjugation.

With these observations in mind, we have estimated the neuroprotective effects (as % neuroprotection) following the approach by Goldstein et al.[25] based on the calculation of orbital energies and lipophilicity. The results suggest that the presence of a *p*-hydroxyl group in nitrones **4** and **5** should lead to excellent levels of neuroprotection. In addition, a quinolinyl group (nitrones **2** and **3**), which gives rise to a high energy HOMO, would also exhibit good levels of protection. On the other hand, the presence of a 3-(nitrooxy) alkoxy) benzylidene group (compounds **8**–**11**) would lead to a poor level of neuroprotection, mainly for short alkyl moieties (nitrones **9** and **11**).

Taking into consideration the theoretical results above, we carried out the corresponding in vitro antioxidant and neuroprotection analyses on these nitrones.

3. Antioxidant Evaluation. In this investigation, α-aryl-*N*-alkyl (methyl, *tert*-butyl, benzyl) and α-hetero (aryl)-*N*-alkyl (*tert*-butyl, benzyl) nitrones, have been tested as antioxidants. Antioxidants are defined as substances that, even at low concentration, significantly delay or prevent oxidation of easily oxidizable substrates. The formation of Reactive Oxygen Species (ROS) is characteristic of aerobic organisms, as an unavoidable consequence of cell metabolism. Their involvement in myocardial and CNS ischemia is under intensive study. The potential role of free radicals in cerebral ischemia is due to their extreme reactivity and their tendency to initiate and participate in chain reactions. Normally, defense against these highly reactive species can be accomplished by the organism using enzymes and naturally occurring antioxidants. For the estimation of the antioxidative potential of chemical components, different experimental approaches were used. The most popular screening assays use commonly available instrumentation. They have been developed to be fast and easy. Most of them require a spectrophotometric measurement and a certain reaction time in order to obtain reproducible results.[26] The used assays do not measure the same chemistry. In this way, factors such as solubility or steric hindrance, which may be of overriding importance in one environment but not in another, can be varied and the antioxidant ability of a compound in a variety of milieus may be evaluated.

3.1. Estimation of Inhibition of Lipid Peroxidation (LP) Induced by AAPH. AAPH induced linoleic acid oxidation has been developed as a quick and reliable method for measuring the antioxidant activity. The water-soluble azo compound AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride] has been extensively used as a clean and controllable source of thermally produced alkyl peroxyl free radicals. Azo compounds generating free radicals through spontaneous thermal decomposition are useful for in vitro studies of free radical production.[27] The use of the free radical reactions initiator AAPH is recommended as more appropriate to measure radical-scavenging activity in vitro because the activity of the peroxyl radicals produced by the action of AAPH shows a greater similarity to cellular activities such as lipid peroxidation.[28] Oxidation of sodium linoleate by a thermal free radical initiator (AAPH) is followed by UV spectrophotometry in a highly diluted sample.[27] In the AAPH assay, the highly reactive alkylperoxyl radicals are intercepted mainly by a hydrogen atom transfer (HAT) from the antioxidant.[29] Therefore, particularly effective HAT agents are compounds with high hydrogen atom donating ability, that is, compounds with low heteroatom-H bond dissociation energies and/or compounds from which hydrogen abstraction leads to sterically hindered radicals as well as compounds from which abstraction of hydrogen leads to C-centered radicals stabilized by resonance.

In Tables 3 and 4 we show the rate of increase in lipid peroxidation by measuring the formation of conjugated dienes as an indirect measure of lipid peroxidation (% inhibition). The induction period time t_{inh} produced by the tested nitrones representing the period where the added antioxidant inhibits lipid peroxidation by reacting with the AAPH derived peroxyl radical, itself being consumed by this reaction, is, in brackets, given in minutes. Thus, nitrone **4** with t_{inh} 88.2–83.0 min is very interesting and close to the inhibition time of trolox used as a reference compound. Compounds **1**, **6**, **7**, and **9** did not show any inhibition value, whereas **5** (9%) presented very limited value (Table 3). The remaining nitrones within this subgroup exhibited significant inhibition of lipid peroxidation, the most potent being nitrones **2** (85% and 56.7 min) and **10** (81% and 36.6 min) which, despite the different substructural characteristics, inhibited similarly the lipid peroxidation. It is

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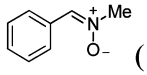
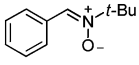
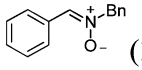
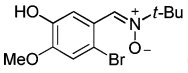
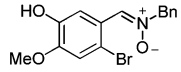
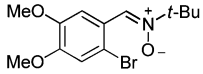
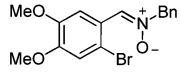
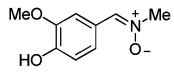
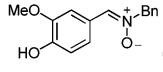
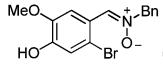
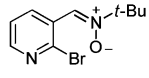
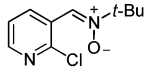
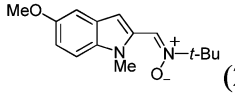
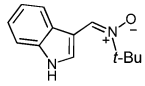
Table 4. In Vitro Antioxidant Activities for Nitrones 12−25

Nitrone	LOX (%)[d]	AAPH(%)[e] (min)[f]	HO•[g,h]	$O_2^{\bullet-}$ (%)[i]	NO (%)[k,(m)]	clog*P*
 **(12)**	22	5 (1)	77	8	No (No)	1.32
 **(13)** (PBN)[a]	83	No (0)	90	15	2 (4.5)	2.56
 **(14)**	No	74 (25.2)	97	24	nd[l] (No)	3.09
 **(15)**	No	67 (7.2)	72	11	2 (3)	2.83
 **(16)**	No	89 (84.6)	No	6	2 (2)	3.36
 **(17)**	No	47 (0.52)	90	15	1 (2)	3.15
 **(18)**	21	No (53.7)	No	16	1 (1)	3.68
 **(19)**	59	90 (18)	No	11	7 (8)	0.5
 **(20)**	No	90 (88.2)	52	No	9 (10)	2.67
 **(21)**	24	87 (41.8)	78	3	5 (6)	3.36
 **(22)**[b]	88	40 (8)	93	10	4 (5.5)	2.01
 **(23)**	59	37 (29.8)	30	5	2 (4)	1.86
 **(24)**	31	80 (43.8)	79.5	5	3 (5)	3.07
 **(25)**[c]	42	57 (21.6)	59	15	3 (4.5)	2.57
NDGA	40					
caffeic acid				15		
trolox		63 (62.1)	73			
SNP					54 (58)	

[a]0 at 0.01 mM, 0 at 0.05 mM. [b]0 at 0.01 mM, 0 at 0.05 mM. [c]0 at 0.01 mM, 0 at 0.05 mM: these results concern the % LOX inhibition. [d]Inhibition at 0.1 mM. [e]Inhibition at 0.1 mM. [f]Induction time t_{inh} produced by the tested nitrones. [g]Inhibition at 0.1 mM. [h]These results concern the ability of nitrones to compete with DMSO for hydroxyl radicals. [i]0.1 mM. [k]0.1 mM + 1 mM cysteine. [l]nd: not determined. [m]Nitrate reductase/NADPH system.

worth pointing out that nitrones **3** and **4**, with close lipophilicity values, have high t_{inh} values. Nitrones **11** (64%), **4** (61%), **8** (49%), and **4** (37%) follow.

Afterward, we carried out similar antioxidant studies with nitrones **12**−**25**, previously prepared in our laboratory (Table 4).[19] With the exception of compounds **13** and **18**, which did

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Table 3. In Vitro Antioxidant Activities for Nitrones 1−11

nitrone	LOX (%)[a]	AAPH (%)[b] (min)[c]	DPPH (RSA) (%)[d]	OH[f]	$O_2^{\bullet}$ (%)[g]	clogP
1	65	no (0)	0.9	60	29	3.44
2	no	85 (56.7)	1.7	100	12	3.74
3	no	37 (78)	42.3	95	23	4.27
4	no	61 (83)	81.9	77	8	5.87
5	no	9 (17.4)	78.6	no	no	5.34
6	17	no (0)	8.6	no	no	3.13
7	39	no (0)	0.0	95	no	2.60
8	57	49 (47)	0.4	54	2	3.29
9	19	no (0)	0.6	98	19	3.04
10	30	81 (36.6)	nd[h]	98	no	3.83
11	21	64 (46)	nd[h]	100	no	3.57
caffeic acid					15	
curcumin			35.0[e]			
quercetin			94.2[e]			
trolox		63 (62.1)				

[a]Determined at 0.1 mM. [b]Determined at 0.1 mM. [c]Induction time t_{inh} produced by the tested nitrones. [d]Determined at 0.5 mM. [e]Determined at 15 μM. [f]Determined at 0.1 mM. [g]Determined at 0.1 mM. [h]nd: not determined.

not show any inhibition, all other compounds caused inhibition of LP. Nitrone **18** did not present any inhibition at zero time. However, it showed 67% after 13 min and t_{inh} value = 53.7 min. Compounds **12**, **17**, **22**, **23**, and **25** showed inhibition values (5−57%) lower than the common standard trolox (63%). Obviously, the absence of hydrogen atoms (phenolic hydroxyl groups) has a direct impact on these results. The standard inhibitor trolox exerts its inhibitory effect on LP mainly through the ability of its 6-hydroxy-5, 7,8-trimethylchromane moiety to break the radical chain. On the other hand, nitrones **14**−**16**, **19**−**21**, and **24** gave higher values (67−90%) than trolox, the most potent inhibitors being the compounds **19** and **20** (90%). Nitrones **15**, **16**, **19**, **20**, and **21** might exert their antioxidative effect through their easily oxidizable hydroxyl unit. The antioxidative effect of nitrone **24** should be attributed through interception of the alkylperoxyl radicals mainly by the indole moiety and possible formation of oxidized derivatives. Lipophilicity, as clogP theoretically calculated values using the CLOG P program,[30] does not seem to influence their interaction.

