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Synthesis, neuroprotective and antioxidant capacity of PBN-related indanonitrones

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ABSTRACT

In this work six **PBN**-related indanonitrones **1**–**6** have been designed, synthesized, and their neuroprotection capacity tested *in vitro,* under OGD conditions, in SH-SY5Y human neuroblastoma cell cultures. As a result, we have identified indanonitrones **1**, **3** and **4** (EC_{50} = 6.64 ± 0.28 μM) as the most neuroprotective agents, and in particular, among them, indanonitrone **4** was also the most potent and balanced nitrone, showing antioxidant activity in three experiments [LOX (100 μM), APPH (51%), DPPH (36.5%)], being clearly more potent antioxidant agent than nitrone **PBN**. Consequently, we have identified (*Z*)-5-hydroxy-*N*-methyl-2,3-dihydro-1*H*-inden-1-imine oxide (**4**) as a hit-molecule for further investigation.

1. Introduction

The formation of Reactive Oxygen Species (ROS) is the result of normal cellular metabolism in living organisms. ROS act at low to moderate concentrations in many physiological cell processes, but at high concentrations they produce adverse effects in lipids, proteins, and DNA [1,2]. Oxidative stress (OS) is involved in diverse pathological conditions, including cancer, and neurological disorders [3,4], and is one of the most important molecular events occurring before and after stroke [5,6]. Consequently, and particularly in what concerns stroke, current research efforts in this area are mainly focused on the identification of new scavenging ROS [7].

In this context, nitrones, organic molecules widely known as radical traps, appear to be interesting drug candidates to treat this pathology [8]. The first proposal of nitrones as therapeutic candidates for stroke was made when Novelli reported that α-phenyl *N*-*t*-butylnitrone (**PBN**) (Fig. 1) prevented and reversed traumatic shock injury in rats [9]. From that starting point, a number of studies have been performed resulting for instance in the development of **NXY-059**, a **PBN** derivative which was the first nitrone to reach clinical trials, but failed in advanced clinical phase III, after showing no significant efficacy when compared with placebo [10]. In spite of this, nitrones, as efficient neuroprotective agents in experimental ischemia studies [11], are still antioxidant agents for potential stroke treatment.

Some years ago our group [12–17] started a project targeted to the synthesis and biological evaluation of new nitrones, as potential drugs for stroke therapy. Among them, various nitrones derived from (hetero) aromatic aldehydes [12,13] related to **PBN** (Fig. 1), quinolylnitrones [14,15], and steroid-nitrone hybrids, such as the “cholesteronitrones”, have been studied [16,17].

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DPPH, 1,1-diphenyl-2-picrylhyrazyl; LP, lipid; LOX, lipoxygenase

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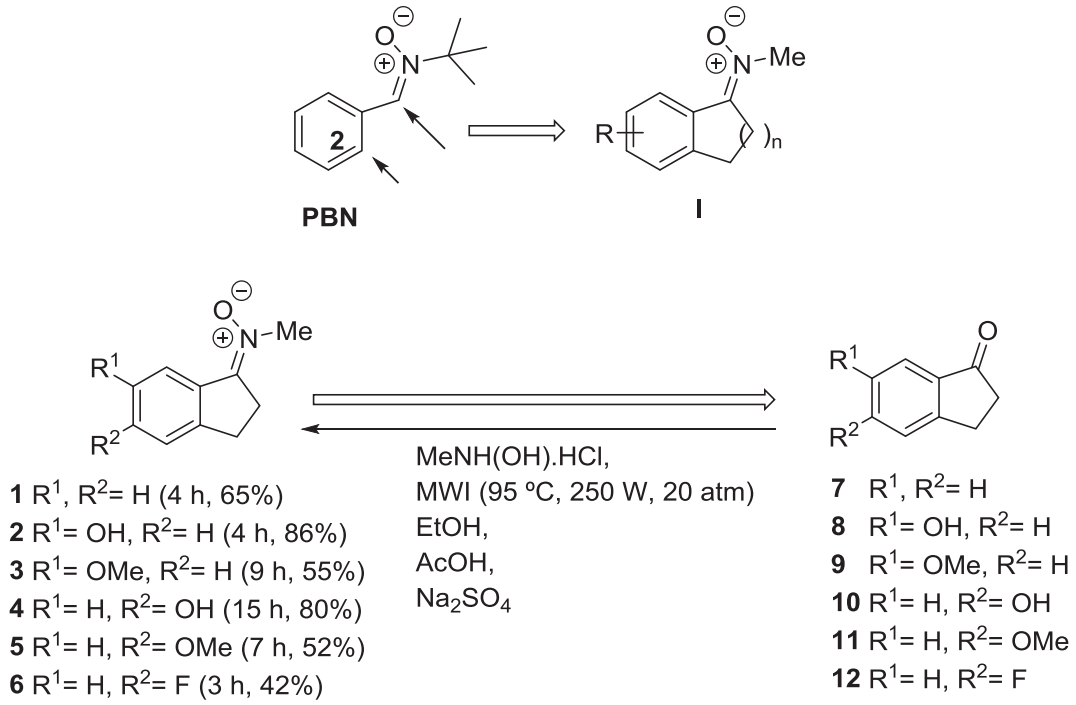


Fig. 1. Structures of **PBN**, **IN** of general type **I**, and synthesis of the **IN1-6** investigated in this work.

