Full Paper

2-Benzazepine Nitrones Protect Dopaminergic Neurons against 6-Hydroxydopamine-Induced Oxidative Toxicity

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A number of C-3 spirocyclic 2-benzazepine analogs of α-phenyl-*N*-*tert*-butyl nitrone (PBN) were synthesized and tested for their activity in protecting rat brain mitochondria and dopaminergic (DA) neurons against 6-hydroxydopamine (6-OHDA), a toxin inducing destruction of the DA nigrostriatal pathway in rodent models of Parkinson's disease. The newly synthesized nitrone derivatives were firstly investigated for their activity in decreasing the level of hydroxyl radicals generated during 6-OHDA oxidation, and inhibit lipid peroxidation (TBARS assay) and protein carbonyl content (PCC) in rat brain mitochondria. Most of the studied 2-benzazepine nitrones showed inhibitory potencies in both TBARS and PCC assays at least two magnitude orders higher than that of PBN. The data obtained usefully complemented the known structure–activity relationships. In particular, **5** and **10**, bearing C-3 spiro cyclopentyl and tetrahydropyranyl moieties, respectively, at 8 μM concentration proved to be significantly more effective than PBN in protecting cultured DA neurons exposed to 6-OHDA, which alone causes about 45% cell loss in 24 h. In addition, we found that **5** inhibited butyrylcholinesterase with an IC_{50} value of 16.8 μM, which would enhance its potential as neuroprotective agent in Alzheimer's neurodegeneration. These findings extend the utility of benzazepine-based PBN analogs in the treatment of age-related free radical-mediated disorders.

Keywords: 2-Benzazepine nitrones / Cholinesterase inhibition / Dopaminergic neurons / 6-Hydroxydopamine / Neuroprotection

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Introduction

Linear and cyclic nitrones (Fig. 1), such as α-phenyl-*N*-*tert*-butyl nitrone (PBN, **1**), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), are among the most commonly used spin-trapping reagents, which not only have contributed to the understanding of free radical-mediated processes in biochemical systems, but have found applications as therapeutic agents in the treatment of pathological disorders caused by unregulated production of reactive oxygen species (ROS) [1], including ischemia-reperfusion injury [2], neurodegeneration, and aging processes [3, 4].

PBN has been used in the treatment of age-related disorders, such as stroke, Parkinson's (PD), and Alzheimer's disease (AD) [1, 5, 6]. Disodium-[(*tert*-butylimino)-methyl]benzene-1,3-disulfonate *N*-oxide (NXY-059), which was the first neuroprotective

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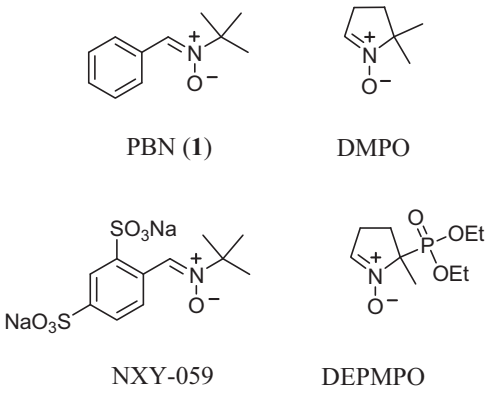


Figure 1. Structures of PBN, **1** and other linear and cyclic nitrones.

agent that entered phase-3 clinical trials [7, 8], showed efficacy in the acute treatment of ischemia injury due to stroke. The cyclic derivative DMPO has shown cardioprotective properties [9]. Recently, new amphiphilic amide conjugates of cyclic and linear nitrones have been reported which protect the cell against oxidative insults, acting not only as radical scavengers but also as bioenergetic agents targeted to the mitochondrial electron and proton transport chain [10, 11].

Based on Floyd's seminal work [1], PBN and analogs are more than just radical scavengers. Mechanisms other than spin-trapping, including antioxidant properties, action on membrane enzymes and ion transport proteins, and anti-inflammatory activity, have been investigated. With respect to the neuroprotection properties, PBN has been also shown to reversibly and dose-dependently inhibit acetylcholinesterase (AChE, EC 3.1.1.7) from mouse brain, with a competitive inhibition constant (K_i) of 0.58 mM [12], whereas it does not interfere with muscarinic or glutamate receptors [13, 14].

Among the cyclic nitrones, a number of 3,3-dimethyl-4,5-dihydro-3*H*-2-benzazepine 2-oxides, such as compound **2** (Fig. 2), and its spiro C-3 cycloalkyl analogs, have been found out as inhibitors of lipid peroxidation. As the electron paramagnetic resonance (EPR) spectroscopy demonstrated, 2-benzazepine nitrone analogs are able to trap radicals, forming more stable spin adducts than PBN in both lipid and apoprotein fractions of low density lipoproteins [15, 16]. A number of 2-benzazepine nitrone derivatives have been also evaluated by us [17] for their activity *in vitro* as protective agents against oxidative stress induced in rat brain mitochondria by 6-hydroxydopamine (6-OHDA), a toxin inducing degeneration of dopaminergic (DA) neurons in the nigro-striatal tract, which is widely used to reproduce a rodent model of PD [18–20].

Once inside DA neurons, 6-OHDA readily oxidizes to form ROS, such as H_2O_2 [21], and the corresponding *p*-quinone, to reduce striatal levels of antioxidant enzymes, such as total glutathione (GSH) [22] or superoxide dismutase [23], to increase iron levels in the substantia nigra [24], and to interact directly with complexes I and IV of the mitochondrial respiratory chain [25, 26], leading as a consequence to respiratory inhibition and further oxidative stress. H_2O_2 may generate hydroxyl radicals ($^{\bullet}OH$) [27], and ultimately lipid peroxidation products. The 6-OHDA quinone triggers a cascade of oxidative reactions resulting in the formation of an insoluble polymeric pigment related to neuromelanin [28]. ROS may be central to the etiology of PD and other disorders with α-synuclein pathology [29]. Transition metals (e.g., iron) are able to catalyze the production of ROS [30], which could induce α-synuclein aggregation, suggesting a possible link between oxidative stress and α-synuclein fibril formation [31]. Besides the age-related alterations in the ubiquitin–proteasome system [32], impaired antioxidative mechanisms may result in oxidative stress. Indeed, human brain copper–zinc superoxide dismutase (SOD1) has been proven to be a major target of oxidative damage in PD and AD [33].

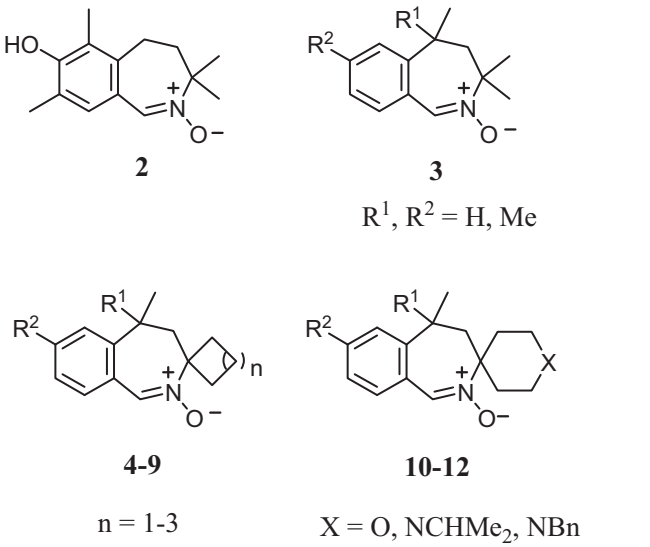


Figure 2. Structures of 3,3,6,8-tetramethyl-4,5-dihydro-3*H*-2-benzazepin-7-ol 2-oxide (**2**) [16] and benz-2-azepine nitrones (**3**–**12**) investigated in this study.