3.2. Antioxidant Activity of Nitrones Determined by DPPH. The in vitro antioxidant activity for nitrones **1**−**11** was determined by the use of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and is given in Table 3. The results referred to nitrones **12**−**25** have been recently described.[19] The stable free radical 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).[31] A freshly prepared DPPH solution exhibits a deep-purple color with an absorption maximum at 517 nm. This purple color 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 into colorless/bleached product. In this assay, we measured the DPPH initial absorbance and the absorbance once the potential antioxidant had been added. The reduction of absorbance is a measure of the free DPPH due to the action of the antioxidant. The antioxidant activity was expressed as the RSA% (Radical Scavenging Activity). We used curcumin and quercetin as reference compounds. The RSA(%) values for nitrones **1**, **2**, and **6**−**9**, at 0.5 mM, are very low (Table 3). As shown, and as expected, nitrones **4** and **5** bearing free phenol groups provided the best RSA(%) values, in the 79−82% range. Quinoline **3** is a moderate antioxidant agent with a RSA value (42%) 24-fold more potent than the analogous quinoline **2**, stressing the critical importance of the *N*-benzyl group compared to the *N*-*tert*-butyl, in order to have higher RSA(%) values. Not surprisingly, nitrone **4** bearing the benzyl moiety is slightly more potent than nitrone **5**.

3.3. Competition of the Tested Compounds with DMSO for Hydroxyl Radicals. Superoxide ($O_2^{-\bullet}$) anion and hydroxyl radical ($^{\bullet}OH$) are free radical species of potential importance in cerebral ischemia. In the acidic conditions of ischemic brain, $O_2^{-\bullet}$ is probably protonated to give $HO_2^{-\bullet}$ species. Iron released from damaged brain cells is more likely to be readily available to catalyze the generation of OH radicals. Between the ROS, the hydroxyl ($^{\bullet}OH$) free radical is possibly the most toxic, as it reacts with a number of biological important molecules such as DNA, lipids, or carbohydrates. Polyunsaturated fatty acids are found in high concentrations in the CNS and are particularly vulnerable by free radicals. Thus, we tried to test the ability of our compounds to scavenge hydroxyl radicals. The competition of the synthesized nitrones with DMSO for HO radicals, generated by the Fe^{3+}/ascorbic acid system, expressed as percent inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity.[32] In this experiment, the majority of the tested nitrones showed remarkable activity at 100 μM, with values higher than the well-known antioxidant trolox (see Tables 3 and 4). Within the subgroup **1**−**11** (Table 3), nitrones **2**, **11**, **9**, **10**, **7**, and **3** exhibited 95−100% scavenging activity, whereas **4** with 77%, **1** with 60%, and **8** with 54% seemed to follow. Two nitrones, namely **5** and **6**, did not present any result under the experimental conditions. Derivatives with low lipophilicity values, such as nitrones **2**, **3**, **7**, **9**, **10**, and **11** (clogP = 2.60−3.74), showed high antioxidant activity. It is evident that the type and substitution pattern in the aromatic rings is important for the HO radical scavenging ability of the tested nitrones, e.g., the presence of the quinolinyl group or of the 3-(nitrooxy)-propoxy)benzylidene moiety.

Regarding nitrones **12**−**25**, (previously prepared in our laboratory[19]), the data are indicated in Table 4. Nitrones **16**, **18**, and **19** did not show any result at 0.1 mM concentration, whereas compound **23** presented 30% scavenging activity. All the other compounds significantly compete with DMSO at 0.1 mM, giving, in some cases, values higher than trolox (73%). It seems that lipophilicity does not play any significant role. However, antioxidants with hydrophilic or lipophilic character are both needed to act as radical scavengers in the aqueous phase or as chain-breaking antioxidants in biological membranes.

3.4. Superoxide Anion Radical ($O_2^{-\bullet}$) Scavenging Activity. The superoxide anion radical ($O_2^{-\bullet}$) is less toxic than the hydroxyl radical but still one of the most known toxic ROS. In addition, this radical suffers dismutation by the superoxide dismutase enzyme (SOD), producing H_2O_2, the precursor for the formation of hydroxyl radicals (see above) in the presence of iron ions.[33] Superoxide anion radicals $O_2^{\bullet}$ and H_2O_2 are

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reactive oxygen species produced in vivo, through a one-electron reduction process of molecular oxygen. The charged superoxide anion probably escapes via an anion channel in the cell membrane to enter the cerebrospinal fluid (CSF) and brain extracellular fluid. The evaluation of superoxide anion radical scavenging activity can be determined using assays involving nonenzymatic or enzymatic production of superoxide anions. Herein, enzymatic superoxide anion radicals were generated by a hypoxanthine and xanthine oxidase (XOD) reaction system. The majority of the nitrones of both groups did not even present any, or presented very low, scavenging activity at 0.1 mM compared to caffeic acid (15%), used as a standard reference compound. Only nitrone **1** showed 29% scavenging activity. At pH 7.4, superoxide anion reduces the tetrazolium blue into formazan blue (λ_{max} = 560 nm). The production of superoxide radical was estimated by the nitroblue tetrazolium method.[34] The superoxide anion scavenging activity ($O_2^{-\bullet}$) (%) of the tested nitrones has been spectrophotometrically measured.

3.5. In Vitro Inhibition of Soybean Lipoxygenase. Cerebral ischemia-reperfusion (IR) triggers lipid peroxidation and inflammation, which exacerbate injury. Recognition of inflammatory components involved in stroke has expanded the list of potential targets for therapy, including inducible nitric oxide synthase (iNOS), nuclear factor kappa B (NF-κB), and 5-lipoxygeanse (5-LOX).[35] LOX is the key enzyme in leukotriene biosynthesis.[36] Leukotrienes, derived from the biotransformation of arachidonic acid catalyzed by 5-lipoxygenase (5-LOX), cause inflammation and are thus involved in the pathobiology of stroke injury. LOXs play a role in membrane lipid peroxidation by forming hydroperoxides in the lipid bilayer.[37] Inhibitors of LOX have attracted attention initially as potential agents for inflammatory diseases treatment, but their therapeutic potential has now been expanded to certain types of cardiovascular diseases.[38] Inhibition of LOX was performed by the UV absorbance based enzyme assay.[32] While one may not extrapolate the quantitative results of this assay to the inhibition of mammalian LOX, it has been shown that inhibition of plant LOX activity by nonsteroidal anti-inflammatory agents (NSAIDs) is qualitatively similar to their inhibition of the rat mast cell LOX and may be used as a simple qualitative screen for such activity.[39] The four classes of direct 5-lipoxygenase inhibitors encompass: (a) redox-active compounds keeping the active site iron in the ferrous state, thereby uncoupling the catalytic cycle of the enzyme, (b) iron ligand inhibitors, (c) nonredox type inhibitors that compete with arachidonic acid or lipid hydroperoxides to bind to 5-LOX,[40] and (d) a novel class of inhibitors that may act in an allosteric manner.[41]

Among the nitrones **1**−**11**, the most potent representative is compound **1** followed by **8**, whereas nitrones **6**, **7**, **9**, **10**, and **11** showed moderate inhibition. In compounds **2** and **3**, which share the common structural feature of a quinolinyl substituent, as well as in nitrones **4** and **5**, in which a 3,5-di-*tert*-butyl-4-hydroxybenzylidene group is incorporated, no inhibitory activity was observed.

Perusal of the % inhibition values in Table 4 shows that the most potent inhibitors are the *N*-*tert*-butyl nitrones **22** (88%) and **13** (83%), presenting higher activity than the reference compound NDGA (40%). We were not able to determine their IC_{50} values under our experimental conditions. Thus, it seems that the inhibitory effect can mainly be attributed to the presence of the *N*-*tert*-butyl moiety (**23**−**25**), as the replacement of *N*-*tert*-butyl by *N*-methyl or *N*-benzyl groups diminishes/suppresses inhibition (e.g., **12** with *N*-methyl and **14** with *N*-benzyl group). Nitrones **12**, **18**, **19**, **21**, and **23**−**25** exhibit weaker inhibition. Nitrones **14**−**17** and **20** do not present any biological activity under the reported experimental conditions. Furthermore, it seems that within the pyridinyl analogues, the physicochemical properties of substituent at C-2 must be taken under consideration. Bulkier and more lipophilic substituents lead to more potent LOX inhibitors. Thus, the 2-Br derivative **22** (π-Br = 0.86, MR-Br = 0.888) exhibits higher activity than the 2-Cl substituted analogue **23** (π-Cl = 0.71, MR-Cl = 0.63).

Compounds incorporating phenol moieties do not seem to be particularly effective agents. Although lipophilicity is referred to as an important physicochemical property for LOX inhibitors,[42] among the tested nitrones, the most potent compound **22** has a % inhibition value of 88% and clogP 2.01, which does not follow this trend. On the contrary, nitrone **18**, with the higher lipophilicity value, shows low antilipoxygenase activity (21%, Table 4).

In general, the inhibitory activity of the nitrones does not seem to be correlated with their antioxidant activity. Because these molecules do not bear a close and chemical similarity to the arachidonic acid (the substrate of LOX), it is possible that they might bound not to the active site but to a separate enzyme site. Further studies will be necessary to confirm their mechanism of action.