In order to improve and explore the potential therapeutic capacity **PBN**, new conformationally restricted **PBN** derivatives (**I**) were designed by inserting a cycle of diverse size, by modifying the value of n, between the ***C***arbon linked at the nitrone motif [***C***═N(O)*t*-Bu] and C2, resulting in the indanonitrones (**IN**) **1–6** (Fig. 1). **IN1** (Fig. 1) is the parent compound, without substituents, while **IN2** and **IN3** (Fig. 1) bear an hydroxyl and a methoxy group, respectively, at C6; **IN4** and **IN5** (Fig. 1) bear an hydroxyl and a methoxy group, respectively, at C5, while **IN6** (Fig. 1) bear a fluor at C5. From this work we have identified **IN4**, as a neuroprotective nitrone, being the most balanced antioxidant agent showing activity in the LOX, APPH, DPPH experiments.

2. Results and discussion

2.1. Chemistry

The synthesis of **IN1-6** (Fig. 1) has been easily achieved by reaction of commercially available indanones **7–12** (Fig. 1), by reacting them with *N*-methylhydroxylamine hydrochloride in the presence of sodium sulfate, sodium acetate, in dry ethanol, in an efficient protocol mediated by microwave irradiation that has afforded the expected **INs** in 3 to 15 h reaction times, and modest to good yields (42–86%) (Fig. 1) (**Experimental Part**). Free phenol **IN2** and **IN4** were transformed into their sodium salts for solubility reasons to deal with their biological analyses. All new compounds **2–4** gave good analytical and spectroscopic data in good agreement with their structures (**Experimental Part**), and with what has been described in the literature, when known compounds, as it was the case of **IN1**, **5**, and **6,** shown in a paper reporting a new method for the preparation of the 1,4-dihydroindeno [1,2-*b*]pyrroles utilizing the 2,3-dihydroisoxazole-pyrrole rearrangement reaction [18]. From the stereochemical point of view **IN1-6** have been isolated as pure *Z*-diastereomers, showing no trace of the corresponding *E*-isomers, a fact confirmed by their nOe analyses in the ^{1}H NMR experiments carried out, and supported by the same assignment in the literature [18]. This is possible due to the significant steric hindrance that would result from the interaction of the H(7) and the methyl group in the presumed *E*-isomer. In support of this hypothesis, we were unable to prepare other **IN** from *N*-*t*-butyl(benzyl) hydroxylamimes. In fact, the synthesis of ketonitrones is not so simple as it would appear, and the literature records examples of clear failures, and solutions, to prepare them [19]. In our case, and for instance, in any case, and regardless of the method used, we could not synthesize the any nitrone from commercially available tetralones.

2.2. Neuroprotection studies

In order to know the neuroprotective activity of the **INs** we have assessed their capacity to recover cell viability when cells were subjected to oxygen and glucose deprivation (OGD) and reperfusion (IR). This study was performed in human neuroblastomas cell line SH-SY5Y, by using the tetrazolium salt XTT test, as indicated in the **Experimental Part**.

In Fig. 2, results on % of cell viability recover (A) and % of neuroprotection (B) are presented when cells were subjected to OGD in the presence of different **IN** concentrations, ranging from 0.01 to 1000 μM. Results indicate that all **INs** were able to increase neuroblastoma cell viability and show a high, dose-dependent neuroprotective effect.

The EC_{50} data and maximal neuroprotective effect are shown in Table 1. As shown, EC_{50} values range from 1.03 ± 0.12 μM (**IN1**) to 82.16 ± 4.35 μM (**IN2**). By comparing with the EC_{50} determined for **PBN**, it is clear that reference nitrone **PBN** and **IN1**, **3** and **4** all have a similar neuroprotective effect.

Among the tested nitrones, the observed neuroprotective power follows this order: **IN1** ≥ **IN3** ≥ **IN4** > > **IN6** > > **IN-5** > > > **IN2**, from the most to less potent. In addition, and based on these results, from the structure-activity relationship point of view, several conclusions can be drawn. Thus, among **IN** bearing an H group at R^2, the incorporation of a OMe group (**IN3**), instead an H at R^1 (**IN1**) does not affect the neuroprotective activity, while the presence of an OH motif decreases very significantly this capacity (**IN2**: EC_{50} = 82.16 ± 4.3 μM), being thus **IN2** the less neuroprotective **IN** on this group. However, while the neuroprotective activity of **IN1** reaches 100% of effect, the maximum efficacy achieved by **IN3** does not reach 100%, at the doses tested. On the other hand, for **INs** bearing an H group at R^1 (**IN4-6**), the maximum efficiency is achieved by **IN4**, bearing an OH group in R.[2] Note also that the neuroprotective capacity