ROS in neuronal cell membranes can initiate lipid peroxidation of polyunsaturated fatty acids (PUFAs). PUFAs, following free radical attacks under oxidative stress conditions, can produce lipid peroxides, which would be fragmented to form reactive carbonyl species (RCS), such as acrolein, malondialdehyde, 4-oxo-2-nonenal (ONE), and 4-hydroxy-2-nonenal (HNE) [34, 35]. These extremely reactive carbonyl compounds, in addition to being cytotoxic themselves, are capable of covalently modifying proteins, altering their normal structure and function. PCC is an irreversible oxidative damage that often leads to formation of high-molecular-weight aggregates, which are resistant to degradation and accumulate as damaged or unfolded proteins [36]. RCS, formed during lipid

peroxidation and sugar glycoxidation, accumulate with ageing and oxidative stress-related diseases, such as atherosclerosis, diabetes, or neurodegenerative diseases [37]. Acrolein-modified α-synuclein was found in PD substantia nigra [38]; increased levels of HNE-modified proteins have been observed in affected areas of PD, dementia with Lewy bodies (DLB), and multiple-system atrophy (MSA) brains [39–41]. Moreover, increased levels of HNE and malondialdehyde have been detected in plasma from AD patients [42].

In a previous study [17], we investigated a number of 4,5-dihydro-3*H*-2-benzazepine *N*-oxide derivatives for their potential as neuroprotective agents, by assessing inhibition of both 6-OHDA-induced lipid peroxidation (formation of thiobarbituric acid (TBA) reactive substances, TBARS) and protein oxidation (assessed as the increase of PCC) in rat brain mitochondria. New 2-benzazepine nitrones were identified which exhibited *in vitro* activity in protecting rat brain mitochondria from oxidative injury triggered by 6-OHDA. In particular, compound **7** (Fig. 2; Table 1), bearing 5-*gem*-dimethyl and spiro C-3 cyclohexyl groups, proved to be orders of magnitude more effective than PBN against 6-OHDA-induced oxidative damage.

With the aim of extending our structure–activity relationship (SAR) study, we synthesized a number of new 2-benzazepine nitrones (Fig. 2), focusing our investigation in particular on spiro C-3 cycloalkyl analogs. For the sake of comparison, the previously reported compounds **5** and **7** were included in the study. Besides the measurements of their ability to antagonize 6-OHDA-induced lipid peroxidation (TBARS assay) and increase of PCC in rat brain mitochondria, the most active compounds were tested for their protective effects in primary mesencephalic DA neurons exposed to 6-OHDA. Furthermore, some of them were studied *in vitro* for the cholinesterase (ChE) inhibition, in order to assess any additional neuroprotective properties not directly related to their radical-trapping activity.

Table 1. Lipophilicity ($\log k'_w$) and activity (IC_{50}) of PBN and related 2-benzazepine nitrones against 6-OHDA-induced hydroxyl radical formation

Compounds	R^1	R^2	n or X	$\log k'_w$ [a)]	IC_{50} (μM)[b)]
1 (PBN)				1.77	785 ± 19
3	Me	Me		3.17	24 ± 0.8*
4	Me	H	1	2.80	135 ± 7.2*
5	H	H	2	3.12	8.1 ± 0.9*
6	Me	H	2	3.40	9.0 ± 1.9*
7	Me	H	3	3.26	22 ± 1.2*
8	Me	Me	3	3.52	11 ± 3.2*
9	H	Me	3	3.32	249 ± 9.5*
10	Me	H	O	2.28	16 ± 0.5*
11	Me	H	$NCHMe_2$	1.72	22 ± 2.9*
12	Me	H	NBn	3.30	157 ± 7.8*

[a)] Log of RP-HPLC polycratic capacity factor, that is capacity factor extrapolated at 100% aqueous mobile phase (see Experimental section).

[b)] Concentration of the test nitrone required to inhibit by 50% the •OH formation, fluorimetrically monitored using terephthalic acid (THA) as a chemical dosimeter. Values are means ± SEM from four independent determinations.

* Statistically significant at $p < 0.05$ (one-way ANOVA and Bonferroni post hoc test) when compared to the control (BPN, **1**). Previously reported IC_{50} values [17] for compounds **5** and **7** were 7.0 ± 1.2 and 19 ± 2.6, respectively.

Results and discussion

We evaluated a number of new derivatives of 4,5-dihydro-3*H*-2-benzazepine *N*-oxide, bearing some diverse spiro C-3 cycloalkyl groups, for their ability to protect DA neurons against the oxidative injury induced by 6-OHDA, a neurotoxin producing experimental model of PD [18, 43–46]. It has been shown that 6-OHDA, under physiological conditions, undergoes rapid oxidation with formation of H_2O_2, which in turn produce cytotoxic species like •OH radicals, and the corresponding oxidation product, namely the deprotonated *p*-quinone, which is the only species detected at pH >5 [47, 48]. Evidence has been provided that 6-OHDA reacts releasing Fe(II) from the iron storage protein ferritin [49], whereas the 6-OHDA quinone intermediate yields dopaminechrome, which is not stable and further reacts to form an insoluble polymeric material known as neuromelanin [50]. The formation of the •OH radical occurs without involvement of the Fe ion, or any other transition metal ion, in Fenton-type reactions [51].

The effects of the newly synthesized nitrones, the reference compound PBN and the already reported **5** and **7** (positive controls) on the •OH generated during 6-OHDA (10 μM) oxidation were firstly assessed by measuring the relative fluorescence after 10 min of incubation, using THA as a chemical dosimeter [17, 20, 48, 52, 53]. The IC_{50} values are listed in Table 1.

The data show that all the test nitrones inhibited 6-OHDA-dependent •OH production more potently than PBN. The activities of the analogs **3**, **5**–**8**, **10**, and **11**, which displayed 30–100-fold improvement over PBN, are worthy of note. The bulkiness of the spiro C-3 cyclic group affects the •OH-scavenging activity. Indeed, their optimal size should be between those of the cyclopentyl (**5** and **6**) and cyclohexyl (**7** and **8**) groups, whereas the bulkiest *N*-benzylpiperidine group (**12**) causes a sharp drop in activity. It was previously observed that, in general, 5,5-dimethyl-substituted 2-benzazepine nitrones are more active than the respective monomethyl derivatives [17]. In this study, this is particularly evident with the spiro C-3 cyclohexyl derivatives (**8** vs. **9**), but not with the cyclopentyl ones (**6** vs. **5**).

The oxidative stress induced by 6-OHDA, which causes lipid peroxidation and protein damage, is thought to be involved in neuronal cell death associated to PD. Using this model, we investigated the effects of compounds **3**–**12** on 6-OHDA-induced oxidative damage in rat brain mitochondrial membrane homogenates [17, 54], assessing both the formation of TBARS and increase of PCC. With the exception of derivatives **9** and **12**, which did not attain 50% TBARS inhibition at the maximum concentration tested (1 mM), all the assayed benzazepine nitrones showed reduction in the TBARS level, with inhibitory potencies 1–2 orders of magnitude higher than that of PBN (Table 2).

The C-3 spirocyclic benzazepine nitrone derivatives **5**–**8**, **10**, and **11**, with IC_{50} values ranging from 17 to 45 μM, showed the most remarkable effects against lipid peroxidation, displaying 150–400-fold increase of the TBARS inhibition potency over PBN. The most lipophilic compound **8** was the most potent one.

Previous findings of others [55, 56] and ourselves [17] proved that lipophilicity contributes to increase the inhibition of lipid peroxidation. We measured the relative lipophilicity ($\log k'_w$) of the 2-benzazepine nitrones through a reversed-phase (RP)-HPLC technique [17, 57] (Table 1), showing that all the investigated compounds, with the exception of **11**, are more lipophilic than the reference PBN. Compound **11** (calculated $pK_a = 9.33$), which should be predominantly protonated at the piperidine nitrogen at physiological pH (like **12**, $pK_a = 8.22$), was nearly isolipophilic with PBN. The whole spread of the measured $\log k'_w$ is 1.80 log units, but most of the examined compounds (i.e., the spiro C-3 cycloalkyl analogs **4**–**9**) vary in just 0.7 log units. Homologation of the spiro C-3 cyclobutyl group (**4**) to the cyclopentyl one (**6**) results in $\log k'_w$ increment of 0.6 units, whereas lipophilicity of the higher cyclohexyl homolog (**7** vs. **6**) no longer increases. The addition of a second CH_3 group at C-5 (compare **6** with **5**, and **8** with **9**), as well as the grafting of a CH_3 at the C-7 position (e.g., **8** vs. **7**), results in a modest $\log k'_w$ increment (0.2–0.3 units).

