Synthesis and Neuroprotective Properties of N-Substituted C-Dialkoxyphosphorylated Nitrones

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Supporting Information

ABSTRACT: Herein, we report the synthesis and neuroprotective power of some N-substituted C-(dialkoxy)phosphorylated nitrones 4a–g, by studying their ability to increase the cell viability, as well as their capacity to reduce necrosis and apoptosis. We have identified (Z)-N-tert-butyl-1-(diethoxyphosphoryl)methanimine oxide (4e) as the most potent, nontoxic, and neuroprotective agent, with a high activity against neuronal necrotic cell death, a result that correlates very well with its great capacity for the inhibition of the superoxide production (72%), as well as with the inhibition of lipid peroxidation (62%), and the 5-lipoxygenase activity (45%) at 100 μM concentrations. Thus, nitrone 4e could be a convenient promising compound for further investigation.

1. INTRODUCTION

Reactive oxygen species (ROSs) exert their physical functions at low to moderate concentrations, but at high concentrations, they become toxic for the living systems, resulting in oxidative stress (OS).1 Oxidative stress (OS) plays a key role in many pathologies, being possibly the most important molecular event occurring before and after ischemic damage.2 Aerobic organisms have integrated antioxidant systems, which include enzymatic and nonenzymatic antioxidants that are usually effective in blocking harmful effects of ROS.3

In this context, free radical scavengers, such as nitrones like NXY-059 (1) (Figure 1) (Cerovive, AstraZeneca),4,5 are efficient neuroprotective agents in experimental ischemia studies.1 However, as NXY-059 (1) failed in advanced clinical trials for stroke, the neuroprotection therapy strategy for cerebral ischemia based on antioxidants and nitrite ROS scavenging agents has been critically analyzed and almost discontinued.6

In spite of this, the impressive results that we have recently published using novel quinolyl nitrones mean that the structure and functional groups incorporated in the nitroline cores have a critical influence on their antioxidant and pharmacological properties and that there is still place for novelty and originality in this area of medicinal chemistry. On the other hand, as the capability of phosphorylated nitrones DEPMPO8 and 2-(diethoxyphosphoryl)-N-(benzylidene)propan-2-amine oxide derivatives 39 (Figure 1) to trap various radicals has been well recognized, all of these observations inspired us to study the antioxidant properties of the N-substituted C-dialkoxyphosphorylated nitrones 4a–g, including the (diethoxyphosphoryl)nitrones (DEOPNs) 4a–e and (dibenzyloxophosphoryl)nitrones (DBOPNs) 4f,g (Figure 1), and their neuroprotective properties.

2. RESULTS AND DISCUSSION

2.1. Chemistry. The phosphorylated nitrones 4a–d were synthesized from hydroxymethylphosphonate (5) by Swern oxidation and subsequent reaction with the respective
hydroxylamine$^{10-12}$ (Scheme 1), whereas nitrones 4e and 4fg have been synthesized here for the first time in an analogous manner starting from diethyl hydroxymethylphosphonate (5) and dibenzyl hydroxymethylphosphonate (6), respectively (Supporting Information). According to our previous observations, crystalline DEOPN 4a exists as a Z isomer, whereas in chloroform-d as well as in benzene-d$_6$ solutions at room temperature, a 15:85 equilibrium mixture of E/Z isomers was noticed ($^1$H NMR analysis).$^{10}$ Similarly, DEOPN 4b exists as a 14:86 mixture of E/Z isomers.$^{10}$ On the other hand, both (R)- and (S-configured DEOPNs 4c and 4d in chloroform-d solution form 7:93 mixtures of E/Z isomers.$^{11}$ Nitrones 4f and 4g in deuterated chloroform solutions form equilibrium mixtures of E/Z isomers, 10:90 and 5:95, respectively, whereas nitrone 4e exists as only a Z isomer. Surprisingly, all of the phosphorylated nitrones 4a–g exist as Z isomers in dimethyl sulfoxide (DMSO) and less than 2% of E-isomers could be detected with a 600 MHz NMR spectrometer.

2.2. Neuroprotective Effect of N-Substituted C-Dialkoxyphosphorylated Nitrones. Following the usual protocols (see the Supporting Information), we obtained the cell viability and neuroprotection values shown in Table 1 and Figure 2. Based on these results, we conclude that all of the nitrones showed moderate neuroprotective values, in the micromolar range, from 10.62 (4e) to 131.21 (4c). Second, by comparing with the EC$_{50}$ determined for the standard compounds $\alpha$-phenyl-tert-butylnitrone (PBN), citicoline (CCh), and N-acetyl-cysteine (NAC), it is clear that most of the nitrones 4, with the exception of 4a and 4c, are more neuroprotective than PBN and CCh but less than NAC.