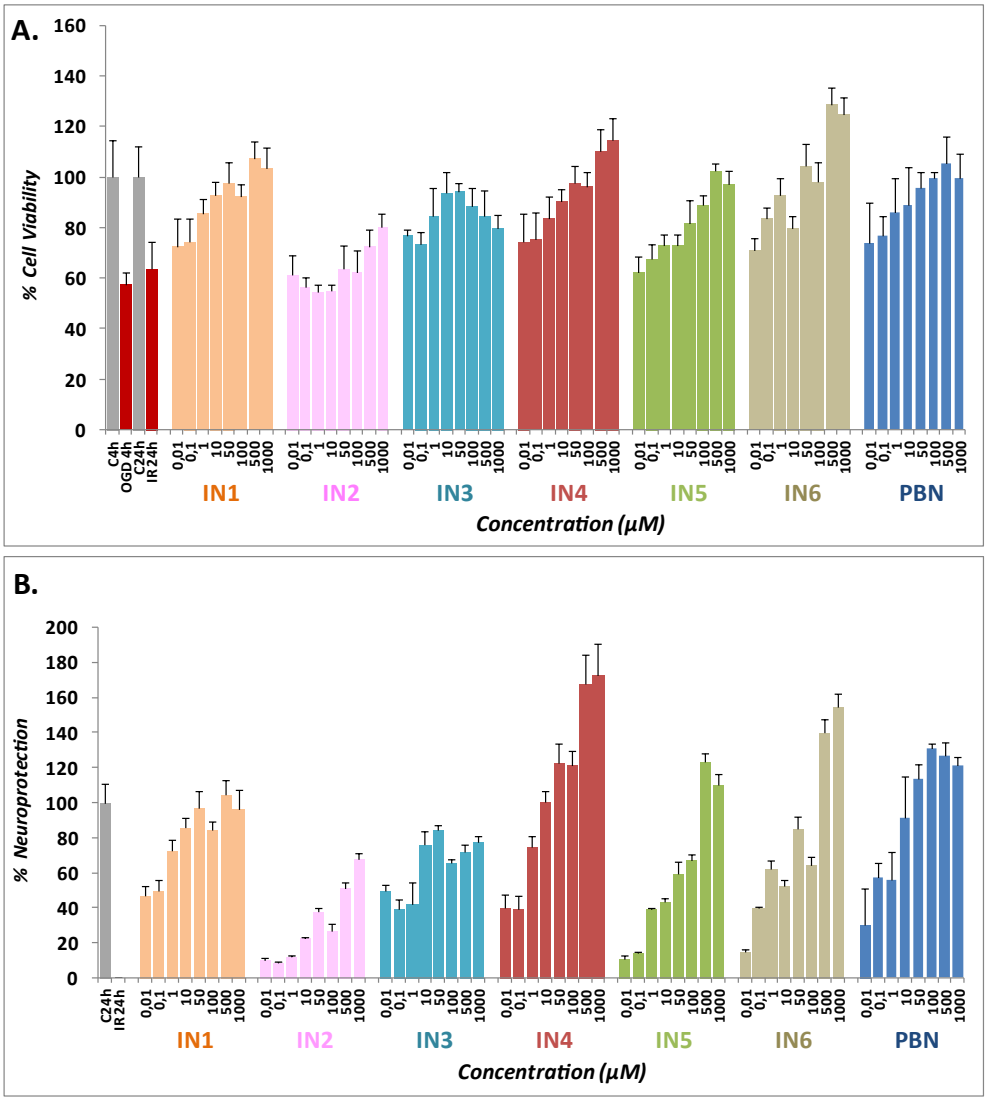


Fig. 2. Effect of IN1-6 on % cell viability (A) and % neuroprotection (B) in SH-SY5Y human neuroblastoma cell cultures exposed to oxygen-glucose deprivation (OGD). Bar chart shows the percentage of cell viability at 24 h of recovery after 4 h OGD (A) or % neuroprotection (B), either untreated (R24h) or treated with different concentrations (μM) of **IN1-6**, or **PBN**. The values induced by OGD at 4 h without recovery period (OGD 4 h) and by IR at 24 h are also indicated. The values represent the average of three independent experiments (mean ± SEM). Multiple statistical comparisons were performed versus control (R24h) by one-way analysis of variance (Holm-Sidak method) and the overall significance level was $P < 0.001$.

decreases when the OH group is substituted by OMe or F in **IN5** and **IN6**, respectively. Moreover, by comparing with **INs** bearing an H at R^2, the maximal neuroprotective activity is higher, which could mean than, at least in part, **IN**s bearing an H at R^1, at high concentrations, may have a certain effect on cell proliferation, under ischemic conditions, which protect cells from death.

Finally, in order to determine whether these **INs** have a certain neurotoxic/ antiporoliferative effect in the absence of ischemia, we tested their effect on the viability of neuroblastoma cells in basal conditions.

As shown in Fig. 3, none of the **INs** tested showed neither a neurotoxic effect, nor proliferative effect, which shows that the observed increase in neuroprotective effect observed for **INs** bearing an H at R^2, in the ischemic conditions (reperfusion), is not totally due to their proliferative capacity, but to a certain neurorepairing effect, under cell damage conditions, which protects them against cell death induced by the ischemic insult.

2.3. Antioxidant assays

Based on the neuroprotection results, we have investigated the antioxidant capacity of the most potent neuroprotective **IN1**, **3** and **4**, using also **PBN** for comparative purposes, in different antioxidants tests, such as the interaction with the free radical 1,1-diphenyl-2-picrylhyrazyl (DPPH), the inhibition of lipid peroxidation (LP) induced by 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH), and the *in vitro* inhibition of soybean lipoxygenase (LOX). Each *in vitro* experiment was performed at least in triplicate, and the standard deviation of absorbance was less than 10% of the mean.

DPPH is a stable free radical in an ethanolic solution. In its oxidized form, the DPPH radical has an absorbance maximum centred at about 517 nm. The DPPH method is described as a simple, rapid and convenient method independent of sample polarity for screening many samples for radical- scavenging activity [20]. Reduction of DPPH stable free radical (50 μM) by the examined compounds at 50 μM and 100 μM, was studied after 20 and 60 min. The most potent nitrone on this assay corresponded to **IN4** (36.5%), very far from the value observed for nordihydroguaiaretic acid (**NDGA**, 93%), used as standard (Table 2).