Our data suggest that all the 2-benzazepine nitrones studied should be lipophilic enough to have a good affinity for and great residence time in the lipid phase, allowing the lipids to be protected from peroxidation in brain mitochondria. While no statistically significant relationship was found between pIC_{50}^{TBARS} and $\log k'_w$, the lipid peroxidation inhibition potency was linearly correlated with the $^{\bullet}OH$-scavenging activity *in vitro*, as demonstrated by the following regression equation:

$$pIC_{50}^{TBARS} = 1.29(\pm 0.093)pIC_{50}^{OH} - 1.71(\pm 0.411)$$
$$n = 11;\ r^2 = 0.955;\ s = 0.197;\ F = 192 \quad (1)$$

In the above regression analysis a truncated pIC_{50}^{TBARS} value of 3.00 for compounds **9** and **12** ($IC_{50} > 1$ mM) was used to retain SAR information.

As for the inhibition of PCC increase, PBN exhibited very low activity ($IC_{50} = 17.3$ mM). In contrast, the examined 2-benzazepine nitrones, with the exception of **9**, significantly reduced the carbonyl content in rat brain mitochondrial proteins with IC_{50} values in the micromolar range (Table 2). Once again, compounds **5**–**8**, **10**, and **11** (IC_{50} values ranging from 45 to 53 μM) showed more than 300-fold increase of the PCC inhibition potency over PBN. The scatter plot of the pIC_{50}^{PCC} versus pIC_{50}^{OH} (not shown) suggested a trend of nonlinear (quadratic or bilinear) correlation; pIC_{50}^{PCC} linearly increases along with the increase of pIC_{50}^{OH} up to the value of ca. 4 (compounds **1**, **4**, **9**, and **12**), while remaining quite constant for the compounds showing $pIC_{50}^{PCC} > 4$ (**3**, **5**–**8**, **10**, and **11**).

Overall, the inhibition data in Table 2 show that, with two exceptions (**9** and **12**), the investigated compounds are able to efficiently inhibit TBARS formation and PCC increase in rat brain mitochondria preparations, with IC_{50} values orders of magnitude lower than those of PBN. The observed correlations between pIC_{50} values in TBARS (linear Eq. 2) and PCC (trend of quadratic or bilinear relationship) assays and $pIC_{50}{}^{OH}$ values would suggest that the neuroprotective properties of the 2-benzazepine nitrones should depend, at least

Table 2. Inhibitory effect (IC_{50}) of PBN and related 2-benzazepine nitrones on lipid peroxidation (TBARS) and protein carbonylation (PCC) induced by 6-OHDA in rat brain mitochondria

Compounds	IC_{50} (μM)[a]	
	TBARS	PCC
1 (PBN)	7245 ± 211	17 329 ± 351
3	43 ± 6.7*	55 ± 6.1*
4	369 ± 9.4*	117 ± 8.1*
5	29 ± 1.8*	49 ± 2.0*
6	27 ± 2.6*	46 ± 1.3*
7	25 ± 3.7[b]	51 ± 3.4*
8	17 ± 2.9*	45 ± 4.4*
9	–[b]	–[b]
10	32 ± 1.5*	49 ± 2.8*
11	45 ± 2.3*	53 ± 3.4*
12	–[b]	276 ± 3.7*

[a] Concentration of the test nitrone required to reduce the formation of thiobarbituric acid reactive substances (TBARS) and increase of protein carbonyl content (PCC). Values are means ± SEM from four independent determinations.

[b] No statistically significant inhibition activity was attained up to the maximum tested concentration (1 mM).

* Statistically significant at $p < 0.05$ (one-way ANOVA and Bonferroni post hoc test) when compared to the control (PBN). The following IC_{50} values were previously reported [17] for compounds **5** and **7**: 36 ± 6.2 and 20 ± 3.5 (TBARS), and 53 ± 3.9 and 48 ± 4.0 (PCC), respectively.

in part, upon their ability to trap damaging •OH radicals or other free radical species. However, the formation of nitroxyl radical species, which act as chain-breaking antioxidants [58], thus preventing propagation of either lipid peroxidation and PCC, may be supposed as additional mechanism accounting for the observed activities.

In terms of SARs, the data obtained in this study support the importance of the size of the spirocycle moiety at C-3, which must be as large as the cyclopentyl (**5** and **6**) and cyclohexyl (**7** and **8**) groups. The spiro C-3 cyclohexyl group may be replaced by saturated six-membered heteroyclic groups of comparable molar volume, such as the tetrahydro-2*H*-pyran-4-yl group (**10**), without significantly decreasing the protective activity in brain mitochondria. In contrast, the replacement of the cyclohexyl group with the 1-alkylpiperidin-4-yl groups (**11** and **12**), predominantly in protonated form at pH 7.4, can be tolerated for relatively small alkyl groups, like the isopropyl one (**11**), whereas the alkylation of the piperidine nitrogen with the bulky benzyl group (**12**) does sharply decrease the activity.

The protective effects of PBN (**1**) and the most active 2-benzazepine nitrones in the TBARS and PCC assays, namely **5**, **6**, **7**, and **10** (and not **8**, which exhibited the lowest solubility in the assay conditions), were tested and compared to PBN in cultured primary mesencephalic DA neurons exposed to 6-OHDA. Cells were incubated with the test compounds, each at three concentrations (8, 40, or 200 μM), 30 min before being exposed to 20 μM 6-OHDA for 24 h. In the cell cultures, DA neurons were identified by TH immunohistochemistry. Control cultures contained numerous TH-immunoreactive (ir) cells showing long and branching processes (Fig. 3A). The ability of the test compounds to prevent the 6-OHDA-induced degeneration and death of the TH-ir cells is shown in Fig. 4; the photomicrographs of TH-ir neurons showing the effects of compounds **5** and **7** at 200 μM are reported in Fig. 3 (C and D).

Twenty-four hours after treatment with 20 μM 6-OHDA, cultures showed a marked reduction (about 45%) in the number of TH-ir neurons relative to the control cultures (Figs. 3B and 4).

Treatment with PBN at 8 and 40 μM concentrations (but not 200 μM) significantly decreased the 6-OHDA-induced DA neuron degeneration (around 25% reduction), but the number of TH-ir neurons was significantly lower than in the control cultures. In contrast, the loss of TH-ir neurons induced by the toxin was blocked (i.e., levels not significantly different to controls) by simultaneous treatment with **5** and **10** at all the concentrations tested. Compound **6** showed significant protective effects only at the highest concentration (200 μM), whereas compound **7** did not significantly reduce the 6-OHDA-induced cell loss even at 200 μM concentration (Figs. 3D and 4).

The differences in cell viability maintenance between the test compounds are not correlated with the differences in their IC_{50}^{OH} values. This lack of correlation could be attributed to the existence of other factors involved in their pharmacological action and/or accumulation and localization of the nitrone compounds in the cell, which could be nonlinearly related to lipophilicity as our data suggest.

Previously reported evidence, showing that the beneficial effects of chronic PBN treatment in retarding of aging and age-related cognitive deficits could be related, besides to its capacity of removing ROS, to its ability of inhibiting AChE [12], prompted us to study the ChE (AChE and BuChE) inhibitory properties of compounds **5**–**7** and **10**. The potential for ChE inhibition by our compounds is somehow supported by the evidence that also chlordiazepoxide, a benzodiazepine containing a nitrone group, is a reversible mixed-type inhibitor of AChE and BuChE, with K_i values of 50 and 0.30 μM, respectively [59].

The effects *in vitro* on the ChE activity of PBN **1** and 2-benzazepine nitrones **5**–**7** and **10** were studied in concentration up to 2 mM, using the spectrophotometric kinetic assay of Ellman et al. [60] with AChE from electric eel (*Electrophorus electricus*) and BuChE from equine serum, as model ChEs. The mean IC_{50} values are given in Table 3.

The ChE inhibition assay revealed that the tested 2-benzazepine nitrones act as BuChE-selective inhibitors, with a potency higher than (or at least comparable with) that of PBN. Compound **5** showed about 70-fold selectivity for BuChE over AChE, with IC_{50} values of 16.8 μM and 1.15 mM, respectively. A comparison between BuChE inhibition data and $\log k'_w$ values suggests that the inhibition potency depends largely on the lipophilicity, since **5**–**7**, with $\log k'_w$ ranging between 3.1 and 3.4, are one order of magnitude more potent than **1** and **10**, having $\log k'_w$ values of 1.8 and 2.3, respectively.

