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 (EC50 = 6.64 ± 0.28 μM) as the most neuroprotective agents, and in particular, among them, indanonitrones 4 was also the most potent and balanced nitrone, showing antioxidant activity in three experiments (LOX (100μM), 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-1H-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-butylnitrooxine (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), quinolinonoitrones [14,15], and steroid-nitrones hybrids, such as the “cholesteronitrones”, have been studied [16,17].

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DPHP, 1,1-diphenyl-2-picrylhyrazy1; LP, lipid; LOX, lipoxygenase
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In order to improve and explore the potential therapeutic capacity of PBN, new conformationally restricted PBN derivatives (I) were designed by inserting a cycle of diverse size, by modifying the value of n, between the Carbon linked at the nitrone motif and C2, resulting in the indanonitrone (IN) 1–6 (Fig. 1). IN1 (Fig. 1) is the parent compound, without substituents, while IN2 and IN3 (Fig. 1) bear a 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 15h 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-dihydropyridindene [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 1H 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) hydroxylamines. 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 EC50 data and maximal neuroprotective effect are shown in Table 1. As shown, EC50 values range from 1.03 ± 0.12 μM (IN1) to 82.16 ± 4.35 μM (IN2). By comparing with the EC50 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 R2, the incorporation of a OMe group (IN3), instead an H at R1 (IN1) does not affect the neuroprotective activity, while the presence of an OH motif decreases very significantly this capacity (IN2: EC50 = 82.16 ± 4.3μM), 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 R1 (IN4-6), the maximum efficiency is achieved by IN4, bearing an OH group in R.2 Note also that the neuroprotective capacity...
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 R², the maximal neuroprotective activity is higher, which could mean that, at least in part, INs bearing an H at R¹, 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/antiproliferative 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 R², 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 antioxidant 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 IN1 and IN3 (60%), IN4 (57%), and IN5 (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].
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 (100μM), but less potent than the value determined for NDGA (0.45μM) (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 (100μM), 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 nitrore motif at para position (Fig. 4). To sum up, we have identified IN4 as a new hit-nitrone for further investigation.

### Table 1

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure R¹</th>
<th>Structure R²</th>
<th>Neuroprotection EC₅₀ ± SEM (µM)</th>
<th>P&lt; (PBN)</th>
<th>P&lt; (IN1)</th>
<th>Maximal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN1</td>
<td>H</td>
<td>H</td>
<td>1.03±0.12</td>
<td>*</td>
<td>-</td>
<td>104.2±8.6</td>
</tr>
<tr>
<td>IN2</td>
<td>OH</td>
<td>H</td>
<td>82.16±4.35</td>
<td>***</td>
<td>***</td>
<td>67.4±4.1</td>
</tr>
<tr>
<td>IN3</td>
<td>OMe</td>
<td>H</td>
<td>3.13±0.09</td>
<td>ns</td>
<td>ns</td>
<td>84.6±8.1</td>
</tr>
<tr>
<td>IN4</td>
<td>H</td>
<td>OH</td>
<td>6.64±0.28</td>
<td>ns</td>
<td>*</td>
<td>167.7±16.6</td>
</tr>
<tr>
<td>IN5</td>
<td>H</td>
<td>OMe</td>
<td>38.02±3.63</td>
<td>***</td>
<td>***</td>
<td>123.5±4.3</td>
</tr>
<tr>
<td>IN6</td>
<td>H</td>
<td>F</td>
<td>13.91±1.12</td>
<td>**</td>
<td>***</td>
<td>154.5±7.9</td>
</tr>
<tr>
<td>PBN</td>
<td></td>
<td></td>
<td>6.03±0.41</td>
<td>-</td>
<td>*</td>
<td>131.3±13.1</td>
</tr>
</tbody>
</table>

*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 EC₅₀'s were considered to be statistically significant when p ≤ 0.05. EC₅₀'s were calculated from data obtained of three experiments made in triplicate on cells from four different cultures. *non significant; *, p < 0.05; **, p < 0.01, ***, p < 0.001, compared with PBN or IN1, as indicated.

![Fig. 3. Effect of INs on SH-SYSY 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).](image-url)
Table 2
Antioxidant activities of IN1, 3 and 4 and PBN, against 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azobis(2-aminopropane) dihydrochloride (APPH), and in vitro inhibition of soybean lipoygenase (LOX). a

<table>
<thead>
<tr>
<th>Nitrones</th>
<th>DPPH (%)</th>
<th>APPH (%)</th>
<th>LOX (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBN</td>
<td>12</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>1</td>
<td>5.5</td>
<td>57</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>60</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>36.5</td>
<td>51</td>
<td>100 μM</td>
</tr>
<tr>
<td>NDGA</td>
<td>93</td>
<td>–</td>
<td>0.45 μM</td>
</tr>
<tr>
<td>Trolox</td>
<td>–</td>
<td>93</td>
<td>–</td>
</tr>
</tbody>
</table>

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.), with Merck Silica Gel (40–63 μm) or by flash chromatography (Biotage Isolera One equipment) and the adequate eluyent for each case.