3.6. NO Donating Activity. Nitric oxide (NO) is a radical acting as a bioregulator in many cells and tissues, a key inter- and intracellular messenger molecule involved in a variety of pathophysiological processes,[43] such as maintenance of the vascular tone, neuronal signaling, and host response to infection.[44] Additionally, NO may reduce inflammation connected to oxidative stress by scavenging ROS, which can adversely increase mucosal permeability and kill cells.[44] Furthermore, it is found that NO as well as NO-derived ROS, interact with peroxidases[45] and lipoxygenases,[46] altering the generation of prostaglandins and leukotrienes, which are signaling molecules involved in inflammation.[47] As a result, nitrones capable of acting as NO donors were introduced and new therapeutic possibilities have been emerged.[6−12]

The nitrones were tested in vitro for their ability to release NO by the action of a thiol cofactor. We tried to delineate, if possible, the influence of nitrone functionality and structure on NO release. NO donation with simultaneous production of cyanide occurs by action of reducing agents like thiols. L-Cysteine was used as a cofactor in this in vitro biological test. The rate of NO release is affected by pH, and the mechanism does not involve the intermediacy of *S*-nitrosothiols. In all experiments, sodium nitroprusside (SNP), a source of NO, as demonstrated by the ability to nitrosate amines and ketones, was used as the reference drug. The screened compounds (final concentration 100 μM) were tested for their ability to release NO when dissolved in a phosphate buffer containing L-cysteine (pH 7.4). Released NO was oxidized in the presence of air, transforming it to nitrites, which were detected by the Griess reaction.[48]

Virtually no formation of NO_2^- was detectable in the absence of L-cysteine for nitrones **1**−**11**. Very low if any formation of NO_2^- was observed from nitrones **19** and **20** (Table 4). The present study demonstrates that under the reported experimental conditions the NO release from the tested compounds

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is very limited and low and seems to be independent of the time.

Oxidation of NO by air can also yield nitrate, which is not detectable by the Griess assay. Therefore, to absolutely conclude that none of the nitrones released NO, we employed nitrate reductase/NADPH system to see if any residual nitrate can be reduced back to nitrite before using the Griess assay again. Very small changes were observed (values in parentheses in Table 4).

3.7. In Vivo Inhibitory Activity of the Carragenan Induced Rat Paw Edema. Inflammation is a key defense mechanism, mediated by a complex cascade of mediators. The inflammatory response in stroke involves not only leukocytes, endothelial and glial cells, but also neurons. After stroke, leukocytes home toward the lesion, and brain parenchymal cells (microglia, astrocytes, endothelia, even neurons) transform to an inflammatory phenotype.[49] Available literature links stroke-induced inflammation to the progression of damage. Thus, inhibition of inflammation would seem to be an obvious therapeutic strategy.

For the in vivo screening, the selection of the compounds was generally based on their good inhibitory activity on LOX, hydroxyl radical scavenging activity, and antilipid peroxidation. Some selected nitrones (**12**−**14**, **22**) were examined by using the functional model of carrageenan-induced rat paw edema. Results were expressed as percentage of weight increase at the right hind paw in comparison to the uninjected left hind paw. The induced edema is a nonspecific inflammation highly sensitive to NSAIDs. Thus, it has been accepted as a useful tool to study new anti-inflammatory agents.[50] It reliably predicts the anti-inflammatory potency of the NSAIDs and detects during the second phase which anti-inflammatory agents may play an inhibitory role in prostaglandin amplification.[51] As shown in Table 5, nitrones **12** and **22** exhibited a comparable effect (26.5 and 23.2%), yet weaker compared to indomethacin (47%). Lower lipophilicity is correlated with a biological effect in vivo (nitrones **12** and **22**). No biological response has been produced by nitrones **13** and **14**.

Table 5. In Vivo Anti-inflammatory Activities for Nitrones 12−14 and 22[a]

nitrone	ICPE %[b] ± SD	ClogP
12	26.5* ± 0.8	1.32
13	no	2.56
14	no	3.09
22	23.2** ± 0.6	2.01
indomethacin	47** ± 1.0	4.18

[a]Percentage of inhibition of carraggenan-induced rat paw edema (ICPE %). Each value represents the mean ± DS obtained from six animals in two independent experiments ($n = 6 \times 2$). In all cases, statistical significance against controls were performed by the Student's t test (*$p < 0.1$, **$p < 0.01$). [b]Dose of the administered compounds: 0.01 mmol/kg.

On the basis of the interesting antioxidant profile obtained by most of the tested nitrones, we next carried out the neuroprotection analyses.

4. Neuroprotection Evaluation against Experimental Ischemia. The neuroprotective effect of nitrones was evaluated on primary neuronal cultures from cerebral cortex, cultured for 6−8 days and subjected to oxygen−glucose deprivation (OGD).

4.1. Nitrones Reduce the Loss of Neuronal Viability Induced by OGD. Cell viability assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) determination was performed to evaluate the potential neuroprotective effect on nitrones **1**−**11** against OGD. Exposure of neuronal cultures to 4 h OGD (OGD 4 h) induced a significant decrease in cell viability (65.2%, $p < 0.0001$ versus 100% control, by one-sample t test), which was partially reversed after 24 h recovery (reperfusion) (R24h, 77.0%; $p < 0.01$ versus OGD 4 h, Student's t test) but without reaching the 24 h control value ($p < 0.0001$ versus 100% control, one-sample t test) (Figure 1).

Nitrones **1**−**11** (ranging from 0.1 μM to 1 mM) were added at the beginning of reperfusion period to evaluate their potential neuroprotective effect. Cytidine-5′-diphosphocholine (citicoline or CDP-choline), a well-known neuroprotective agent,[52] and PBN, described as protective against free radicals,[17,18] were used as reference compounds to evaluate the neuroprotection on neuronal cultures. Citicoline was assayed from 1 μM to 1 mM, and we found a neuroprotective effect at 10 and 100 μM (87.4 and 91.5%, respectively, compared to 100% control) (Figure 2). PBN did not induce neuroprotective effect in the range of assayed concentration (0.1−10 mM; Figure 2).

The addition of 100−250 μM nitrone **1**, 1 μM nitrone **2**, or 10−100 μM nitrone **3** and **10**, significantly increased cell viability during reperfusion and returned near control values (100.3−95.7%, 93.4%, 94.4−101.2%, and 94.9−96.1%, respectively, compared to 100% control) (Figure 1). The neuroprotection induced by nitrones **1**−**3** and **10** was compared with citicoline and PBN, these nitrones providing significantly higher neuroprotection than citicoline (Table 6). Conversely, and very interestingly, selected compounds from Table 4, such as nitrones **12**, **13**, **16**, **19**, and **20**, showed no neuroprotective activity (Figure 2).

4.2. Nitrones Attenuate Necrotic Neuronal Death Induced by OGD. To confirm the neuroprotective effects of nitrones **1**−**11** on cell viability, the effect of these compounds on necrotic cellular death was measured by monitoring LDH release from the cultured neuronal cells. Results from Figure 3 show that exposure of neuronal cultures to 4 h OGD (OGD 4 h) followed by 24 h reperfusion (R24h) induced a significant increase in LDH release (3.23 ± 0.26 times the control values; $p < 0.001$, Student's t test) (Figure 3). These increasing LDH release values are in keeping with the loss in cell viability observed under this condition (about 35%, Figure 1). Exposure of neuronal cultures to just 4 h OGD (OGD 4 h) did not induce a statistically significant increase in LDH release (38.05 ± 8.69%) when compared with control of 24 h reperfusion (C) (30.96 ± 5.31%). However, this difference was statistically significant ($p < 0.05$, Student's t test) when compared with control of 4 h by itself (19.77 ± 4.10%) (not shown). This means that, during the OGD treatment, neurons suffered a cellular membrane damage, which not only cannot be repaired but is enhanced during reperfusion. However, the presence of some nitrones during this period reversed the necrotic death to a great extent (Figure 3).

Nitrones neuroprotective effects (ranging from 1 to 250 μM) on necrotic cell death were assayed by adding them at the beginning of the reperfusion period, and their effect was compared with the neuroprotective effect of citicoline, as a known neuroprotective agent,[52] and PBN, as a neuroprotective agent against free radicals.[17,18]

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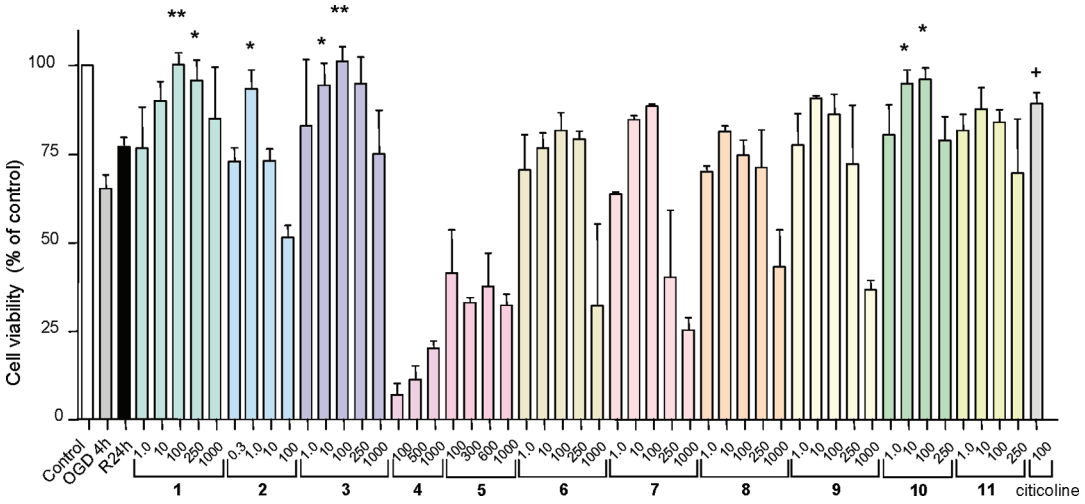

Figure 1. Effect of nitrones on primary neuronal cultures exposed to oxygen–glucose deprivation (OGD). Bar chart showing the percentage of cell viability at 24 h of recovery after 4 h OGD, either untreated (R24h) or treated with different concentrations (μM) of nitrones **1**–**11**, or citicoline. The value induced by OGD at 4 h without recovery period (OGD 4 h) is also indicated. Cell viability corresponding to control cells (1.534 ± 0.09 AU) was considered as 100%. The values represent the average of three to four independent experiments; error bars representing the SEM *P < 0.05, and **P < 0.01 versus R24h by one-way analysis of variance following Dunnett's post test, when analysis of variance was significant. +P < 0.05 versus R24h by Student's t test. Statistical significances below R24h value were not shown.