While a more in-depth SAR study on cyclic nitrones could help in the understanding of the molecular factors modulating their ChE inhibition potency and selectivity, our preliminary data highlighted the anti-BuChE activity as a possible additional mechanism contributing to improve the potential of the benzazepine nitrone analogs for the treatment of AD neurodegeneration. Indeed, it is known that in healthy human brain AChE predominates over BuChE activity, but, as AD progresses, the levels of AChE in the brain decrease by as much as 90%, whilst the levels of BuChE, mainly in the G_1 form (i.e., globular form of monomer structure), increase [61–63]. This suggests that the inhibition of BuChE may be useful in ameliorating the cholinergic transmission, which likely worsen in AD due to the BuChE increased activity [64].

In conclusion, this study led us to identify some new nitrone-based compounds with good activity, much stronger than that exerted by PBN, in protecting rat brain mitochondria from lipid peroxide formation and protein oxidative

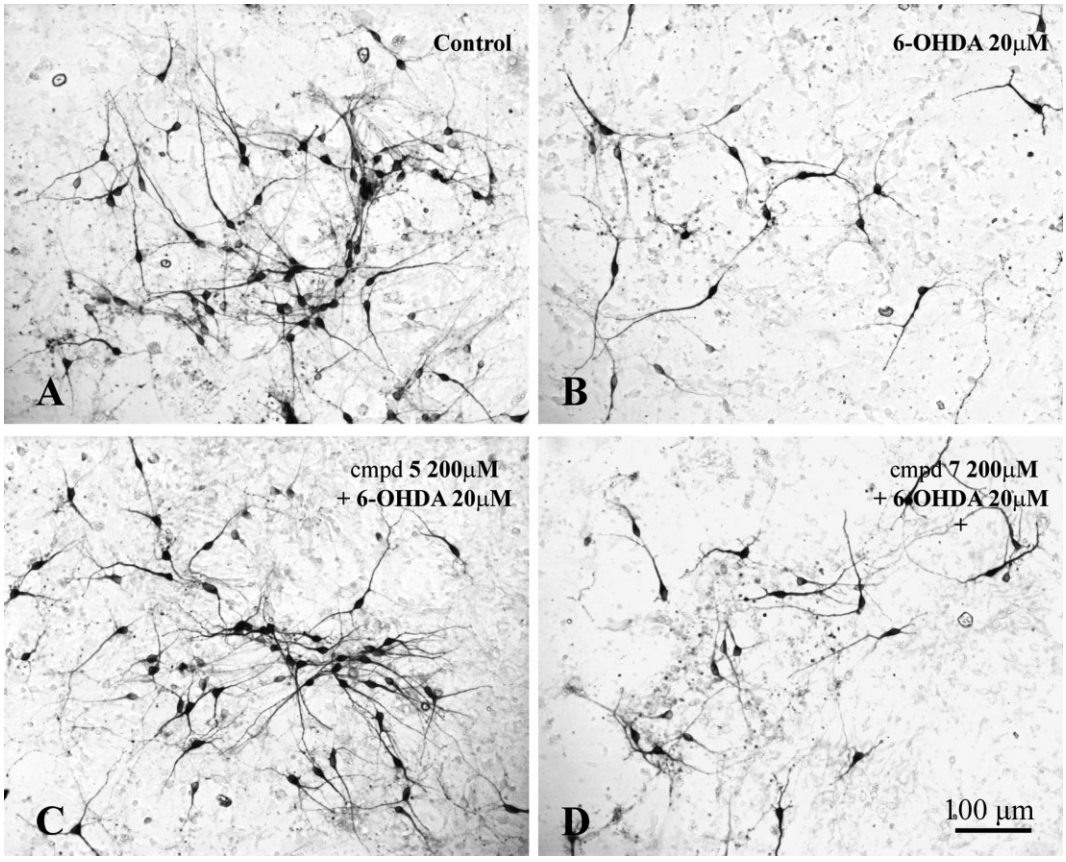


Figure 3. Photomicrographs of representative TH-immunoreactive (-ir) neurons: control culture (A); culture treated with 20 μM 6-OHDA (B); cultures simultaneously treated with 6-OHDA (20 μM) and compounds **5** (C), or **7** (D) at 200 μM concentration. Scale bar: 100 μm.

damage induced by the neurotoxin 6-OHDA. A major outcome was the demonstration that two compounds, namely **5** and **10**, bearing as spirocyclic moieties at C-3 the cyclopentyl and tetrahydropyranyl groups, respectively, are significantly more effective than PBN in protecting cultured DA neurons against 6-OHDA-induced toxicity, maintaining their function and viability. The observed inhibitory effects on BuChE activity, with IC_{50} in the micromolar range, may contribute to improve the therapeutic potential of 2-benzazepine nitrones in age-related neurodegenerative diseases.

Experimental

Chemicals and reagents

PBN, 6-OHDA hydrobromide, terephthalic acid (THA), TBA, butylated hydroxytoluene crystalline, 2,4-dinitrophenylhydrazine hydrochloride, desferrioxamine, 1,1,3,3-tetraethoxypropane, 5,5′-dithiobis-(2-nitrobenzoic acid), sodium dodecylsulfate (SDS), EDTA, hydrogen peroxide, bovine serum albumin (BSA), mouse monoclonal antibody to tyrosine hydroxylase (TH), 3,3′-diaminobenzidine, trypsin, DNase, poly-L-lysine, laminin, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Avidin–biotin-peroxidase complex and biotinylated secondary antibody were purchased from Vector (Burlingame, CA, USA). Guanidine hydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F12 (HAMS F12) were obtained from Gibco (Paisley, UK). Fetal bovine serum was from Biochrom KG (Berlin, Germany). The water used for the preparations of solutions was of 18.2 MΩ (Milli-RiOs/QA10 grade, Millipore Corp., Bedford, MA, USA). All remaining chemicals used were of analytical grade and were purchased from Fluka Chemie AG (Buchs, Switzerland).

Synthesis

All solvents were distilled and dried before use. Melting points (mp) were determined in a capillary tube and are uncorrected. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ or in DMSO-d_6 solutions using a Bruker WM 400 or Jeol JNM-ECA 600 NMR spectrometers. Chemical shift values are expressed in δ (ppm) and the coupling constants J in Hertz. Abbreviations used for signal patterns are as follows: s, singlet; d, doublet; t, triplet; q, quartet, dd, doublet of doublets; m, multiplet; brd, broad; signals due to NH and OH protons were located by deuterium exchange with D_2O. Mass spectra were obtained by the EI technique (Finnigan-MAT 95 XL engine), ESI method (Agilent 1100 Series

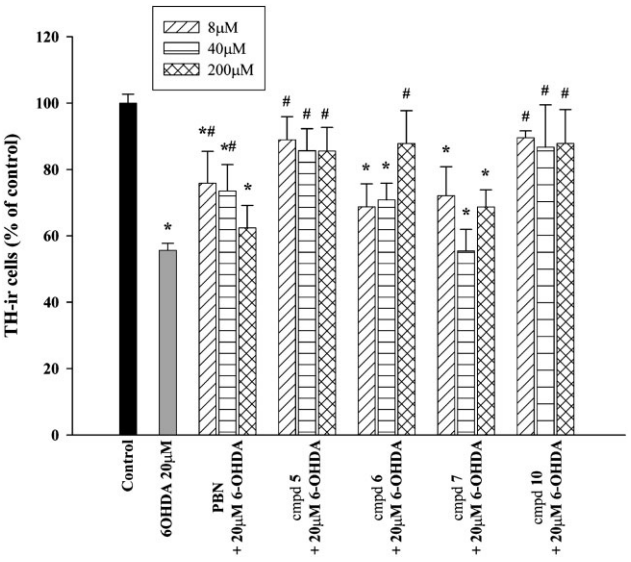


Figure 4. Effects of treatment with 6-OHDA (20 μM) and PBN or compounds **5**–**7** and **10** (8, 40, and 200 μM) on the number of TH-immunoreactive (-ir) cells. The data are expressed as percentages of the number of TH-ir cells obtained in the respective control cultures (100%) and represent means ± SEM; $^{*}p < 0.05$ versus control group (untreated cells), $^{\#}p < 0.05$ versus 20 μM 6-OHDA group (one-way ANOVA and Bonferroni post hoc test).