The use of AAPH is possibly the most recommended method for measuring 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 LP [21]. LP is one of the major outcomes of ROS mediated injury that directly damages membranes and generates a number of secondary products that possess neurotoxic activity. The highest anti-LP values were presented by nitrones **3** (60%), **1** (57%), and **4** (51%), but showing values lower than Trolox (93%), the reference compound in this test (Table 2).

LOX is the key enzyme in leukotriene biosynthesis [22].

Table 1
Neuroprotective effects of **PBN** and **IN1-6** on the cell viability decrease induced by OGD and reperfusion in SH-SY5Y neuroblastoma cells.

Compound	Structure R^1	Structure R^2	Neuroprotection $EC_{50} \pm SEM$ (μM)	P< (PBN)	P< (IN1)	Maximal Activity
IN1	H	H	1.03±0.12	*	-	104.2±8.6
IN2	OH	H	82.16±4.35	***	***	67.4 ±4.1
IN3	OMe	H	3.13±0.09	ns	ns	84.6±8.1
IN4	H	OH	6.64±0.28	ns	*	167.7±16.6
IN5	H	OMe	38. 02±3.63	***	***	123.5±4.3
IN6	H	F	13. 91±1.12	**	***	154.5±7.9
PBN		-	6.03±0.41	-	*	131.3 ±13.1

[a]Data are given as means ± S.E.M. Statistical differences against **PBN** or **IN1** were determined by applying a one-way ANOVA test. Differences between $EC_{50's}$ were considered to be statistically significant when $p \leq 0.05$. $EC_{50's}$ were calculated from data obtained of three experiments made in triplicate on cells from four different cultures. [ns]non significant; *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$, compared with **PBN** or **IN1**, as indicated.

Leukotrienes, derived from the biotransformation of arachidonic acid catalyzed by 5-lipoxygenase (5-LOX), cause inflammation and are thus involved in the stroke injury. LOXs play a role in membrane lipid peroxidation by forming hydroperoxides in the lipid bilayer [23], whereas cerebral ischemia-reperfusion triggers LP and inflammation, which exacerbate injury. Inhibition of LOX was performed by the UV absorbance based enzyme assay [14]. As shown, only **IN4** showed a significant LOX inhibition power (100 μM), but less potent than the value determined for **NDGA** (0.45 μM) (Table 2).

Very interestingly, **IN1, 3** and **4,** the most potent neuroprotective **INs**, show a very high capacity to inhibit the LP induced by AAPH, results which correlate very well with their neuroprotective capacity, that indicating that the neuroprotective effect of these **INs** could be due, at least in part, to their antioxidant capacity.

3. Conclusions

Herein, we have reported the synthesis and neuroprotective properties of **IN1-6**, related to **PBN** (Fig. 1), in order to ascertain the effect of preventing the free conformational freedom by inserting a five membered-ring system on their antioxidant power, and consequently in their neuroprotective potential. From the neuroprotective studies we conclude that one the most potent neuroprotective agent found here, **IN4** is also the most balanced antioxidant compound, showing activity in three experiments [LOX (100 μM), APPH (51%), DPPH (36.5%)], being clearly more potent than **PBN**. This result can be explained by the fact that **IN4** bears a hydroxyl free group at C5, and that the homolytic loss of a hydrogen in the reaction with a ROS leads to an oxygen radical stabilized in a very stable *p*-quinonoid radical species of type **II**, by conjugation with the nitrone motif at *para* position (Fig. 4). To sum up, we have identified **IN4** as a new hit-nitrone for further investigation.

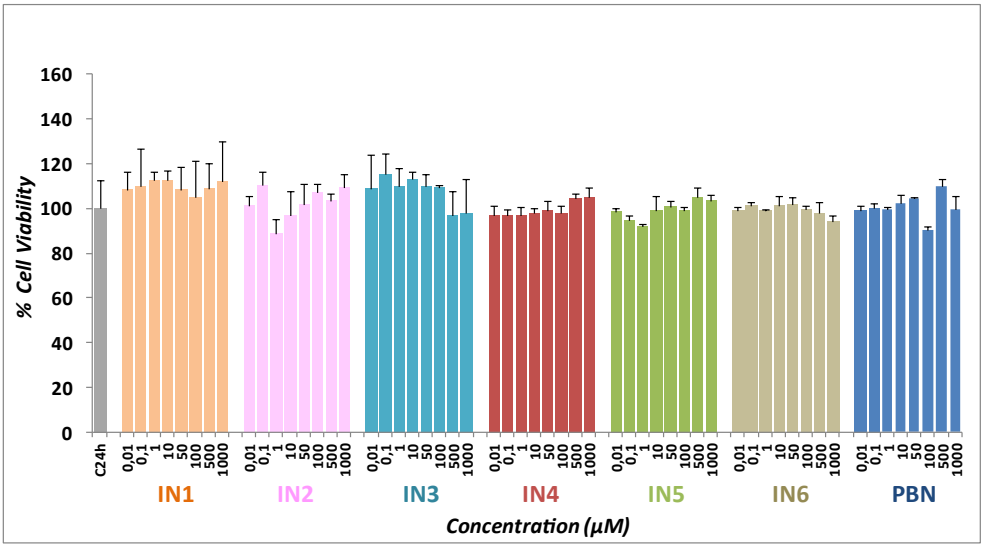
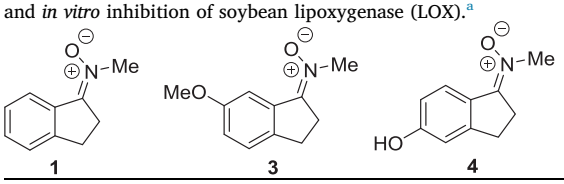