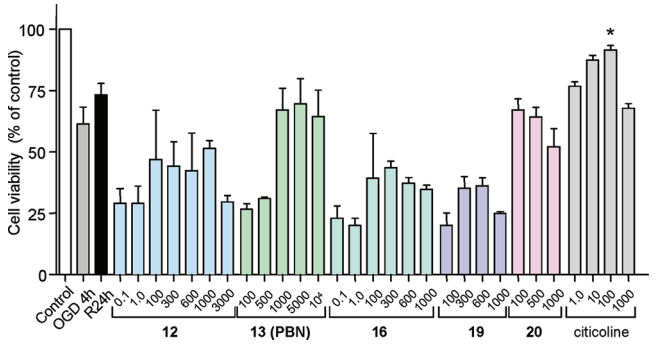

Figure 2. Effect of nitrones on primary neuronal cultures exposed to oxygen–glucose deprivation (OGD). Bar chart showing the percentage of cell viability at 24 h of recovery after 4 h OGD, either untreated (R24h) or treated with different concentrations (μM) of nitrones **12**, **13** (PBN), **16**, **19**, **20**, or citicoline. The value induced by OGD at 4 h is also indicated. Cell viability corresponding to control cells (1.497 ± 0.05 AU) was considered as 100%. The values represent the average of three to five independent experiments; error bars representing the SEM *P < 0.05 versus R24h by Student's t test. Statistical significances below R24h value were not shown.

Citicoline, tested from 1 to 250 μM, decreased LDH release in a dose-dependent manner, having significant neuroprotective effect at concentrations between 10 and 250 μM (35, 43 and 59%, respectively) (Figure 3). PBN also induced neuroprotective effects at the same concentrations, from 30 to 50%. The addition of 1–250 μM of nitrone **1**, 1–10 μM of nitrone **2** or 1–250 μM of nitrone **3**, 100–250 μM of nitrone **4** or 250 μM of nitrone **5**, 1–10 μM of nitrone **6** or 250 μM of nitrone **7**, 100 μM or 250 μM of nitrones **8** and **9**, respectively, and 1 μM nitrones of **10** and **11**, significantly decreased the LDH release during recovery.

Maximal neuroprotective effects (statistically significant when higher than 50% neuroprotection) were obtained at 250 μM nitrone **1**, 1–10 μM nitrone **2**, 10–100 μM nitrone **3**, 1–10 μM nitrone **6**, and 250 μM nitrone **7**, an effect comparable with citicoline or PBN at 100–250 μM concentrations (Table 7). As shown in the table, a smaller efficiency (near to 50% neuroprotection) was also obtained at 100–250 μM nitrone **4**, 100 μM nitrone **8**, 250 μM nitrone **9**, and 1–10 μM nitrones **10** and **11**.

Table 6. Neuroprotective Effect for Nitrones 1–3 and 10 in Neuronal Cultures Exposed to Oxygen–Glucose Deprivation (OGD)[a]

molecule	concentration	neuroprotection (%)
nitrone		
1	100 μM	101.3 ± 3.3***
	250 μM	81.2 ± 5.0***
2	1 μM	71.2 ± 4.0**
3	10 μM	75.7 ± 4.9**
	100 μM	105.2 ± 4.3***
10	10 μM	77.6 ± 3.1***
	100 μM	83.0 ± 2.7***
PBN	5 mM	−13.4 ± 1.9***
citicoline	100 μM	53.2 ± 1.8

[a]Neuroprotection was defined as the percentage to reach the control value, defined as 100%, from R24h value, defined as 0%. Data were obtained from Figure 1 (Figure 2 for PBN). **P < 0.01, and ***P < 0.001 compared to citicoline, by Student's t test.

5. In Vitro Blood–Brain Barrier Permeation Assay. In today's drug discovery research, screening for the BBB penetration is of great importance. One major problem for successful CNS drugs is to cross the BBB and reach their therapeutic targets. On the other hand, for drugs acting in peripheral tissues, penetration into the CNS might cause unwanted side effects. In the last years, several in vitro methods have been performed to predict the BBB permeation of investigational drugs. Among them, the parallel artificial membrane permeation assays (PAMPA) have the advantage of predicting passive blood–brain barrier permeation with high success, high throughput, and reproducibility.

To evaluate brain penetration of compounds here described, we used the PAMPA-BBB method described by Di et al.,[53] and

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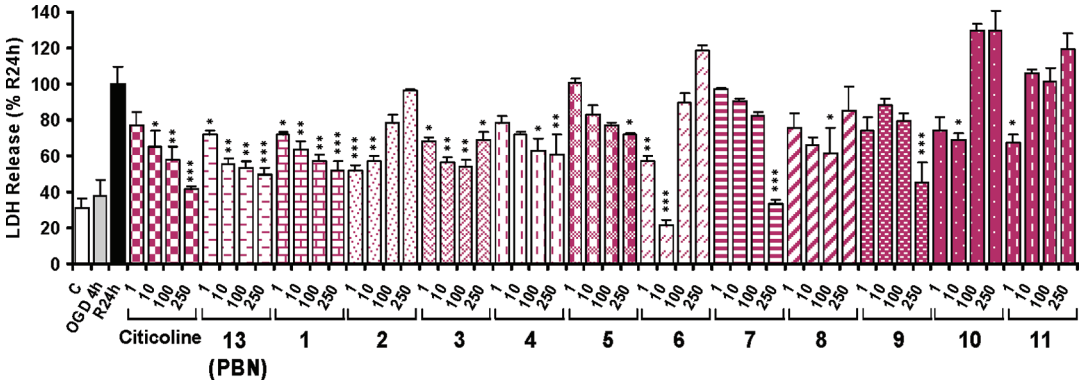

Figure 3. Effect of nitrones on LDH release in primary cortical neuronal cultures exposed to oxygen–glucose deprivation (OGD). Bar chart shows the percentage of LDH release at 24 h of recovery after 4 h OGD, either untreated (R24h) or treated with different concentrations (μM) of nitrones **1**–**11**, or citicoline or PBN. The value induced by OGD at 4 h without recovery period (OGD 4 h) is also indicated. LDH release corresponding to R24h cells exposed to 4 h OGD and 24 h of reperfusion (R24h) (35.42 ± 3.49%) was considered as 100%. The values represent the average of four independent experiments; error bars representing the SEM *P < 0.05, **P < 0.01, and ***P < 0.001 versus R24h by one-way analysis of variance followed by Holm–Sidak's post test, when analysis of variance was significant. Statistical significances above R24h value were not shown.

Table 7. Neuroprotective Effect for Nitrones 1–11 against Necrotic Cell Death in Neuronal Cultures Exposed to Oxygen–Glucose Deprivation (OGD)[a]

nitrone	concentration (μM)	neuroprotection against necrotic cell death (%)
1	250	70.08 ± 7.52*
2	1	70.17 ± 3.95**
	10	62.22 ± 3.36**
3	10	63.01 ± 3.26**
	100	66.82 ± 4.57**
4	100	53.80 ± 6.01
	250	56.95 ± 10.6
6	1	61.87 ± 2.81**
	10	114.17 ± 15.69**
7	250	96.26 ± 6.62***
8	100	55.57 ± 12.51
9	250	79.21 ± 19.55
10	10	45.37 ± 2.41
11	1	47.48 ± 3.33
citicoline	100	61.26 ± 8.26
	250	84.64 ± 3.48***
PBN	100	67.82 ± 4.70**
	250	73.20 ± 5.83**

[a]Neuroprotection against necrotic cell death was defined as the percentage to reach the control *C* value (defining as 100% neuroprotection the difference between R24h and *C* percentages). Data were obtained from Figure 3. *P < 0.05, **P <0.01, and **P < 0.001 compared to 50% neuroprotection, by one-sample Student's t test.

subsequently optimized by Rodríguez-Franco and colleagues for molecules with limited water solubility.[54−56] In the last years, this method was successfully applied by some of us to different classes of compounds.[57−64] The in vitro permeability (P_e) values of derivatives **1**, **2**, **10**, and **11** through a lipid extract of porcine brain were determined by using PBS/ethanol (70:30) (Table 8). In the same assay, 15 commercial drugs of known CNS penetration were also tested and their experimental values were compared to reported values, giving a good lineal correlation. All tested nitrones showed good permeability values, close to the reported for verapamil (P_e = 16 × 10^{-6} cm s^{-1}), which is generally used as a high permeability standard.[53] Thus, it is expected that they could penetrate into the CNS and reach their biological targets located in the CNS.

Table 8. Experimental Permeability (P_e 10^{-6} cm s^{-1})[a] in the PAMPA-BBB Assay for Nitrones 1, 2, 10, and 11 with Their Predictive Penetration in the CNS

nitrone	P_e	prediction
1	27.0 ± 0.4	cns+
2	15.0 ± 0.1	cns+
10	22.0 ± 1.3	cns+
11	16.8 ± 0.3	cns+

[a]PBS:EtOH (70:30). Data are the mean ± SD of three independent experiments.