LC/MSD Trap System VL) or GC-MS method (Thermo Focus DSQ II, EI, 70 eV, ion source temperature 200°C, gas chromatographic inlet probe with Varian FactorFour VF-5ms column). IR spectra were recorded using a Perkin-Elmer Spectrum One FT-IR spectrophotometer, and the most significant absorption bands expressed in cm^{-1} are listed. Elemental analyses (C, H, and N) were performed on an Euro EA3000 analyzer, and the results agreed to within 0.4% of the theoretical values.

3,3-Dimethyl-4,5-dihydro-3*H*-2-benzazepine *N*-oxide derivatives and their spiro *C*-3-cycloalkyl analogs were prepared through the reactions shown in Scheme 1. Accordingly, 2,3,4,5-tetrahydro-1*H*-2-benzazepines were synthesized from readily available imines. They were then converted into 2-benzazepine nitrones **3**–**12** via hydrogen peroxide oxidation in the presence of catalytic amounts of sodium tungstate, under mild conditions.

Table 3. Cholinesterase (ChE) inhibition data of PBN and selected 2-benzazepine nitrones

Compounds	ChE inhibition, IC_{50} (μM)[a]	
	AChE	BuChE
1 (PBN)	>2000 (12%)	478 ± 38
5	1147 ± 34	16.8 ± 0.54
6	925 ± 42	27.5 ± 3.3
7	>1000 (6%)[b]	73.0 ± 1.0
10	>2000 (21%)	418 ± 33

[a] IC_{50} values (means ± SEM of at least three experiments) of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), or % inhibition (average value of three measurements in parentheses) at the maximum inhibitor concentration assayed (2 mM). [b] Due to its limited solubility, compound **7** was tested up to 1 mM concentration.

Compounds **5** and **7** have been reported earlier [17, 65]. Compounds **3**–**10** and **12** were obtained and tested as hydrochlorides. Chiral compounds **5** and **9** were tested as racemic mixtures.

General synthesis procedures and/or suitable references, along with selected spectral and physical data, for the newly synthesized target nitrone derivatives (**3**, **4**, **6**, and **8**–**12**) are given below. Data for the respective homoallylamine and 2,3,4,5-tetrahydro-1*H*-2-benzazepine intermediates are reported in Supporting Information.

Homoallylamines

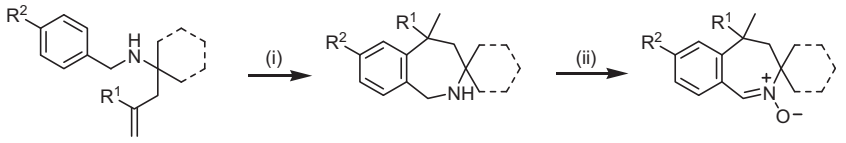
2,4-Dimethyl-*N*-(4-methylbenzyl)pent-4-en-2-amine (for **3**), 1-(2-methylprop-2-en-1-yl)-*N*-(phenylmethyl)cyclobutanamine (for **4**), 1-(2-methylprop-2-en-1-yl)-*N*-(phenylmethyl)cyclopentanamine (for **6**), *N*-(4-methylbenzyl)-1-(2-methylprop-2-en-1-yl)cyclohexanamine (for **8**), 1-allyl-*N*-(4-methylbenzyl)cyclohexanamine (for **9**), 4-(2-methylprop-2-en-1-yl)-*N*-(phenylmethyl)tetrahydro-2*H*-pyran-4-amine (for **10**), 1-isopropyl-4-(2-methylprop-2-en-1-yl)-*N*-(phenylmethyl)piperidin-4-amine (for **11**), and 4-(2-methylprop-2-en-1-yl)-*N*-di(phenylmethyl)piperidin-4-amine (for **12**) were obtained in 51–71% yields, according to reported procedures [65–67] from the corresponding imines and allyl(methallyl) magnesium bromide (chloride).

Tetrahydrobenz-2-azepines

3,3,5,5,7-Pentamethyl-2,3,4,5-tetrahydro-1*H*-2-benzazepine (for **3**), 5,5-dimethyl-1,2,4,5-tetrahydrospiro[2-benzazepine-3,1′-cyclobutane] (for **4**), 5,5-dimethyl-1,2,4,5-tetrahydrospiro[2-benzazepine-3,1′-cyclopentane] (for **6**), 5,5,7-trimethyl-4,5-dihydrospiro-[2-benzazepine-3,1′-cyclohexane] (for **8**), 5,7-dimethyl-4,5-dihydrospiro[2-benzazepine-3,1′-cyclohexane] (for **9**), 5,5-dimethyl-1,2,2′,3′,4,5,5′,6′-octahydrospiro[2-benzazepine-3,4′-pyran] (for **10**), 1′-isopropyl-5,5-dimethyl-1,2,4,5-tetrahydrospiro[2-benzazepine-3,4′-piperidine] (for **11**), and 1′-benzyl-5,5-dimethyl-1,2,4,5-tetrahydrospiro[2-benzazepine-3,4′-piperidine] (for **12**) were prepared according by analogy to reported procedures [17, 65–68] and obtained in 33–82% yields.

*Oxidation of benz-2-azepines to nitrones **3**, **4**, **6**, and **8**–**12**: Typical procedure*

To a solution of 50 mmol of benz-2-azepine and $Na_2WO_4 \times 2H_2O$ (0.83 g, 2.5 mmol) in acetone/water mixture (9:1 v/v, 100 mL) 50% H_2O_2 (12 mL, 200 mmol) was added dropwise at 0°C in half an hour. The resulting mixture was stirred at room temperature for 1–4 days (TLC monitoring). The reaction mixture was then poured into water (300 mL) and extracted with CH_2Cl_2 (5 × 30 mL). The combined organic layers were washed with H_2O (2 × 50 mL), dried over $MgSO_4$ and concentrated *in vacuo*. Crystallization of the resulting solid from hexane/ethyl acetate mixture gave white crystals of nitrones (**4**, **8**, **10**, and **12**). In case of compounds **6** and **11** the oily residue was purified by column chromatography on Al_2O_3 (using hexane/ethyl acetate mixture, 5:1 v/v, as eluent). In case of poorly crystallizing nitrones, oils obtained after the workup were converted into hydrochloride salts by mixing their ether solutions (ca. 1 g of nitrone in 50 mL of dry diethyl ether) with saturated HCl/Et_2O solution until pH 5 was achieved. Resulting



R^1, R^2 = H or Me

Reagents and conditions: (i) H_2SO_4, 70°C, 1–4 h; (ii) 50% v/v H_2O_2, $Na_2WO_4{\times}2H_2O$, Me_2CO/H_2O (9:1), 0°C, 30 min, then r.t. 1–4 d (TLC monitoring).

Scheme 1. General synthesis scheme of the examined spiro C-3 cycloalkyl 2-benzazepine nitrones.

white precipitates were filtered off, washed with absolute ether, and dried on air.

3,3,5,5,7-Pentamethyl-4,5-dihydro-3H-2-benzazepine 2-oxide ***3***

White crystals, 64% yield, mp 62–63°C (from hexane/ethyl acetate mixture); hydrochloride – white crystals, mp 107–108°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.42 (s, 6H), 1.55 (s, 6H), 2.22 (s, 2H), 2.35 (s, 3H), 7.06 (m, 2H), 7.18 (brs, 1H), 8.00 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150.9 MHz) δ 21.6, 29.1 (2C), 31.7 (2C), 38.2, 54.6, 71.0, 125.2, 126.9, 127.3, 131.7, 139.2, 139.4, 147.6 ppm; GC-MS (70 eV) *m/z* (rel. intensity): 231 (33, M^+), 215 (5), 199 (13), 176 (23), 158 (100), 143 (86), 128 (66), 115 (65), 91 (34), 77 (19), 65 (16), 55 (20); IR (KBr, ν cm^{-1}) 1565, 1548 (C=N); Anal. Calc. for $C_{15}H_{21}NO$: C, 77.88; H, 9.15; N, 6.05. Found: C, 78.19; H, 9.09; N, 5.88.