Among the DEOPNs 4a–e, on going from nitrone 4a (R = Me, EC$_{50}$ = 98.30 $\mu$M) to nitrones 4b or 4c, the incorporation of benzyl or tert-butyl motifs, respectively, instead of the methyl group, produces strong positive neuroprotective responses, affording the two most neuroprotective agents discovered in this work. Very interestingly, the $\alpha$-substitution on nitrone 4b by a methyl group significantly decreases the neuroprotection observed for this nitrone, more strongly in the (R)-enantiomer 4c than in the (S)-enantiomer 4d. However, nitrones 4b and (S)-4d have a lower maximal activity than (R)-4c, which shows a lower neuroprotection. This is possibly due to the fact that at high concentrations both nitrones are more toxic than (R)-4c. This result is very interesting, showing that the absolute configuration at the stereogenic center has an influence on the neuroprotection, but unfortunately we have no explanation for this fact.

This could also be supported by the results of the effect of these nitrones on the necrotic and apoptotic cell death tests (Figure 3a,b, see below). By comparing DEOPNs 4a,b with the corresponding DBOPNs 4fg, we conclude that nitrone 4f is 2.2-fold more potent than nitrone 4a ($P < 0.01$) but that nitrone 4b has a neuroprotective potency very similar to that of nitrone 4g (nonstatistically significant). However, DBOPNs 4fg have a maximal neuroprotective effect higher than those of their corresponding DEOPNs 4a,b, indicating that the addition of a benzoyl group, instead of an ethoxy group, affords less toxicity at high concentration.

To shed light on the neuroprotective effects of nitrones 4a–g, the effect of these compounds on necrotic and apoptotic cellular death was measured by monitoring lactate dehydrogenase
by applying a one-way analysis of variance (ANOVA) test. Differences were considered to be statistically significant when \( P \leq 0.05 \); \( P \), \( P < 0.01 \); \( ** P \), \( P < 0.001 \); compared with the basal value.

During reperfusion, parallel to a reduction in cell viability loss of about 30% during the IR condition. The presence of some nitrones \( 4 \) during this period reversed the necrotic death in a concentration-dependent manner (Figure 3A). The neuroprotective effects of nitrones, at concentrations from 1 to 500 \( \mu \text{M} \), on necrotic cell death were assayed by adding them at the beginning of the reperfusion period. Their effect was compared with the neuroprotective effect of NAC, from 1 to 500 \( \mu \text{M} \) doses, showing a decrease in LDH release in a dose-dependent manner, having then a significant neuroprotective effect at concentrations between 10 and 500 \( \mu \text{M} \) (ranging from 59 to 100% of maximal reduction) (Figure 3A).

The addition of 10–500 \( \mu \text{M} \) nitrone \( 4e \) produced a reduction of necrotic cell death, in the same range as NAC (from 55 to 100% LDH release inhibition), thus being the most potent neuroprotective nitrone, followed by nitrone \( 4a \) (52–70% inhibition), indicating that the substitution of Me by the \( t\)-Bu group increases the neuroprotective and antinecrotic effect. Nitrones \( 4b–d \) were also able to reduce LDL release induced by IR in a dose-dependent manner, although nitrone \( 4c \), nitrone \( 4b \), at 100 \( \mu \text{M} \) dose, or nitrone \( 4d \), at 100 and 500 \( \mu \text{M} \) concentrations, did not reduce the LDH release, indicating that, similar to the observed antioxidant and neuroprotective effects, both nitrones are toxic at these concentrations. In general, DBOPNs \( 4a,b \) have a neuroprotective effect higher than that of their corresponding DBOPNs, indicating that the addition of a benzoxyl group, instead of the ethoxy, decreases their antinecrotic cell death activity.

The observed high antinecrotic cell death capacity of these nitrones contrasts with their weak capacity to reduce apoptotic cell death. An increase in caspase-3 activity is correlated with apoptosis. Thus, to know whether these nitrones reduce apoptosis induced by experimental ischemia, the caspase-3 activity was measured.

As shown in Figure 3B, the reduction in caspase-3 activity induced by the nitrones, NAC, and PBN was very small, and only at high concentrations (100–500 \( \mu \text{M} \)), an antiapoptotic effect was observed. In general, DBOPNs \( 4f,g \) seem to be less antiapoptotic than their analogues DBOPNs, indicating that the incorporation of a benzoxyl group, instead of an ethoxy group, decreases not only their antinecrotic but also their antiapoptotic cell death activity. It is interesting to note that the introduction of a \( t\)-Bu group in nitrone \( 4e \), the best neuroprotective nitrone in our hands, has an opposite antinecrotic or antiapoptotic effect. Thus, while this chemical modification increases the antinecrotic effect, it decreases its antiapoptotic effect, as shown by comparing these activities between nitrones \( 4a \) and \( 4e \).