Fig. 3. Effect of INs on SH-SY5Y neuroblastomas cells cultures in basal conditions. Bars chart represent the percentage of cell viability in the presence or absence (control; C) of the indicated concentrations of the different compounds. The cell viability corresponding to the control cells (untreated) was considered as 100%. The values represent the mean ± SEM of three independent experiments, each performed in triplicate, in different cultures. The statistics show non-significant neurotoxic or neuroprotective effects of these compounds against the control (one-way ANOVA).

Table 2
Antioxidant activities of **IN1**, **3** and **4** and **PBN**, against 1,1-diphenyl-2-picrylhyrazyl (DPPH), 2,2-azobis(2-amidinopropane) dihydrochloride (APPH), and *in vitro* inhibition of soybean lipoxygenase (LOX).[a]



1 **3** **4**

Nitrones	DPPH (%)	APPH (%)	LOX (%) (IC_{50})
PBN	12	11	23
1	5.5	57	38
3	4	60	no
4	**36.5**	**51**	**100 μM**
NDGA	93	–	0.45 μM
Trolox	–	93	–

[a] **INs** were tested at 100 μM; Values are means of three or four different determinations; "no" indicates no activity under the experimental conditions; -, not determined; means within each column differ significantly ($p < 0.05$).

4. Experimental Part

4.1. Chemistry

Compound purification was performed by column chromatography with Merck Silica Gel (40–63 μm) or by flash chromatography (Biotage Isolera One equipment) and the adequate eluyent for each case. Reaction course was monitored by thin layer chromatography (t.l.c.), revealing with UV light (λ = 254 nm) and ethanolic solution of vanillin or ninhydrin. Melting points were determined using a Reichert Thermo Galen Kofler block and are uncorrected. Samples were dissolved in $CDCl_3$ or DMSO-d_6 using TMS as internal standard for ^{1}H NMR spectra. In ^{13}C NMR spectra, $CDCl_3$ central signal (77.0 ppm) and DMSO-*d* (39.5 ppm) were used as references. ^{1}H NMR and ^{13}C NMR spectra were obtained in Bruker Avance 300 (300 MHz) and Bruker Avance 400 III HD (400 Hz) spectrometers. Chemical shifts (δ) are given in ppm. Coupling constants (*J*) are given in Hz. Signal multiplicity is abbreviated as: singlet (s), doublet (d), triplet (t), quartet (c), doublet of doublets (dd), triplet of doublets (td), or multiplet (m). IR spectra were recorded on a Perkin-Elmer Spectrum One B spectrometer. Units are cm^{-1}. Low resolution mass spectra were recorded on an Agilent HP 1100 LC/MS Spectrometer, whereas High Resolution mass spectrometry (Exact Mass) was performed in an AGILENT 6520 Accurate-Mass QTOF LC/MS Spectrometer. Elemental analysis were performed in an Elementary Chemical Analyzer LECO CHNS-932.

4.1.1. General method for the synthesis of *IN1-6*

A mixture of corresponding 1-indanone (1 equiv), Na_2SO_4 (4 equiv), anhydrous NaOAc (2 equiv), and *N*-methylhydroxylamine hydrochloride (2 equiv), in dry EtOH, was irradiated in a microwave apparatus (Biotage) at 95 °C, 250 W, and 20 atm, for the time indicated in each case. Then, the mixture was cooled, the solvent was removed, toluene was added to eliminate traces of AcOH, and the residue purified by column chromatography to give the desired indanonitrone, that was recrystallized from AcOEt.

4.1.1.1. (Z)-N-Methyl-2,3-dihydro-1H-inden-1-imine oxide (*1*).

Following the general method, a mixture of 1-indanone (**7**) (132 mg, 1 mmol), *N*-methylhydroxylamine hydrochloride (124.5 mg, 1.5 mmol), NaOAc (198 mg, 2.4 mmol) and Na_2SO_4 (568 mg, 4 mmol), in EtOH (7 mL), was irradiated for 4 h. After work-up and chromatography eluting with mixtures of CH_2Cl_2 and MeOH, from 0% to 5%, and recrystallization, indanonitrone **1** [18] (104.2 mg, 65%) was isolated: mp 141–3 °C; IR (KBr) *ν* 2995, 2918, 1590, 1198 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 8.63 (d, *J* = 7.7 Hz, 1H, H4), 7.41–7.29 (m, 2H, H6, H7), 7.28–7.20 (m, 1H, H5), 3.65 (s, 3H, H8), 3.10–3.02 (m, 2H, H3), 3.01–2.93 (m, 2H, H2); ^{13}C NMR (101 MHz, DMSO-d_6) *δ* 148.1 (C3a), 147.3 (C1), 135.3 (C7a), 130.6 (C6), 126.8 (C5), 125.8 (C4), 125.2 (C7), 50.7 (C8), 29.0 (C2), 28.8 (C3); MS (ES) *m/z* (%): 162 [M^+ + 1], 184 [M^+ + Na]; HRMS. Calcd. for $C_{11}H_{13}NO_2$: 161.08406. Found: 161.08356. Anal. Calcd for $C_{10}H_{11}NO.¼\ H_2O$: C, 72.48; H, 7.00; N, 8.45. Found: C, 72.75; H, 7.18; N, 8.68.