5,5-Dimethyl-4,5-dihydrospiro[2-benzazepine-3,1′-cyclobutane]-2-oxide ***4***

White crystals; hydrochloride – white crystals, 85% yield, mp 97–98°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.39 (s, 6H), 1.60 (m, 2H), 1.70 (m, 2H), 2.53 (m, 2H), 2.55 (s, 2H), 7.13 (dd, J = 7.6, 1.0 Hz, 1H), 7.23 (dt, J = 7.6, 1.0 Hz, 1H), 7.28 (dt, J = 7.8, 1.3 Hz, 1H), 7.32 (brdd, J = 7.8 Hz, 1H), 7.78 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 12.8, 31.8 (2C), 32.7 (2C), 36.9, 56.6, 72.1, 125.5, 126.3, 129.0, 129.2, 129.6, 134.8, 146.8 ppm; EI-MS (70 eV) *m/z* (rel. intensity): 229 (78, M^+), 212 (100), 184 (36), 162 (69), 156 (93), 143 (53), 128 (87), 115 (52), 91 (38), 77 (26); IR (KBr, ν cm^{-1}) 1550 (C=N); Anal. Calc. for $C_{15}H_{19}NO$: C, 78.56; H, 8.35; N, 6.11. Found: C, 79.08; H, 8.16; N, 6.38.

5,5-Dimethyl-4,5-dihydrospiro[2-benzazepine-3,1′-cyclopentane]-2-oxide ***6***

Pale-yellow viscous oil, 80% yield; hydrochloride – white crystals, mp 171–172°C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.40 (s, 6H), 1.77–1.63 (m, 8H), 2.38 (s, 2H), 7.43 (brdt, J = 7.3 Hz, 1H), 7.55–7.52 (m, 2H), 7.63 (brdd, J = 7.8 Hz, 1H), 8.49 (s, 1H) ppm; ^{13}C NMR (DMSO-d_6, 100.6 MHz) δ 25.9 (2C), 29.4 (2C), 37.8, 39.9 (2C), 51.8, 80.8, 124.4, 125.2, 127.0, 134.2, 137.0, 152.2, 155.1 ppm; EI-MS (70 eV) *m/z* (rel. intensity): 243 (47, M^+), 226 (97), 211 (45), 162 (100), 144 (57), 128 (98),115 (44), 91 (35), 77 (24); IR (KBr, ν cm^{-1}) 1559 (C=N); Anal. Calc. for $C_{16}H_{21}NO$: C, 78.97; H, 8.70; N, 5.76. Found: C, 79.13; H, 8.47; N, 5.63.

5,5,7-Trimethyl-4,5-dihydrospiro[2-benzazepine-3,1′-cyclohexane]-2-oxide ***8***

White crystals, 76% yield, mp 117–118°C (from hexane/ethyl acetate mixture); hydrochloride – white crystals, mp 123–124.5°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.39 (s, 6H), 1.43–1.75 (m, 8H), 2.21–2.27 (m, 2H), 2.27 (s, 2H), 2.35 (s, 3H), 7.02 (m, 2H), 7.16 (d, J = 1.3 Hz, 1H), 8.00 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150.9 MHz) δ 21.5, 22.5 (2C), 24.9, 31.7 (2C), 35.9 (2C), 37.4, 54.4, 73.6, 126.4, 126.6, 127.2, 129.8, 137.8, 138.9, 146.9 ppm; GC-MS (70 eV) *m/z* (rel. intensity): 271 (38, M^+), 239 (40), 197 (15), 183 (16), 176 (29), 171 (25), 158 (89), 144 (91), 143 (91), 128 (66),115 (55), 105 (22), 95 (34), 91 (47), 77 (37), 67 (48), 55 (40), 41 (100); IR (KBr, ν cm^{-1}) 1549 (C=N); Anal. Calc. for $C_{18}H_{25}NO$: C, 79.66; H, 9.28; N, 5.16. Found: C, 79.62; H, 9.37; N, 5.36.

5,7-Dimethyl-4,5-dihydrospiro[2-benzazepine-3,1′-cyclohexane]-2-oxide ***9***

White crystals, 68% yield, mp 111–111.5°C (from hexane/ethyl acetate mixture); hydrochloride – white crystals, mp 146–147.5°C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.40 (d, J = 6.8 Hz, 3H), 1.32–1.92 (m, 8H), 1.85 (dd, J = 10.1, 15.3 Hz, 1H), 2.32 (m, 1H), 2.33 (s, 3H), 2.42 (dd, J = 2.5, 15.3 Hz, 1H), 2.51 (m, 1H), 3.07 (ddq, J = 2.5, 6.8, 10.1 Hz, 1H), 7.00 (dd, J = 1.0, 7.6 Hz, 1H), 7.03 (d, J = 7.6 Hz, 1H), 7.05 (d, J = 1.0 Hz, 1H), 7.94 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$, 150.9 MHz) δ 20.7, 21.3, 22.1, 22.7, 24.8, 31.7, 32.4, 36.3, 43.8, 75.4, 126.13, 126.03, 126.9, 130.6, 138.93, 139.0, 144.4 ppm; GC-MS (70 eV) *m/z* (rel. intensity): 257 (1, M^+), 240 (6), 224 (15), 215 (17), 198 (7), 173 (6), 162 (41), 144 (100), 130 (21),115 (37), 95 (30), 91 (26), 77 (23), 67 (27); IR (KBr, ν cm^{-1}) 1529 (C=N); Anal. Calc. for $C_{17}H_{23}NO$: C, 79.33; H, 9.01; N, 5.44. Found: C, 79.14; H, 9.20; N, 5.69.

5,5-Dimethyl-2′,3′,4,5,5′,6′-hexahydrospiro[2-benzazepine-3,4′-pyran]-2-oxide ***10***

White crystals, 48% yield, mp 109–110°C (from hexane/ethyl acetate mixture); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.39 (s, 6H), 1.52 (m, 2H), 2.20 (m, 2H), 2.33 (s, 2H), 3.60 (dt, J = 12.0, 4.4 Hz, 2H), 3.77 (ddd, J = 12.0, 9.4, 2.7 Hz, 2H), 7.07 (dd, J = 7.6, 1.3 Hz, 1H), 7.23 (dt, J = 7.6, 1.3 Hz, 1H), 7.27 (ddd, J = 7.9, 7.6, 1.3 Hz, 1H), 7.37 (brdd, J = 7.8 Hz, 1H), 7.95 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 32.2 (2C), 36.1 (2C), 37.2, 58.5, 64.2 (2C), 70.4, 126.0, 126.7, 128.9, 129.0, 129.6, 136.6, 146.2 ppm; EI-MS (70 eV) *m/z* (rel. intensity): 259 (26, M^+), 242 (37), 198 (80), 186 (78), 156 (27), 143 (50), 129 (100), 115 (79), 81 (99), 77 (28); IR (KBr, ν cm^{-1}) 1546 (C=N); Anal. Calc. for $C_{16}H_{21}NO_2$: C, 74.10; H, 8.16; N, 5.40. Found: C, 74.28; H, 8.37; N, 5.14.

1′-Isopropyl-5,5-dimethyl-4,5-dihydrospiro[2-benzazepine-3,4′-piperidine]-2-oxide ***11***

Pale-yellow oil, 32% yield (~10% impurities of the corresponding *N*,*N*-dioxide, 1′-isopropyl-5,5-dimethyl-4,5-dihydrospiro[2-benzazepine-3,4'-piperidine]-1′,2-dioxide, as determined by ^{1}H NMR).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.34 (d, J = 6.5 Hz, 6H), 1.42 (s, 6H), 2.15 (m, 2H), 2.39 (s, 2H), 2.52 (m, 2H), 2.97 (m, 2H), 3.33 (spt, J = 6.5 Hz, 1H), 3.54 (m, 2H), 7.08 (dd, J = 7.5, 1.2 Hz, 1H), 7.25 (ddd, J = 8.1, 7.5, 1.2 Hz, 1H), 7.32 (dt, J = 7.5, 1.2 Hz, 1H), 7.44 (dd, J = 8.1, 1.2 Hz, 1H), 7.92 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 16.7 (2C), 30.7 (2C), 32.8 (2C), 37.7, 57.9 (2C), 58.9, 69.7, 70.7, 127.0 (2C), 129.5 (2C), 129.8, 137.6, 146.9 ppm; IR (KBr, ν cm^{-1}) 1545 (C=N); Anal. Calc. for $C_{19}H_{28}N_2O$: C, 75.96; H, 9.39; N, 9.32. Found: C, 75.59; H, 9.58; N, 9.43.