CONCLUSIONS

Continuing with our nitrones for stroke project development,[19] we have now investigated and report here the in vitro antioxidant activity of nitrones **1**–**25**. From data in Tables 3 and 4, and regarding their ability to interact with AAPH, the highest activity was observed for nitrones **19** and **20** followed by **21**, which incorporated moieties 3-MeO and 4-OH on the phenyl ring, as well as nitrones **2** and **10** with a 3-(nitrooxy) propoxy) benzylidene group. The *N*-substituent does not seem to influence either the antilipid peroxidation activity or the overall lipophilicity. From the results, it is obvious that inhibition of lipid peroxidation activity is not always accompanied by radical scavenging ability and vice versa. This can be attributed to the different chemical reactions involved in the various assays. Particularly effective antioxidants are the phenoxide anions from phenolic compounds. As shown, and as expected, nitrones **4** and **5** bearing free phenol groups provided the best RSA (%) values, in the 79–82% range, in DPPH test. The incorporation of *N*-*tert*-butyl or benzyl groups, as well as 3-(nitrooxy) propoxy) benzylidene and quinoline moieties, provide nitrones with increased antilipid peroxidation activity, hydroxyl radical scavenging activity, and LOX inhibition. Overall, and among the different nitrones investigated, **2**, **4**, **10**, **11**, **13**, and **22** showed interesting antioxidant activities.

We assayed the nitrones for neuroprotection activity by MTT determination on neuronal cultures. In these experiments, well-known neuroprotective agents, such as nitrone PBN[17,18] and citicoline,[52] were included. Citicoline had

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significant neuroprotective effect at 100 μM (89.3%), in accordance with other studies.[65] However, PBN, ranging from 100 μM to 10 mM, had no neuroprotective effect, even at the concentrations described as protective (1−10 mM) in other studies.[66,67] However, nitrones **1**−**3** and **10** exhibited the highest neuroprotective effect. In neuronal cultures treated with these nitrones, the cell viability was restored during reperfusion period against OGD-induced damage. The neuroprotection induced by nitrones **1**−**3** and **10** was compared with citicoline and PBN and shown in Table 6. From these data, some simple structure−activity relationships can be inferred. First of all, it seems that both the presence of a strong electron-withdrawing group (CF_3) in the benzene ring, or the quinoline heterocyclic ring system conjugated with the nitrone moiety, are key structural motifs for the remarkable neuroprotective capacity observed in compounds **1**−**3**. Conversely, the presence of a highly hindered phenol moiety in nitrones **4** and **5**, or the bromofuran ring system in nitrones **6** and **7**, are associated with strong toxicity along with no activity, or toxicity at high concentrations, respectively. Finally, in nitrones **8**−**11**, the type of the *N*-alkyl residue on the nitrogen bearing the nitrone motif, determined their neuroprotective profile: nitrones **8** and **9**, bearing the *tert*-butyl chain, are more toxic at high concentrations than nitrones **10** and **11** bearing the benzyl group. This is similar to what we observed in the antioxidant activity for the same nitrones determined by DPPH (see above).

In general, the effect of these nitrones on necrotic cellular death, measured by the LDH release method, is in good agreement with data from neuroprotective activity on cell viability shown above. Thus, the trifluoromethyl group in the benzene ring of nitrone **1** and the quinoline ring in nitrones **2** and **3** seem to provide nitrones with maximal neuroprotective capacity. For nitrones **8**−**11**, only nitrone **9**, bearing a *tert*-butyl substituent on the nitrone motif, was significantly neuroprotective. However, and at least as far as necrotic cell death is concerned, small concentrations of nitrones **10** and **11**, bearing a benzyl group, could result in a good neuroprotective capacity. On the contrary, at high concentrations, these nitrones become toxic.

It is also worth pointing out that the theoretical calculations for the neuroprotection are in good agreement with the experimental data for nitrones **2**, **3**, **6**−**9**, and **11**, unlike nitrones **1**, **4**, **5**, and **10**. Similarly, for the BBB prediction, the theoretical calculations are in agreement with the experimental data for nitrones **1**−**7**, but not for nitrones **8**−**11**. The theoretical analyses were utterly successful in identifying the quinolinyl nitrones **2** and **3** as potent neuroprotective agents able to cross the BBB.

Overall, and among the different molecules investigated, permeable quinolinyl nitrones **2** and **3** show potent combined antioxidant and neuroprotective properties and consequently can be considered as new lead compounds for further development in specific tests for potential stroke treatment.

EXPERIMENTAL SECTION

General Methods. Reactions were monitored by TLC using precoated silica gel aluminum 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. The purity of compounds was checked by elemental analyses, conducted on a Carlo Erba EA 1108 apparatus, and confirmed to be ≥95%. 2,2-Diphenyl-1-picrylhyrazyl (DPPH) radical, sodium benzoate, $FeSO_4{\cdot}7H_2O$, EDTA, 30% H_2O_2, nitro blue tetrazolium chloride, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), and nitrate reductase from *Arabidopsis thaliana*, were purchased from Sigma-Aldrich. Phosphate buffer (0.1 M and pH 7.4) was prepared mixing an aqueous KH_2PO_4 solution (50 mL, 0.2 M) and an aqueous of NaOH solution (78 mL, 0.1 M), and the pH (7.4) was adjusted by adding a solution of KH_2PO_4 or NaOH. Nordihydroguaiaretic acid (NDGA), trolox, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), sodium nitroprusside (SNP), and caffeic acid (CA) were purchased from the Aldrich Chemical Co. Milwaukee, WI, (USA). Soybean LOX, linoleic acid sodium salt, xanthine, xanthine oxidase, and indomethacin were obtained from Sigma Chemical, Co. (St. Louis, MO, USA), and carrageenan, type K, was commercially available. For the in vivo experiments, male and female Fischer-344 rats (180−240 g) were used. For the in vitro tests, a Lambda 20 (Perkin−Elmer) UV−vis double beam spectrophotometer was used.

General Procedure for Nitrone Synthesis. Method A: In a 20 mL glass tube equipped with septa, the aldehyde, dry Na_2SO_4 (3 mol equiv), and triethylamine (2 mol 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 90 °C during the time indicated for each compound. When the reaction was over (TLC analysis), the reaction mixture was diluted with water, extracted with AcOEt, dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated. The resultant solid was purified by column chromatography to give pure compounds. Method B: Methyl-2-nitropropane (2 mol equiv) and zinc (3 mol equiv) were added to a solution of the aldehyde in EtOH (0.14M). 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 14 h and then was kept at 0 °C for 2 h. The precipitate was filtered and washed with EtOH. The solvent was evaporated and the resultant solid was purified by column chromatography to give pure compounds.

(*Z*)-2-Methyl-*N*-(3-(trifluoromethyl)benzylidene)propan-2-amine Oxide (1).[21] Following the general procedure (method B), reaction of commercial 3-(trifluoromethyl)benzaldehyde (100 mg, 0.58 mmol), 2-methyl-2-nitropropane (0.125 mL, 1.15 mmol), Zn (115 mg, 1.73 mmol), and acetic acid (0.20 mL, 3.45 mmol) in EtOH (3 mL), after the specified reaction time, and column chromatography (hexane/EtOAc, 8:2, v/v), gave nitrone **1** (54 mg, 39%) as a solid: mp 52−4 °C. IR (KBr) ν_{max} 3436, 2985, 1567, 1341, 1173, 1116 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.69 (s, 1H, H-2′, Ph), 8.38 (d, 1H, J = 8.0 Hz, H-4′, Ph), 7.64−7.62 (m, 2H, N=CH, H-6′, Ph), 7.52 (t, 1H, J = 8.0 Hz, H-5′, Ph), 1.62 [s, 9H, $C(CH_3)_3$]. ^{13}C NMR (101 MHz, $CDCl_3$) δ 131.6 (C-6′, Ph), 130.8 (q, J = 32.9 Hz, CF_3), 128.8 (C-5′, Ph), 128.6 (C=N), 127.9 (C-3′ Ph), 126.4 (C-4′ Ph), 125.2 (C-2′ Ph), 122.5 (C-1′, Ph), 71.5 [$C(CH_3)_3$], 28.3 [$C(CH_3)_3$]. MS (EI) m/z: 245 $(M)^+$, 205, 163, 121, 79, 57. MS (ESI): 246.0 $(M + H)^+$. Anal. Calcd for $C_{12}H_{14}F_3NO$: C, 58.77; H, 5.75; N, 5.71. Found: C, 58.83; H, 6.02; N, 5.99.

(*Z*)-*N*-[(2-Chloro-6-methylquinolin-3-yl)methylene]-2-methylpropan-2-amine Oxide (2). Following the general procedure (method B), reaction of commercial 2-chloro-6-methylquinoline-3-carbaldehyde (300 mg, 1.46 mmol), 2-methyl-2-nitropropane (0.32 mL, 2.92 mmol), Zn (290 mg, 4.38 mmol), and acetic acid (0.50 mL, 8.75 mmol) in EtOH (8 mL), after the specified reaction time, and column chromatography (hexane/EtOAc, 85:15, v/v) gave nitrone **2** (112 mg, 28%) as a solid: mp 120−3 °C. IR (KBr) ν_{max} 3436, 2967,

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1548, 1364, 1333, 1185, 1131 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 10.29 (s, 1H, H-4′, Ar), 8.27 (s, 1H, N=CH), 7.85 (d, 1H, J = 8.8 Hz, H-8′, Ar), 7.63 (s, 1H, H-5′, Ar), 7.56 (d, 1H, J = 8.8 Hz, H-7′, Ar), 2.52 (s, 3H, CH_3), 1.67 [s, 9H, $C(CH_3)_3$]. ^{13}C NMR (101 MHz, $CDCl_3$) δ 148.0 (Ar), 145.5 (Ar), 137.6 (Ar), 136.5 (C-4′, Ar), 133.7 (C-7′, Ar), 127.8, 127.7 (C-5′, C-8′, Ar), 127.2 (Ar), 125.4 (C=N), 122.7 (Ar), 72.3 [$C(CH_3)_3$], 28.3 [$C(CH_3)_3$)], 21.6 (CH_3). MS (EI) m/z: 276 $(M)^+$, 241, 185, 167, 57. MS (ESI) m/z: 277.0 $(M + H)^+$. Anal. Calcd for $C_{15}H_{17}ClN_2O$: C, 65.10; H, 6.19; N, 10.12; Cl, 12.81. Found: C, 65.37; H, 5.91; N, 9.93; Cl, 13.07.