4.1.1.2. (Z)-6-Hydroxy-N-methyl-2,3-dihydro-1H-inden-1-imine oxide (*2*).

Following the general method, a mixture of 6-hydroxy-1-indanone (**8**) (148 mg, 1 mmol), *N*-methylhydroxylamine hydrochloride (124.5 mg, 1.5 mmol), NaOAc (198 mg, 2.4 mmol) and Na_2SO_4 (568 mg, 4 mmol), in EtOH (7 mL), was irradiated for 4 h. After work-up and chromatography eluting with mixtures of CH_2Cl_2 and MeOH, from 5% to 20%, and recrystallization, indanonitrone **2** (151.6 mg, 86%) was isolated: mp > 230 °C; IR (KBr) *ν* 2935, 2698, 1475, 1252 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) *δ* 7.83 (d, *J* = 2.4 Hz, 1H, H7), 7.41 (d, *J* = 8.4 Hz, 1H, H4), 7.18 (dd, *J* = 8.4, 2.5 Hz, 1H, H5), 3.85 (s, 3H, H8), 3.25 (m, 2H, H3), 3.10 (m, 2H, H2); MS (ES) *m/z* (%): 178 [M^+ + 1], 200 [M^+ + Na]; HRMS. Calcd for $C_{10}H_{11}NO_2$: 177.07913. Found: 177.07898. Anal. Calcd para $C_{10}H_{11}NO_2.5/8H_2O$: C: 63.73; H, 6.55; N, 7.43. Found: C, 63.89; H, 6.25; N, 7.71. A suspension of indanonitrone **2** (40.71 mg) in MeOH (5 mL), treated with an aqueous solution of NaOH (0.5 mL, 1 equiv, 0.46 M), was stirred at room temperature (rt) for 1 h. The solvent was removed to give the sodium salt of indanonitrone **2** (38.6 mg): ^{1}H NMR (300 MHz, D_2O) *δ* 7.71 (d, *J* = 2.6 Hz, 1H, H7), 7.17 (d, *J* = 8.3 Hz, 1H, H4), 6.85 (dd, *J* = 8.3, 2.5 Hz, 1H, H5), 3.64 (s, 3H), 2.98 (s, 4H, H2, H3). Anal. Calcd. for $C_{10}H_{10}NNaO_2.11/3H_2O$: **C,** 45.28; H, 6.59; N, 5.28. Found: C, 45.46; H, 6.74; N, 5.56.

4.1.1.3. (Z)-6-Methoxy-N-methyl-2,3-dihydro-1H-inden-1-imine oxide (*3*).

Following the general method, a mixture of 6-methoxy-1-indanone (**9**) (81.1 mg, 0.5 mmol), *N*-methylhydroxylamine hydrochloride (83.5 mg, 1 mmol), NaOAc (82 mg, 1 mmol) and Na_2SO_4 (213.1 mg, 1.5 mmol), in EtOH (7 mL), was irradiated for 9 h. After work-up and chromatography eluting with mixtures of CH_2Cl_2 and MeOH, from 100:0 a 9:1, and recrystallization, indanonitrone **3** (31.1 mg, 55%) was isolated: mp 111–4 °C; IR (KBr) *ν* 3002, 2936, 1575, 1424 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) *δ* 8.46 (d, *J* = 2.6 Hz, 1H, H7), 7.16 (d, *J* = 8.3 Hz, 1H, H4), 6.97 (dd, *J* = 8.3 Hz, *J* = 2.6 Hz, 1H, H5), 3.86 (s, 3H, H9), 3.79 (s, 3H, H8), 3.11–3.08 (m, 2H, H3), 3.02–2.99 (m, 2H, H2); ^{13}C NMR (400 MHz, $CDCl_3$) *δ* 158.7 (C6), 150.5 (C1), 140.3 (C3a), 135.5 (C7a), 125.2 (C4), 119.7 (C5), 110.0 (C7), 55.6 (C9), 49.7 (C8), 30.3 (C2), 28.1 (C3); EM (ES) *m/z* (%): 192 [M^+ + 1], 214 [M^+ + Na]; HRMS (ESI+) *m/z*: Calcd. for $C_{11}H_{13}NO_2$: 191.09466. Found: 191.09463. Anal. Calcd. for $C_{11}H_{13}NO_2$: C, 69.09; H, 6.85; N, 7.32. Found: C, 68.82; H, 6.85; N, 7.34.

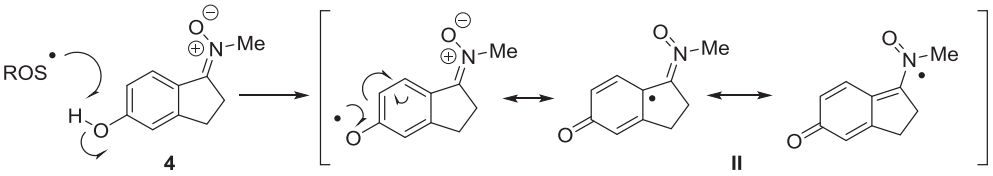


Fig. 4. A possible reaction of a ROS with **IN4**.