1′-Benzyl-5,5-dimethyl-4,5-dihydrospiro[2-benzazepine-3,4′-piperidine]-2-oxide **12**

White crystals (hydrochloride), 60% yield; mp 179°C (with decomposition); ^{1}H NMR ($CDCl_3$, 400 MHz) 1.37 (s, 6H), 2.11 (m, 2H), 2.80 (m, 2H), 2.94 (s, 2H), 2.6 (m, 2H), 3.65 (m, 2H), 4.89 (s, 2H), 7.16–7.21 (m, 3H), 7.23 (m, 1H), 7.02 (brdt, J = 7.3 Hz, 2H), 7.42 (brdt, J = 7.3 Hz, 1H), 7.47 (brdd, J = 7.3 Hz, 2H), 8.00 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 29.1, 30.6, 31.7, 36.7 (2C), 38.9, 50.8, 58.7 (2C), 74.3, 88.3, 126.4, 126.5, 128.0 (2C), 128.9 (2C), 130.5, 133.2, 140.2, 141.3, 162.8, 178.6 ppm; IR (KBr, ν cm^{-1}) 1541 (C = N); EI-MS (70 eV) m/z (rel. intensity): 350 (10, M^+), 327 (10), 312 (29), 186 (14), 145 (21), 131 (7), 115 (8), 96 (15), 91 (100), 77 (17), 65 (8), 51 (6); Anal. Calc. for $C_{23}H_{28}N_2O$: C, 79.27; H, 8.10; N, 8.04. Found: C, 78.99; H, 9.27; N, 8.31.

Determination of $\log k'_w$ values by RP-HPLC

Lipophilicity parameters of the examined derivatives were measured by an RP-HPLC technique [17, 57]. Methanol solutions of the 2-benzazepine nitrones (0.25 mg/mL) were injected into a HPLC equipped with a Symmetry C18 column (150 mm × 3.9 mm id, 5 μm) from Waters Assoc. (Milford, MA, USA). The nitrones were eluted at different mobile phase compositions (0.05 increments of MeOH volume fraction in 40 mM phosphate buffer at pH 5, ranging between 0.75 and 0.45). All the RP-HPLC measurements were carried out at 25 ± 1°C, flow-rate of 1.0 mL/min, at both 254 and 320 nm on a Waters HPLC 1525 multisolvent delivery system, equipped with a Waters 2487 variable wavelength UV detector (Waters Assoc., Milford).

The capacity factors (k') at different mobile phase compositions were calculated as: $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the solute and t_0 is the elution time of MeOH, which is not retained on the column. For each nitrone compound a linear regression analysis (r^2 >0.96) was performed on at least five data points (the lowest MeOH fractions) and the resulting line extrapolated to 100% aqueous mobile phase to give the $\log k'_w$ values (Table 1).

Lipophilicity was also calculated using the ACDLabs software, release 9.0 (Advanced Chemistry Development, Inc., Toronto, Canada). The comparison between $\log k'_w$ and calculated log P (clog P values of the basic compounds **11** and **12**, with calculated pK_a of 9.33 and 8.22, respectively, were corrected for ionization) showed that a linear relation between the two sets of parameters holds up to clog P of ca. 3.5, a value beyond which $\log k'_w$ no longer increases (Fig. 5).

Omitting from the regression analysis the most lipophilic derivatives **7**–**9**, the following linear equation was obtained:

$$\log k'_w = 0.79(\pm 0.080)\ \text{clog}\,P + 0.78(\pm 0.204)$$
$$n = 8;\ r2 = 0.942;\ s = 0.178;\ F = 97.1 \qquad (2)$$

where n represents the number of data points, r^2 the squared correlation coefficient, s the standard deviation of the regression equation, and F is the statistical significance of fit; 95% confidence intervals of the regression coefficient are given in parentheses.

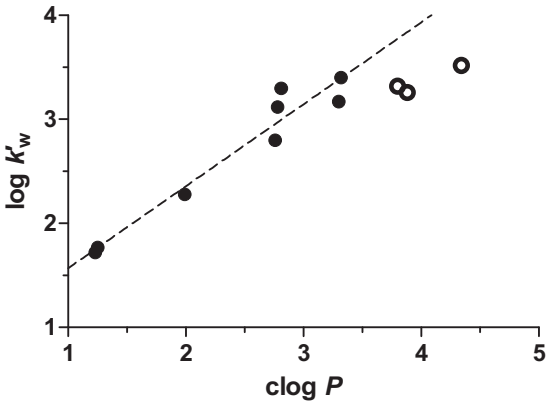


Figure 5. Graphical comparison between RP-HPLC lipophilicity parameter $\log k'_w$ and 1-octanol/water log P calculated by the ACDLabs software. For the basic compounds **11** and **12**, which should be predominantly in the protonated form at physiological pH (7.4), the calculated log D values were used.

Animals

Sprague–Dawley rats (250–300 g) obtained from the breeder of the Universidad de Santiago de Compostela were used for the studies in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. All the procedures were approved by the Animal Ethics Committee of the Universidad de Santiago de Compostela. Rats were housed at 22°C on a 12-h light–dark cycle with food and water available *ad libitum*. For sacrifice, animals were stunned with carbon dioxide and killed by decapitation.

Rat brain mitochondrial preparations

After sacrifice, brains were immediately removed and washed in ice-cold isolation medium (pH 7.4, Na_2PO_4/KH_2PO_4 isotonized with sucrose). Then, brain mitochondria were obtained by differential centrifugation with minor modifications to a previously published method [54]. After removing blood vessels and pial membranes, the brains were manually homogenized with four volumes (w/v) of the isolation medium. Then, the homogenate was centrifuged in an Avanti J-25 centrifuge (Beckman Instruments, Palo Alto, USA) at 1000 × g for 5 min at 4°C. The supernatant was centrifuged at 12 500 × g for 15 min. The mitochondrial pellet was then washed once with isolation medium and recentrifuged under the same conditions. Finally, the mitochondrial pellet was reconstituted in a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) and stored in aliquots under liquid nitrogen. The protein concentration of mitochondrial preparations was determined according to the method of Markwell et al. [69], using BSA as the standard.

Monitoring of hydroxyl radical formation

Hydroxyl radical (•OH) formation was investigated using a previously reported method [17], in which THA is used as a chemical

dosimeter of $^{\bullet}OH$ *in vitro*. The formation of $^{\bullet}OH$ during 6-OHDA autoxidation was monitored fluorimetrically using a luminescence spectrometer Model LS50B (Perkin-Elmer, Norwalk, CT, USA). Briefly, a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) containing 10 mM THA was incubated for 5 min to reach the working temperature (37°C). Then, the test 2-benzazepine nitrone (final concentration ranging from 1 μM to 1 mM) or water was incorporated into the incubation followed by addition of 6-OHDA (10 μM) and 1 mM KCl (pH 2.0). Monitoring of $^{\bullet}OH$ formation was performed for 10 min. The excitation and emission wavelengths used were 312 and 426 nm, respectively. Fluorescence measurements were all relative to the initial reading and the peak of relative fluorescence (ΔF_{max}) used to express the amount of the produced $^{\bullet}OH$. The test compound concentration that inhibited $^{\bullet}OH$ formation by 50% (IC_{50}) was calculated from a concentration-response curve generated with seven different concentrations of the 2-benzazepine nitrone, using the program OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA). The data are reported in Table 1.

Lipid peroxidation assay

The inhibitory effects of test 2-benzazepine nitrones on the lipid peroxidation were determined by monitoring the formation of TBARS, using a spectrophotometric method [17].