(*Z*)-*N*-[(2-Chloro-6-methylquinolin-3-yl)methylene]-1-phenylmethanamine Oxide (3). Following the general procedure (method A), reaction of commercial 2-chloro-6-methylquinoline-3-carbaldehyde (200 mg, 0.97 mmol), Na_2SO_4 (410 mg, 2.92 mmol), Et_3N (0.30 mL, 1.95 mmol), and *N*-benzylhydroxylamine hydrochloride (310 mg, 1.95 mmol) in THF (4 mL), after 30 min, and column chromatography (hexane/EtOAc, 7:3, v/v), gave nitrone **3** (192 mg, 64%) as a solid: mp 154−156 °C. IR (KBr) ν_{max} 3436, 2918, 1569, 1555, 1429, 1342, 1180, 1146, 1048 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 10.20 (s, 1H, H-4′, Ar), 8.10 (s, 1H, N=CH), 7.84 (d, 1H, J = 8.3 Hz, H-8′, Ar), 7.62 (s, 1H, H-5′, Ar), 7.57 (d, 1H, J = 8.3 Hz, H-7′, Ar), 7.54−7.51 (m, 2H, Ph), 7.47−7.41 (m, 3H, Ph), 5.16 (s, 2H, CH_2Ph), 2.52 (s, 3H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 147.4, 145.6, 137.7 (3Ar), 136.8 (C-4′, Ar), 133.9 (C-7′, Ar), 132.7 (Ph), 129.5, 129.4, 129.3, 129.1 (C=N, 3Ph), 127.8 (2Ar), 127.0 (C-8′, Ar), 122.2 (Ar), 72.2 (CH_2Ph), 21.6 (CH_3). MS (EI) m/z: 310 $(M)^+$, 275, 91. MS (ESI) m/z: 311.0 $(M + H)^+$. Anal. Calcd for $C_{18}H_{15}ClN_2O$: C, 69.57; H, 4.86; N, 9.01; Cl, 11.41. Found: C, 69.52; H, 5.14; N, 9.30; Cl, 11.63.

(*Z*)-*N*-(3,5-Di-*tert*-butyl-4-hydroxybenzylidene)-1-phenylmethanamine Oxide (4).[22] Following the general procedure (method A), reaction of commercial 3,5-di-*t*-butyl-4-hydroxybenzaldehyde (200 mg, 0.82 mmol), Na_2SO_4 (470 mg, 3.29 mmol), Et_3N (0.25 mL, 1.64 mmol), and *N*-benzylhydroxylamine hydrochloride (260 mg, 1.64 mmol) in THF (4 mL), after 40 min, and column chromatography (hexane/EtOAc, 6:4, v/v), gave nitrone **4** (264 mg, 95%) as a solid: mp 225−6 °C. IR (KBr) ν_{max} 3623, 3430, 2956, 1593, 1438, 1412, 1239, 1146, 1119 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.16 (s, 2H, H-2′, Ar), 7.50−7.34 (m, 6H, N=CH, 5Ph), 5.61 (s, 1H, OH), 5.03 (s, 2H, CH_2Ph), 1.45 (s, 18H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.9 (C-4′, Ar), 135.8 (C-3′, Ar), 135.1 (C=N), 133.5, 129.1, 128.8, 128.7 (CH_2Ph), 126.4 (C-2′, Ar), 122.2 (C-1′, Ar), 70.6 (CH_2Ph), 34.3 [$C(CH_3)_3$], 30.1 [$C(CH_3)_3$]. MS (EI) m/z: 339 $(M)^+$, 323, 266, 91. MS (ESI) m/z: 340.3 $(M + H)^+$. Anal. Calcd for $C_{22}H_{29}NO_2$: C, 77.84; H, 8.61; N, 4.13. Found: C, 77.51; H, 8.43; N, 4.30.

(*Z*)-*N*-(3,5-Di-*tert*-butyl-4-hydroxybenzylidene)-2-methylpropan-2-amine Oxide (5).[22] Following the general procedure (method A), reaction of commercial 3,5-di-*t*-butyl-4-hydroxybenzaldehyde (120 mg, 0.49 mmol), Na_2SO_4 (280 mg, 1.95 mmol), Et_3N (0.15 mL, 0.98 mmol), and *N*-*t*-butylhydroxylamine hydrochloride (123 mg, 0.98 mmol) in THF (4 mL), after 16 h, and column chromatography (hexane/EtOAc, 6:4, v/v), gave nitrone **5** (105 mg, 70%) as a solid: mp 230−3 °C. IR (KBr) ν_{max} 3436, 2969, 1438, 1360, 1195, 1093 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.21 (s, 2H, H-2′, Ar), 7.44 (s, 1H, N=CH), 5.56 (s, 1H, OH), 1.60 [s, 9H, $NC(CH_3)_3$], 1.46 [s, 18H, $ArC(CH_3)_3$]. ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.6 (C-4′, Ar), 135.7 (C-3′, Ar), 130.6 (C=N), 126.5 (C-2′, Ar), 122.9 (C-1′, Ar), 69.9 [$NC(CH_3)_3$], 34.4 [$ArC(CH_3)_3$], 30.2 [$ArC(CH_3)_3$], 28.5 [$NC(CH_3)_3$]. MS (EI) m/z: 305 $(M)^+$, 249, 234, 57. MS (ESI) m/z: 306.3 $(M + H)^+$. Anal. Calcd for $C_{19}H_{31}NO_2$: C, 74.71; H, 10.23; N, 4.59. Found: C, 74.83; H, 10.12; N, 4.81.

(*Z*)-*N*-[(5-Bromofuran-2-yl)methylene]-1-phenylmethanamine Oxide (6). Following the general procedure (method A), reaction of commercial 5-bromofuran-2-carbaldehyde (110 mg, 0.63 mmol), Na_2SO_4 (360 mg, 2.51 mmol), Et_3N (0.19 mL, 1.25 mmol), and *N*-benzylhydroxylamine hydrochloride (200 mg, 1.25 mmol) in THF (4 mL), after 30 min, and column chromatography (hexane/EtOAc, 6:4, v/v), gave nitrone **6** (142 mg, 81%) as a solid: mp 108−110 °C. IR (KBr) ν_{max} 3436, 1477, 1222, 1134, 1010 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, 1H, J = 3.0 Hz, H-3′, Ar), 7.44−7.43 (m, 6H, N=CH, 5Ph), 6.46 (d, 1H, J = 3.0 Hz, H-4′, Ar), 5.01 (s, 2H, CH_2Ph). ^{13}C NMR (101 MHz, $CDCl_3$) δ 148.8 (C-2′, Ar), 132.4, 129.5, 129.3, 129.1 (Ph), 124.4, 124.2 (C=N, C-5′, Ar), 117.5 (C-3′, Ar), 114.2 (C-4′, Ar), 69.6 (CH_2Ph); MS (EI) m/z: 281, 279 $(M)^+$, 91. MS (ESI) m/z: 282.0, 280.0 $(M + H)^+$. Anal. Calcd for $C_{12}H_{10}BrNO_2$: C, 51.45; H, 3.60; N, 5.00; Br, 28.53. Found: C, 51.74; H, 3.69; N, 5.16; Br, 28.08.

(*Z*)-*N*-[(5-Bromofuran-2-yl)methylene]-2-methylpropan-2-amine Oxide (7). Following the general procedure (method A), reaction of commercial 5-bromofuran-2-carbaldehyde (150 mg, 0.86 mmol), Na_2SO_4 (730 mg, 5.14 mmol), Et_3N (0.52 mL, 3.43 mmol), and *N*-*t*-butylhydroxylamine hydrochloride (430 mg, 3.42 mmol) in THF (5 mL), after 1 h, and column chromatography (hexane/EtOAc, 6:4, v/v), gave nitrone **7** (150 mg, 71%) as a solid: mp 93−5 °C. IR (KBr) ν_{max} 3436, 2978, 1476, 1357, 1202, 1116, 1013 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, 1H, J = 3.3 Hz, H-3′, Ar), 7.69 (s, 1H, N=CH), 6.46 (d, 1H, J = 3.3 Hz, H-4′, Ar), 1.57 (s, 9H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 149.6 (C-2′, Ar), 123.9 (C=N), 120.6 (C-3′, Ar), 117.0 (C-4′, Ar), 114.0 (C-5′, Ar), 69.9 [$C(CH_3)_3$], 28.0 [$C(CH_3)_3$]. MS (EI) m/z: 247, 245 $(M)^+$, 191, 189, 79, 57. MS (ESI) m/z: 248.0, 246.0 $(M + H)^+$. Anal. Calcd for $C_9H_{12}BrNO_2$: C, 43.92; H, 4.91; N, 5.69; Br, 32.47. Found: C, 43.80; H, 4.75; N, 5.81; Br, 31.93.