*4.1.1.4. (Z)-5-Hydroxy-N-methyl-2,3-dihydro-1H-inden-1-imine oxide (**4**).* Following the general method, a mixture of 5-hydroxy-1-indanone (**10**) (148 mg, 1 mmol), *N*-methylhydroxylamine hydrochloride (124.5 mg, 1.5 mmol), NaOAc (198 mg, 2.4 mmol) and Na_2SO_4 (568 mg, 4 mmol), in EtOH (7 mL), was irradiated for 15 h. After work-up and chromatography eluting with mixtures of CH_2Cl_2 and MeOH, from 5% a 20%, and recrystallization, indanonitrone **4** (141.1 mg, 80%) was isolated: pf > 230 °C; IR (KBr) ν 3426, 2926, 1586, 1181 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 8.26 (d, J = 9.6 Hz, 1H, H4), 7.00–6.85 (m, 2H, H6, H7), 3.79 (s, 3H, H8), 3.23 (m, 2H, H3), 3.20–3.07 (m, 2H, H2); EM (ES) m/z (%): 178 [M^+ + 1], 200 [M^+ + Na]; HRMS. Calcd for $C_{10}H_{11}NO_2$: 177.07879. Found: 177.07898. Anal. Calcd for $C_{10}H_{11}NO_2.1/3H_2O$: C, 65.56; H, 6.42; N, 7.65. Found: C, 65.65; H, 6.46; N, 7.89. A suspension of indanonitrone **4** (49.56 mg), in MeOH (5 mL), treated with an aqueous solution NaOH (0.5 mL, 1 equiv, 0.56 M), was stirred at rt for 1 h. The solvent was removed to give the sodium salt of indanonitrone **4** (58.4 mg): ^1H NMR (300 MHz, D_2O) δ 8.17 (d, J = 8.5 Hz, 1H, H4), 6.56–6.47 (m, 2H, H6, H7), 3.48 (s, 3H, H8), 2.90 (m, 2H, H3), 2.86 (m, 2H, H2). Anal. Calcd. for $C_{10}H_{10}NNaO_2.9/4H_2O$: C, 50.10; H, 6.10; N, 5.84. Found: C, 50.39; H, 6.37; N, 6.05.

*4.1.1.5. (Z)-5-Methoxy-N-methyl-2,3-dihydro-1H-inden-1-imine oxide (**5**).* Following the general method, a mixture of 5-methoxy-1-indanone (**11**) (81.1 mg, 0.5 mmol), *N*-methylhydroxylamine hydrochloride (83.5 mg, 1 mmol), NaOAc (82 mg, 1 mmol) and Na_2SO_4 (284.1 mg, 2 mmol), in EtOH (7 mL), was irradiated for 7 h. After work-up and chromatography eluting with mixtures of CH_2Cl_2 and MeOH, from 100:0 a 9:1, and recrystallization, indanonitrone **5** [18] (54.7 mg, 52%) was isolated: mp 163–6 °C; IR (KBr) ν 2941, 2919, 1593, 1485, 1443, 1257, 1059 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.78 (d, J = 8.7 Hz, 1H, H7), 6.83 (dd, J = 8.7 Hz, J = 2.4 Hz 1H, H6) 6.77 (d, J = 2.4 Hz, 1H, H4), 3.82 (s, 3H, H9), 3.73 (s, 3H, H8), 3.16–3.02 (m, 2H, H3), 3.01–2.86 (m, 2H, H2); ^{13}C NMR (400 MHz, $CDCl_3$) δ 162.2 (C5), 150.2 (C3a), 149.0 (C1), 128.5 (C7), 127.7 (C7a), 112.9 (C6), 109.8 (C4), 55.4 (C9), 49.2 (C8), 29.9 (C2), 28.9 (C3); MS (ES) m/z (%): 192 [M^+ + 1], 214 [M^+ + Na], 383.2 [$2M^+$ + 1], 405.2 [$2M^+$ + Na]; HRMS (ESI +) m/z: Calcd. for $C_{11}H_{13}NO_2$: 191.09466. Found: 191.09463.

*4.1.1.6. (Z)-5-Fluoro-N-methyl-2,3-dihydro-1H-inden-1-imine oxide (**6**).* Following the general method, a mixture of 5-fluoro-1-indanone (**12**) (75.1 mg, 0.5 mmol), *N*-methylhydroxylamine hydrochloride (83.5 mg, 1 mmol), NaOAc (82 mg, 1 mmol) and Na_2SO_4 (284.1 mg, 2 mmol), in EtOH (7 mL), was irradiated for 3 h. After work-up and chromatography eluting with mixtures of CH_2Cl_2 and MeOH, from 100:0 a 94:6, and recrystallization, indanonitrone **6** [18] (36.8 mg, 42%) was isolated: mp 135–7 °C; IR (KBr) ν 2919, 1584, 1472, 1362, 1248, 1200, 1063 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.79 (dd, $J_{H,H}$ = 8.6 Hz, $J_{H,F}$ = 5.7 Hz, 1H, H7), 7.05–6.82 (m, 1H, H4, 1H, H6), 3.70 (s, 3H, H8), 3.08 (dd, J = 8.4 Hz, J = 4.5 Hz, 2H, H3), 3.01–2.86 (m, 2H, H2); ^{13}C NMR (400 MHz, $CDCl_3$) δ 164.43 (d, $J_{C,F}$ = 252.4Hz, C5), 150.46 (d, $J_{C,F}$ = 9.1 Hz, C3a), 148.2 (C1), 130.97 (d, $J_{C,F}$ = 2.3 Hz, C7a), 128.85 (d, $J_{C,F}$ = 9.1 Hz, C7), 114.3 (d, $J_{C,F}$ = 22.4 Hz, C6), 111.8 (d, $J_{C,F}$ = 23.0 Hz, C4), 49.6 (C8), 29.9 (C2), 28.8 (C3); MS (ES) m/z (%): 180 [M^+ + 1], 202 [M^+ + Na], 359.2 [$2M^+$ + 1], 381.2 [$2M^+$ + Na]; HRMS (ESI +) m/z: Calcd. for $C_{10}H_{10}FNO$: 179.07474. Found: 179.07464.