A brain mitochondrial preparation (1 mg protein/mL), in a Na_2PO_4/KH_2PO_4 buffer (pH 7.4) isotonized with KCl, was incubated at 37°C for 5 min to reach the working temperature. Then, the test 2-benzazepine nitrone (final concentration ranging from 1 μM to 1 mM) or water was incorporated into the incubation cell, followed by addition of 6-OHDA (10 μM) or 1 mM KCl (pH 2.0), and the mixture incubated for 20 min exactly. Butylated hydroxytoluene (20 μM) and desferrioxamine (20 μM) were immediately added, in order to prevent amplification of the lipid peroxidation during the assay.

A sample aliquot (200 μL) was treated with SDS (8.1%, w/v), followed by addition of acetic acid (20%), and the mixture vortexed for 1 min. Then, TBA (0.8%) was added and the resulting mixture incubated at 95°C for 60 min. After cooling to room temperature, 3 mL of *n*-butanol was added and the mixture shaken vigorously. After centrifugation at 2500 × *g* for 10 min, the absorbance of the supernatant (organic layer) was measured at 532 nm using an Ultrospec III spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). For calibration, a standard curve (5–150 nM) was generated using the malondialdehyde (MDA), prepared via acid-catalyzed hydrolysis (H_2SO_4; 1.5% v/v) of 1,1,3,3-tetraethoxypropane. The protein concentration of the samples was determined according to a previously reported method [69], using BSA as the standard, and the TBARS results were expressed as nmol MDA/mg protein. The IC_{50} values are listed in Table 2.

Assessment of protein carbonyl content

The ability of the test 2-benzazepine nitrones to inhibit the protein oxidative damage caused by the autoxidation of 10 μM 6-OHDA in the rat brain mitochondrial preparation was assayed by determining PCC, according to the previously reported method [17]. Brain mitochondria (1 mg protein/mL) were incubated in Na_2PO_4/KH_2PO_4 buffer (pH 7.4, isotonized with KCl), at 37°C for 5 min. Then, the test nitrone compound (final concentrations ranging from 1 μM to 1 mM) or water was incorporated into the incubation, followed by 6-OHDA (10 μM) or 1 mM KCl (pH 2.0), and the mixture was incubated for exactly 20 min.

A sample aliquot (200 μL) was then immediately submitted to protein precipitation, by addition of trichloroacetic acid (TCA 20% w/v), followed by centrifugation at 15 000 × *g* for 5 min. The resulting pellet was reconstituted in 0.5 M NaOH with sonication (Branson Sonic Corp., Danbury, CT, USA) for 5 s. Then, 10 mM 2,4-dinitrophenylhydrazine in 2 M chloric acid was added and the mixture was incubated at room temperature for 1 h, in darkness with continuous agitation. After the addition of TCA (20% w/v), the mixture was centrifuged at 15 000 × *g* for 5 min. The resulting pellet was washed twice with ethyl acetate/EtOH (1:1 v/v). Then, the washed pellet was reconstituted with 6 M guanidine in a 20 mM KH_2PO_4 buffer (pH 2.3) and centrifuged at 15 000 × *g* for 5 min. The absorbance of the resulting solution was measured at 370 nm. The carbonyl content was calculated from the absorbance data and expressed as nmol carbonyls/mg protein. Because of the numerous washing steps, protein content in the final pellet was estimated on HCl blank pellet processed simultaneously, using a BSA standard curve in 6 M guanidine, and reading the absorbance at 280 nm. The IC_{50} values are listed in Table 2.

Primary mesencephalic cell cultures

Ventral mesencephalic tissue was dissected from rat embryos of 14 days gestation (E14). The tissue was incubated in 0.1% trypsin, 0.05% DNase and DMEM for 20 min at 37°C and then washed in DNase/DMEM and mechanically dissociated. The resulting cell suspension was centrifuged at 50 × *g* for 5 min, the supernatant was removed carefully and the pellet was resuspended in 0.05% DNase/DMEM to the final volume required. The number of viable cells in the suspension was estimated by acridine orange/ethidium bromide staining, and cells were plated onto 35-mm culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) previously coated with poly-L-lysine (100 μg/mL) and laminin (4 μg/mL). The cells were seeded at a density of 1.5×10^5 cells/cm^2 and maintained under control conditions (DMEM/HAMS F12, 1:1, containing 10% fetal bovine serum). The cell cultures were maintained in a humidified CO_2 incubator (5% CO_2, 37°C) for 7 days *in vitro*. The medium was totally removed on day 2 and replaced with fresh culture medium.

Treatment and immunohistochemistry of cell cultures

Cultures were treated with the test 2-benzazepine nitrone (8, 40, or 200 μM concentrations) 30 min before the treatment with 6-OHDA (20 μM in 0.02% saline ascorbate) on 6 days *in vitro* for 24 h. Cultures were fixed with 4% *p*-formaldehyde in DPBS at pH 7.4 for 20 min, and the endogenous peroxidase activity was quenched by incubation for 5 min with 3% hydrogen peroxide in DPBS. Cultures were preincubated with a blocking solution containing 10% normal serum in DPBS with 1% BSA and 0.3% Triton X-100 for 1 h, and then incubated overnight at 4°C with a mouse monoclonal anti-tyrosine hydroxylase (anti-TH; 1:30,000) as the DA marker. Cultures were then washed and incubated for 1 h with the corresponding biotinylated secondary antibody (horse anti-mouse) diluted 1:500. The cultures were washed and incubated for 90 min with avidin–biotin-peroxidase complex (1:500). Finally, the labeling was revealed with 0.04% H_2O_2 and 0.05% 3,3′-diaminobenzidine as the chromogen.

Cell counting in primary mesencephalic cultures

The cultured cells, observed with phase contrast microscopy (TH-immunoreactive (ir) cells; Eclipse, Nikon; ×100 magnification),

were counted in five randomly chosen longitudinal and transverse microscopic fields along the diameter of the culture dish away from the curve edge. The operator was blind to the treatment condition. The microscopic field was defined by a 0.5 cm × 0.5 cm reticule (i.e., a total of 1.25 cm^2). The average number of TH-positive cells in a control culture dish was 2321 ± 165. The results from at least three different experiments were recorded, with a minimum sample size of four wells per group and per run. The results were normalized to the counts of the control group in the same batch (i.e., expressed as the percentage over the control group counts), in order to account for possible variability among batches.

Cholinesterase inhibition assay

Compounds were evaluated using AChE (EC 3.1.1.7) from electric eel (Electrophorus electricus), and butyrylcholinesterase (BuChE, EC 3.1.1.8) from equine serum (Sigma) following the method of Ellman et al. [60]. The AChE activity was determined in a reaction mixture containing 200 μL of a solution of AChE (0.415 U/mL in 0.1 M phosphate buffer, pH 8.0), 100 μL of a solution of 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB 3.3 mM in 0.1 M phosphate buffered solution, pH 7.0, containing 6 mM $NaHCO_3$), 100 μL of a solution of the inhibitor (0.25–2.0 mM), and 500 μL of phosphate buffer, pH 8.0. After incubation for 20 min at 25°C, acetylthiocholine iodide (100 μL of 0.05 mM water solution) was added as the substrate, and AChE activity was determined by UV spectrophotometry from the absorbance changes at 412 nm for 3.0 min at 25°C. The concentration of compound which produced 50% inhibition of the AChE activity (IC_{50}) was calculated by nonlinear regression of the response–concentration (log) curve. BuChE inhibitory activity determinations were carried out similarly using butyrylthiocholine iodide (0.05 mM) as the substrate. IC_{50} values are reported in Table 3 as means ± SEM of at least three independent measurements.

Statistical analysis

Multiple comparisons were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. The normality of populations and homogeneity of variances were tested before each ANOVA. Differences at $p < 0.05$ were considered as statistically significant. Statistical analyses were carried out with SigmaStat 3.0 (Jandel Scientific, San Rafael, CA, USA).

Supporting Information

Supplementary data to this article (physicochemical and spectral data for homoallylamine and 2,3,4,5-tetrahydro-1*H*-2-benzazepine intermediates of the synthesis of nitrone derivatives **3**, **4**, **6**, and **8**–**12**) can be found online.

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The authors have declared no conflict of interest.

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