(*Z*)-2-Methyl-*N*-(4-(3-(nitrooxy)propoxy)benzylidene)-propan-2-amine Oxide (8). Following the general procedure (method A), reaction of 3-(4-formylphenoxy)propyl nitrate[20] (67 mg, 0.30 mmol), Na_2SO_4 (128 mg, 0.90 mmol), Et_3N (0.09 mL, 0.60 mmol), and *N*-*t*-butylhydroxylamine hydrochloride (75 mg, 0.60 mmol) in THF (4 mL), after 18 h, and column chromatography (hexane/EtOAc, 2:8, v/v), gave nitrone **8** (56 mg, 63%) as a solid: mp 99−101 °C. IR (KBr) ν_{max} 3436, 2982, 1623, 1609, 1513, 1281, 1269, 1177, 1109 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.28 (d, 2H, J = 8.5 Hz, H-2′, Ph), 7.48 (s, 1H, N=CH), 6.92 (d, 2H, J = 8.5 Hz, H-3′, Ph), 4.67 (t, 2H, J = 6.0 Hz, CH_2ONO_2), 4.12 (t, 2H, J = 6.0 Hz, CH_2OAr), 2.23 (q, 2H, J = 6.0 Hz, $CH_2CH_2CH_2$), 1.61 [s, 9H, $C(CH_3)_3$]. ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.6 (C-4′, Ph), 130.8 (C-2′ Ph), 129.6 (C=N), 124.4 (C-1′, Ph), 114.2 (C-3′ Ph), 70.2 [$C(CH_3)_3$], 69.8 (CH_2ONO_2), 63.6 (CH_2OAr), 28.3 [$C(CH_3)_3$], 26.9 ($CH_2CH_2CH_2$). MS (EI) m/z: 296 $(M)^+$, 240, 194, 177, 136, 57. MS (ESI) m/z: 297.0 $(M + H)^+$. Anal. Calcd for $C_{14}H_{20}N_2O_5$: C, 56.75; H, 6.80; N, 9.45. Found: C, 56.76; H, 6.50; N, 9.24.

(*Z*)-2-Methyl-*N*-(4-(2-(nitrooxy)ethoxy)benzylidene)propan-2-amine Oxide (9). Following the general procedure (method A), reaction of 2-(4-formylphenoxy)ethyl nitrate[20] (120 mg, 0.57 mmol), Na_2SO_4 (480 mg, 3.41 mmol), Et_3N (0.35 mL, 2.27 mmol), and *N*-*t*-butylhydroxylamine hydrochloride (290 mg, 2.27 mmol) in THF (4 mL), after 20 h, and column chromatography (hexane/EtOAc, 2:8, v/v), gave nitrone **9** (72 mg, 45%) as a solid: mp 66−9 °C. IR (KBr) ν_{max} 3436, 2981, 1636, 1604, 1285, 1259, 1111 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, 2H, J = 8.6 Hz, H-2′, Ph), 7.47 (s, 1H, N=CH), 6.91 (d, 2H, J = 8.6 Hz, H-3′, Ph), 4.82 (t, 2H, J = 4.4 Hz, CH_2ONO_2), 4.29 (t, 2H, J = 4.4 Hz, CH_2OAr), 1.60 [s, 9H, $C(CH_3)_3$]. ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.2 (C-4′, Ph), 131.0 (C-2′ Ph), 129.6 (C=N), 125.1 (C-1′, Ph), 114.4 (C-3′ Ph), 71.0 (CH_2ONO_2), 70.5 [$C(CH_3)_3$], 64.2 (CH_2OAr), 28.5 [$C(CH_3)_3$]. MS (EI) m/z: 282 $(M)^+$, 180, 163, 57. MS (ESI) m/z: 283.0 $(M + H)^+$. Anal. Calcd for $C_{13}H_{18}N_2O_5$: C, 55.31; H, 6.43; N, 9.92. Found: C, 55.54; H, 6.18; N, 9.92.

(*Z*)-*N*-(4-(3-(Nitrooxy)propoxy)benzylidene)-1-phenylmethanamine Oxide (10). Following the general procedure (method A), reaction of 3-(4-formylphenoxy)propyl nitrate[20] (80 mg, 0.36 mmol), Na_2SO_4 (200 mg, 1.42 mmol), Et_3N (0.11 mL, 0.71 mmol), and *N*-benzylhydroxylamine hydrochloride (113 mg, 0.71 mmol) in THF (3 mL), after 4 h, and column chromatography (hexane/EtOAc, 3:7, v/v), gave nitrone **10** (102 mg, 87%) as a solid: mp 96−98 °C. IR (KBr) ν_{max} 3436, 1624, 1605, 1282, 1256, 1174 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.20 (d, 2H, J = 8.0 Hz, H-2′, Ph), 7.50−7.31 (m, 6H, N=CH, 5CH_2Ph), 6.90 (d, 2H, J = 8.0 Hz, H-3′, Ph), 5.03 (s, 2H, CH_2Ph), 4.66 (t, 2H, J = 6.0 Hz, CH_2ONO_2), 4.10 (t, 2H, J = 5.6 Hz, CH_2OAr), 2.22 (t, 2H, J = 6.0, 5.6 Hz, $CH_2CH_2CH_2$). ^{13}C NMR (101

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MHz, $CDCl_3$) δ 159.8 (C-4′, Ph), 133.8 (C═N), 133.3 (CH_2Ph), 130.6 (C-2′ Ph), 129.2, 129.0, 128.9 ($3CH_2Ph$), 123.8 (C-1′, Ph), 114.2 (C-3′ Ph), 70.8 (CH_2Ph), 69.8 (CH_2ONO_2), 63.5 (CH_2OAr), 26.9 ($CH_2CH_2CH_2$). MS (EI) m/z: 330 $(M)^+$, 153, 91;. MS (ESI) m/z: 331.0 $(M + H)^+$. Anal. Calcd for $C_{17}H_{18}N_2O_5$: C, 61.81; H, 5.49; N, 8.48. Found: C, 62.02; H, 5.38; N, 8.70.

(*Z*)-*N*-(4-(2-(Nitrooxy)ethoxy)benzylidene)-1-phenylmethanamine Oxide (11). Following the general procedure (method A), reaction of 2-(4-formylphenoxy)ethyl nitrate[20] (70 mg, 0.33 mmol), Na_2SO_4 (190 mg, 1.33 mmol), Et_3N (0.10 mL, 0.66 mmol), and *N*-benzylhydroxylamine hydrochloride (106 mg, 0.66 mmol) in THF (4 mL), after 3 h, and column chromatography (hexane/EtOAc, 3:7, v/v), gave nitrone **11** (102 mg, 87%) as a solid: mp 133−5 °C. IR (KBr) ν_{max} 3436, 1618, 1606, 1284, 1255, 1176, 1147 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, 2H, J = 8.8 Hz, H-2′, Ph), 7.49−7.32 (m, 6H, N═CH, $5CH_2Ph$), 6.91 (d, 2H, J = 8.8 Hz, H-3′, Ph), 5.03 (s, 2H, CH_2Ph), 4.82 (t, 2H, J = 4.4 Hz, CH_2ONO_2), 4.28 (t, 2H, J = 4.4 Hz, CH_2OAr). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.5 (C-4′, Ph), 133.9 (C═N), 133.5 (CH_2Ph), 130.9 (C-2′ Ph), 129.5, 129.2 ($3CH_2Ph$), 124.5 (C-1′, Ph), 114.5 (C-3′ Ph), 71.1 (CH_2Ph), 71.0 (CH_2ONO_2), 64.2 (CH_2OAr). MS (EI) m/z: 316 $(M)^+$, 91. MS (ESI) m/z: 317.0 $(M + H)^+$. Anal. Calcd for $C_{16}H_{16}N_2O_5$: C, 60.75; H, 5.10; N, 8.86. Found: C, 60.92; H, 4.97; N, 8.96.

Chemical Determination of Antioxidant Activity. We used different types of assays to measure in vitro antioxidant activity of nitrones, such as the inhibition of lipid peroxidation (LP) induced by AAPH, the DMSO method for the hydroxyl radical scavenging activity, the superoxide anion radical activity, and the in vitro inhibition of soybean lipoxygenase (LOX). All require a spectrophotometric measurement and a certain reaction time in order to obtain reproducible results. Each in vitro experiment was performed at least in triplicate, and the standard deviation of absorbance was less than 10% of the mean.

Inhibition of Linoleic Acid Lipid Peroxidation.[27,68] Production of conjugated diene hydroperoxide by oxidation of linoleic acid in an aqueous dispersion was monitored at 234 nm. AAPH was used as a free radical initiator. This assay can be used to follow oxidative changes and to understand the contribution of each tested nitrone. Ten μL of the 16 mM linoleic acid sodium salt solution was added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4, prethermostated at 37 °C. The oxidation reaction was initiated at 37 °C under air by the addition of 50 μL of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (10 μL). In the assay without antioxidant, lipid oxidation was measured in the presence of the same level of DMSO. The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. Trolox was used as an appropriate standard.

Antioxidant Activity of Nitrones Determined by DPPH Radical Method.[31] 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) freshly prepared 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 were 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% (radical scavenging activity), calculated as follows: $RSA\% = 100[(A_o - A_i)/A_o) \times 100$, where A_o and A_i are the DPPH absorbance in absence and in presence of added nitrone concentration i, respectively. Data are expressed as means.

Competition of the Tested Nitrones with DMSO for Hydroxyl Radicals.[31] The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system were detected by the determination of formaldehyde produced from the oxidation of DMSO. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested nitrones (concentration 0.1 mM), and ascorbic acid (10 mM). After 30 min of incubation (37 °C), the reaction was stopped with CCl_3COOH (17% w/v) (Table 3). Trolox was used as an appropriate standard.

Scavenging Activity of Superoxide Anion Radical.[34] The superoxide anion was generated by the xanthine−xanthine oxidase system and measured by the nitroblue tetrazolium (NBT) method. NBT and tested nitrone (0.1 mM final concentration) and xanthine oxidase 40 μL (1.4 mL/60 mg, 0.07U/mL) were added to the reaction mixture in phosphate buffer pH 7.4 (0.1M) containing xanthine. After incubating for 10 min at rt, the absorbance was recorded at 560 nm. Caffeic acid was used as an appropriate standard.