4.1.1. General method for the synthesis of IN1–6

A mixture of corresponding 1-indanone (1 equiv), Na2SO4 (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 Na2SO4 (568 mg, 4 mmol), in EtOH (7 mL), was irradiated for 4 h. After work-up and chromatography eluting with mixtures of CH2Cl2 and MeOH, from 0% to 5%, and recrystallization, indanonitrone 1 [18] (104.2 mg, 65%) was isolated: mp 141–3 °C; IR (KBr) ν 2935, 2920, 1457, 1252 cm−1; 1H NMR (300 MHz, DMSO-d6) 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); 13C NMR (100 MHz, DMSO-d6) δ 148.1 (C3a), 147.3 (C1), 135.3 (C7a), 130.6 (C6), 128.6 (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 C11H12NO: 161.0849. Found: 161.0846. Anal. Calcd. for C11H12NO.H2O: 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 Na2SO4 (568 mg, 4 mmol), in EtOH (7 mL), was irradiated for 4 h. After work-up and chromatography eluting with mixtures of CH2Cl2 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; 1H NMR (300 MHz, DMSO-d6) δ 7.38 (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 (s, 3H, H9), 3.10 (m, 2H, H3), 3.00 (m, 2H, H2); MS (ES) m/z (%): 178 [M+1], 200 [M+Na]; HRMS. Calcd. for C10H11NO: 161.0791. Found: 161.0798. Anal. Calcd para C10H11NO.H2O: C, 69.09; H, 6.85; 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): 1H NMR (300 MHz, D2O) 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, H8), 2.98 (s, 4H, H2, H3). Anal. Calcd. for C10H13NO.H2O: C, 73.26; H, 6.59; N, 5.28. Found: C, 73.19; H, 6.41; N, 5.21.

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 Na2SO4 (213.1 mg, 1.5 mmol), in EtOH (7 mL), was irradiated for 9 h. After work-up and chromatography eluting with mixtures of CH2Cl2 and MeOH, from 100% a 9:1, and recrystallization, indanonitrone 3 (31.1 mg, 66%) was isolated: mp 111–4 °C; IR (KBr) ν 3002, 2936, 1575, 1424cm−1; 1H NMR (300 MHz, CDCl3) 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, 2.6 Hz, 1H, H5), 3.86 (s, 3H, H9), 3.79 (s, 3H, H8), 3.10–3.02 (m, 2H, H3), 2.98 (s, 4H, H2, H3). Anal. Calcd. for C11H13NO.H2O: C, 72.48; H, 7.00; N, 8.45. Found: C, 72.75; H, 7.18; N, 8.68.


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 Na2SO4 (568 mg, 4 mmol), in EtOH (7 mL), was irradiated for 15 h.

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, 0.15 mmol), N-methylhydroxylamine hydrochloride (83.5 mg, 1 mmol), NaOAc (82 mg, 1 mmol) and Na2SO4 (284.1 mg, 2 mmol), in EtOH (7 mL), was irradiated for 7 h.

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 Na2SO4 (284.1 mg, 2 mmol), in EtOH (7 mL), was irradiated for 3 h. After work-up and chromatography eluting with mixtures of CH2Cl2 and MeOH, from 100:0 a 9:1, recrystallization, indanonitrone 1586, 1181 cm⁻¹; 1H NMR (300 MHz, DMSO-d6) δ 8.26 (d, J = 9.6 Hz, 1H, H4), 7.00–6.85 (m, 2H, H2, H3), 3.23 (2H, H1), 3.20–3.07 (m, 2H, H2, H3) and MeOH, from 20% a 5%, and recrystallization, indanonitrone 1586, 1181 cm⁻¹; 1H NMR (300 MHz, DMSO-d6) δ 8.26 (d, J = 9.6 Hz, 1H, H4), 7.00–6.85 (m, 2H, H2, H3), 3.23 (2H, H1), 3.20–3.07 (m, 2H, H2, H3).

4.2.1. Neuroblastoma cell cultures

The human neuroblastomas cell line SH-SYSY 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-Antimotic (Gibco; Life Technologies, Madrid, Spain) (containing 100 ui/mL penicillin, 100 mg/mL de streptomycin and 0.25 mg de amphotercine B), 1% gentamicina 15 mg/mL (Sigma-Aldrich, Madrid, España) and 10% Fetal 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% CO2 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 × 10⁵ cells per well, respectively. When the SHSY5Y cells reached 80% confluence, the medium was replaced with fresh medium containing 0.10–1000 µM compound concentrations or PBS in the controls, as indicated in each assay.
4.3.1. Interaction of the nitrones with the stable radical 1,1-diphenylpicrylhydrazyl (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-aminopropane) dihydrochloride (AAPH) is used. The final solution in the UV cuvette consisted of ten microliters of the 16 mM linoleate sodium salt 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 to 100 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 × 10^{-4} w/v in saline). The conversion of soy-linoleate to 13-hydroperoxylinoleic acid at 234nm was recorded and compared with the appropriate standard inhibitor NDGA.

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