4.2. Neuroprotection studies

4.2.1. Neuroblastoma cell cultures

The human neuroblastomas cell line SH-SY5Y were cultured in Dulbecco's: Ham's F12, 1:1 [vol/vol] containing 3.15 mg/mL glucose, 2.5 mM Glutamax and 0.5 mM sodium pyruvate DMEM/F-12, GlutaMAX™; GIBCO, Life Technologies, Madrid (Spain), 1% Antibiotique-Antimitotic (Gibco; Life Technologies, Madrid, Spain) (containing 100 ui/mL penicillin, 100 mg/mL de streptomycin and 0.25 mg de amphoterycine B), 1% gentamicina 15 mg/mL (Sigma-Aldrich, Madrid, España) and 10% Foetal Calf Serum (FCS) (Gibco; Life Technologies, Madrid, Spain) as described [24]. Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Culture media were changed every 2 d. Cells were sub-cultured after partial digestion with 0.25% trypsin-EDTA. For assays, SHSY5Y cells were subcultured in 96 or 48-well plates at a seeding density of 0.50–1 or 2–2.5 × 10^5 or cells per well, respectively. When the SHSY5Y cells reached 80% confluence, the medium was replaced with fresh medium containing 0.01–1000 μM compound concentrations or PBS in the controls, as indicated in each assay.

4.2.2. Neuroblastoma cell cultures exposure to Oxygen-Glucose deprivation (OGD)

Neuroblastoma cell cultures were exposed to OGD so as to induce cellular damage (experimental ischemia). 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), as indicated. 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, **IN** and **PBN** (0.01 μM − 1 mM) were added at the beginning of recovery period (see below). Control cultures in Dulbecco's medium containing glucose were kept in the normoxic incubator for the same period of time as the OGD (C4h), and then culture medium was replaced with fresh medium and cells were returned to the normoxic incubator until the end of recovery period (C24h). Control experiments included the same amounts of vehicle (final concentration < 0.01% dimethyl sulfoxide). 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 triplicate.

4.2.3. Assessment of cell viability. Measurements of cell viability in human SHSY5Y

neuroblastoma cells, were carried out into 96- well culture plates as described [24]. Briefly, control and treated SH-SY5Y neuroblastoma cells (about 0.75–1 × 10^5 cells/well) were incubated with the XTT solution (Cell Proliferation Kit II (XTT), Sigma, Aldrich, Madrid) at 0.3 mg/ml final concentration for 3 h in a humidified incubator at 37 °C with 5% CO_2 and 95% air (v/v) and the soluble orange formazan dye formed was spectrophotometrically quantified, using a Biotek PowerWave XS spectrophotometer microplate-reader at 450 nm (reference 650 nm). All XTT assays were performed in triplicate in cells of different batches. Control cells treated with DMEM alone were regarded as 100% viability. Controls containing different DMSO concentrations (0.001–1% DMSO) were performed in all assays.

4.3. Antioxidant assays

1,1-Diphenyl-2-picrylhyrazyl (DPPH) radical, Nordihydroguaiaretic acid (NDGA), Trolox, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), Soybean LOX linoleic acid sodium salt were purchased from the Aldrich Chemical Co. Milwaukee, WI, (USA). 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); the pH (7.4) was adjusted by adding a solution of KH_2PO_4 or NaOH). A tris buffer solution with pH 9 was also prepared (3.029 g/L) For the *in vitro* tests a Lambda 20 (Perkin–Elmer-PharmaSpec 1700) UV–Vis double beam spectrophotometer was used.

4.3.1. Interaction of the nitrones with the stable radical 1,1-diphenyl-picrylhydrazyl

(DPPH) [20]. To a solution of DPPH in absolute ethanol the appropriate volume of the compounds (0.1 mM final concentrations) dissolved in DMSO was added. The absorbance was recorded at 517 nm after 20 and 60 min at rt.

4.3.2. Inhibition of linoleic acid peroxidation [21]

For initiating the free radical, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) is used. The final solution in the UV cuvette consisted of ten microliters of the 16 mM linoleate sodium solution 0.93 mL of 0.05 M phosphate buffer, pH 7.4, thermostatted at 37 °C. 50 μL of 40 mM AAPH solution was added as a free radical initiator at 37 °C under air and 10 μL of the tested compounds. The oxidation of linoleic acid sodium salt results a conjugated diene hydroperoxide. The reaction is monitored at 234 nm.

4.3.3. In vitro inhibition of soybean-lipoxygenase

In vitro study was evaluated as reported previously [14]. The tested compounds (several concentrations from 1 μM to100 μM, from the stock solution 10 mM were used for the determination of IC_{50}) dissolved in DMSO were incubated at rt with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor NDGA.

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