Soybean LOX Inhibition Study in Vitro.[69] The tested nitrones, dissolved in DMSO, were incubated at rt with sodium linoleate (0.1 mL) and 0.2 mL of enzyme solution (0.1 mg/mL in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor (NDGA). The type of inhibition was studied. The nitrones (in a standard concentration) were added to a series of different substrate concentrations (sodium linoleate). The reactions are started by adding the same amount of enzyme to each of the substrate concentrations containing the tested nitrone. The results support competitive inhibition.

Quantitative Nitrite Assays for NO Release.[70] A solution of the examined nitrones (final concentration 100 μM (Table 1) (20 μL), dissolved in DMSO, was added to 2 mL of 50 mmol phosphate buffer (pH = 7.4) containing the appropriate amount of thiol cofactor 1 mM (L-cysteine). After 5 or 60 min at 37 °C, 1 mL of the reaction mixture was treated with 250 μL of the Griess reagent (4 g sulfanilamide, 0.2 g *N*-naphthyl-ethyldiamine dihydrochloride, 10 mL of 85% phosphoric acid in distilled water, final volume 100 mL). After the mixture stood for 10 min at rt, absorbance was recorded at 540 nm. Sodium nitroprusside (SNP) was used as standard reference compound (100 μM). The yield in nitrite (NO_2^- release) for each nitrone, as function of L-cysteine (mM concentration) is given in Table 4, expressed as % NO_2^- (mol/mol). The nitrite determination was repeated, using the nitrate reductase (0.1 U/mL)/NADPH (1 mM) system[71,72] to see if any residual nitrate could be reduced back to nitrite before using the Griess assay. Values are given as means ± SD of four to six experiments. SD values were always lower than 10%. Calibration curve 10−100 nmol/mL sodium nitrite standard solutions were used for the calibration curve.

In Vivo Assays. Acute Toxicity. In acute toxicity preliminary experiments (data not given), in vivo the nitrones **12**, **13**, **14** and **22** were examined in doses from 0.05 up to 0.1 mmol/kg body weight (lethal range 30−80% after 24 h).

Inhibition of the Carrageenan-Induced Edema..[69] Edema was induced in the right hind paw of Fisher 344 rats (150−200 g) by the intradermal injection of 0.1 mL 2% carrageenan in water. Both sexes were used. Females pregnant were excluded. Each group was composed of six animals. For each nitrone, the experiment was performed twice for validation. The animals, which had been bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water ad libitum during the maintenance but were entirely fasted during the experiment period. In our studies, we followed the recognized national and European ethical guidelines on animal experimentation. The experiments conform to the law for the protection of experimental animals (Republic of Greece) and are registered at the Veterinary Administration of the Republic of Greece. The tested nitrones, 0.01 mmol/kg body weight, were diluted in water with a few drops of Tween 80 and ground in a mortar before use, and they were intraperitoneally simultaneously given with the carrageenan injection. The rats were euthanized 3.5 h after carrageenan administration. The difference between the weight of the injected and uninjected paws was calculated for each animal. The change in paw weight was compared with that in control animals (treated with water or Tween−water) and expressed as a percent inhibition of the edema % ICPE values (Table 5). Indomethacin was tested as a reference compound in 0.01 mmol//kg (47%). Values % ICPE are the mean from two different experiments (n = 6 animals

each time) with a standard error of the mean less than 10%.

$$\%\text{ICPE inhibition} = (A_c - A_{treat}/A_c) \times 100$$

A_c = mean weight of the injected and uninjected (carageenan injection) paws of the group of the control animals, and A_{treat} = mean weight of the injected and uninjected paws (carageenan injection) of the group of the treated animals with each examined compound.

Primary Neuronal Cultures. Primary neuronal cultures from rat cerebral cortex were prepared as previously described.[73,74] All procedures associated with animal experiments were approved by the Ethics Committee of the Hospital Ramón y Cajal and Universidad Complutense de Madrid (UCM, Madrid (Spain). Cell suspensions from cerebral cortex were prepared from 16- to 17-day-old Sprague–Dawley rat embryos. Living cells in cell suspension were counted by trypan blue exclusion method. Cells were seeded on plastic multidishes precoated with 0.05 mg/mL poly-D-lysine at a density of 2.5×10^5 cells/cm^2 and were kept at 37 °C in a 6.5% CO_2 atmosphere in high-glucose Dulbecco's medium supplemented with 15% heat-inactivated (56 °C for 30 min) fetal calf serum. After 24 h, cultured cells were placed in, and maintained in, serum-free medium (Dulbecco's: Ham's F12, 1:1 [vol/vol], 5 mg/mL glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate, and supplemented with 100 μg/mL transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM sodium selenite, and 5 μg/mL insulin), as described by Bottenstein and Sato.[75] Six- to eight-day cultures were used in the experiments and contained 90% β-III-tubulin-positive mature neurons, as described previously.[76]

Exposure of Cell Cultures to Oxygen–Glucose Deprivation and Treatments. Primary neuronal cultures were exposed to OGD so as to induce cellular damage. Cultured cells were washed and placed in glucose-free Dulbecco's medium (bubbled with 95% N_2/5% CO_2 for 30 min) and maintained in an anaerobic chamber containing a gas mixture of 95% N_2/5% CO_2 and humidified at 37 °C at a constant pressure of 0.15 bar. Cells were exposed to OGD for a period of 4 h (OGD 4 h). At the end of the OGD period, culture medium was replaced with oxygenated serum-free medium, and cells were placed and maintained in the normoxic incubator for 24 h to recovery (R24h). In the neuroprotection experiments, citicoline or nitrones (0.1 μM–1 mM) were added at the beginning of recovery period. Control cultures in Dulbecco's medium containing glucose were kept in the normoxic incubator for the same period of time as the OGD, and then culture medium was replaced with serum-free medium and cells were returned to the normoxic incubator until the end of recovery period. Control experiments included the same amounts of vehicle (final concentration <0.1% dimethyl sulfoxide or ethanol). The experimental procedures were blindly performed, assigning a random order to each assayed nitrone. Nitrones were analyzed independently three–five times with different batches of cultures, and each experiment was run in quadruplicate.

Cell Viability Assay. Cell viability was evaluated by quantification of living, metabolically active cells, as determined by a colorimetric assay using the photometric reduction of MTT to a blue formazan product. The assay of living, metabolically active cells was performed by incubating with 0.2 mg/mL MTT in the culture medium for 1.5 h in an incubator at 37 °C in a 6.5% CO_2 atmosphere. After the incubation period, cells were lysed with an equal volume of 10 mM HCl and 10% SDS overnight. Values were quantified by absorbance (test 595 nm, reference 690 nm). Decreased MTT activity denotes impairment of mitochondrial function and is considered to be an index of cell damage. Primary neuronal cultures were collected at times indicated for MTT determination.

Measurement of LDH Activity. For these assays, cultured neurons grown in 24-well culture dishes at a density of 0.5×10^6 cells/well were used. LDH activity was measured as the rate of decrease of the absorbance at 340 nm, resulting from the oxidation of NADH to NAD$^+$ as described.[77,78] Briefly, the culture medium was collected and the neurons were lysed with 0.1 M Tris-HCl (pH 7.4) containing 0.1% Triton X-100, cell suspensions were centrifuged at 13000g for 5 min, and LDH activity was measured in both preparations, culture medium and cell supernatants. Data are given as percentage of LDH release with respect to the total LDH content (LDH in the culture medium plus LDH inside the cells). Increased LDH release denotes increase in plasma membrane damage produced by necrotic cell death.

In Vitro BBB Permeation Assay. Prediction of the brain penetration was evaluated using a parallel artificial membrane permeation assay (PAMPA), in a similar manner as previously described.[53−64] Commercial drugs, phosphate buffered saline solution at pH 7.4 (PBS), and dodecane were purchased from Sigma, Aldrich, Acros, and Fluka. Millex filter units (PVDF membrane, diameter 25 mm, pore size 0.45 μm) were acquired from Millipore. PBL was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μm) and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 180 μL of PBS/ethanol (70:30), and the filter surface of the donor microplate was impregnated with 4 μL of PBL in dodecane (20 mg mL^{-1}). Compounds were dissolved in PBS/ethanol (70:30) at 100 μg mL^{-1}, filtered through a Millex filter, and then added to the donor wells (180 μL). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which was left undisturbed for 240 min at 25 °C. After incubation, the donor plate is carefully removed and the concentration of compounds in the acceptor wells was determined by UV spectroscopy. Every sample is analyzed at five wavelengths, in four wells and at least in three independent runs, and the results are given as the mean ± standard deviation. In each experiment, 15 quality control standards of known BBB permeability were included to validate the analysis set.

AUTHOR INFORMATION

Corresponding Author

*(D.H.-L.) Phone: +30 2310997627. Fax: +30 2310997852. E-mail: hadjipav@pharm.auth.gr. (A.A.) Phone: +34 913368400. Fax: +34 91336-9016. E-mail: alberto.alcazar@hrc.es. (M.J.O.-G.) Phone: 34-1-3941788. Fax: 34-1-3941779. E-mail: mjoset@farm.ucm.es. (J.M.-C.) Phone: +34 915622900. Fax: +34 915644853. E-mail: iqoc21@iqog.csic.es.

Author Contributions

▽These authors have contributed equally to this work.

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ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; BBB, blood–brain barrier; citicoline, cytidine-5′-diphosphocholine; LDH, lactic acid dehydrogenase; LOX, lipoxygenase; LP, lipid peroxydation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; OGD, oxygen–glucose

deprivation; PBN, phenyl-*tert*-butylnitrone; PBL, porcine brain lipid; ROS, reactive oxygen species

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