To sum up, taken together all of these results of neuroprotection, antinecrotic, and antiapoptotic activities of the nitrones described here, we conclude that the mechanism of their neuroprotective activity might be related to their antinecrotic actions more than their antiapoptotic activity.

### 2.3. Antioxidant Assays: ROS Production in Cultures of SHSY5Y Human Neuroblastoma Cells

It is well documented that nitrones NXY-059 and PBN have good antioxidant properties. \(^{3,14} \)

To analyze the antioxidant capacity of nitrones \( 4 \) and explore whether ROS could be involved in their neuroprotective actions, ex vivo experiments were carried out, following the usual protocols (Supporting Information), to evaluate the effect of these nitrones on ROS production in cultures of SHSY5Y human neuroblastoma cells, under oxygen glucose deprivation (OGD) conditions, using PBN, NAC, and

### Table 1. Neuroprotective Effects of Nitrones 4a–g on the Cell Viability Decrease Induced by Oxygen–Glucose Deprivation (OGD) and Reperfusion in SHSY5Y Neuroblastoma Cells

| Compound | Structure (R) | Neuroprotection EC50 ± SEM (μM) | Maximal Activity | P< (PBN) | P< (4e) |
|----------|---------------|---------------------------------|-----------------|---------|--------|
| 4a       | Me            | 98.30 ±1.94                     | 76.35 ±0.50     | *       | **     |
| 4b       | Bn            | 13.46 ±7.95                     | 65.64 ±1.17     | **      | as     |
| 4c       | (R)-CH(Me)Ph  | 131.21 ±11.27                  | 106.37 ±7.13    | **      | **     |
| 4d       | (S)-CH(Me)Ph  | 30.52 ±7.46                    | 52.51 ±2.39     | **      | **     |
| 4e       | t-Bu          | 16.62 ±3.87                    | 11.75 ±11.19    | **      | -      |
| 4f       | Me            | 64.04 ±17.56                   | 102.12 ±9.50    | *       | **     |
| 4g       | Bn            | 23.8 ±10.70                    | 142.39 ±14.05   | **      | *      |
| PBN      | -             | 81.92 ±16.81                   | 52.48 ±6.88     | -       | **     |
| CCh      | -             | 66.96 ±19.46                   | 49.70 ±5.40     | *       | **     |
| NAC      | -             | 1.48 ±0.19                     | 103.20 ±1.52    | **      | *      |

<Figure 2. EC50 values of the neuroprotective effect of DEOPNs 4a–e and DBOPNs 4f,g.>
CCh for comparative purposes and sodium nitroprusside (SNP) as the positive control.

To address this issue, we monitored superoxide production by a fluorimetric assay. SH-SY5Y neuroblastoma cells in the culture had a basal superoxide production rate of 0.76 ± 0.072 AFU/min × 2 × 10⁵ cells (n = 10; Figure 4A). OGD (3 h) and IR (3 h reperfusion after 3 h OGD) caused an increase in the rate of O₂⁻ production (Figure 4A), reaching values of 1.73 ± 0.18 and 2.59 ± 0.278 AFU/min × 2 × 10⁵ cells (n = 10), respectively, values similar to that obtained for 3 h of cell culture had a basal superoxide production rate of 0.76 ± 0.072 AFU/min × 10⁵ cells (at 1 mM concentration). In the case of nitrones 4a−f, values higher than those observed for 3 h OGD and 24 h of reperfusion (R24h) (3.78 ± 0.48%) was considered as 100%. The values represent the average of three independent experiments (mean ± SEM) at *P < 0.05, **P < 0.01, and ***P < 0.001 vs R24h by one-way analysis of variance followed by Holm–Sidak’s post-test, when analysis of variance was significant. Statistical significances above the R24h value are not shown.

![Graph A](image1)

![Graph B](image2)

**Figure 3.** Effect of nitrones on (A) lactate dehydrogenase (LDH) release or (B) caspase-3 activity in SH-SY5Y human neuroblastoma cell cultures exposed to oxygen-glucose deprivation (OGD). The bar chart shows the percentage of LDH release at 24 h of recovery after (A) 3 h OGD or (B) caspase-3 activity (AFU/μg protein/h), either untreated (R24h) or treated with different concentrations (μM) of nitrones 4a−g or NAC and PBN. The value induced by OGD at 3 h without the recovery period (OGD3h) is also indicated. LDH release corresponding to R24h cells exposed to 3 h OGD and 24 h of reperfusion (R24h) (3.78 ± 0.48%) was considered as 100%. The values represent the average of three independent experiments (mean ± SEM) at *P < 0.05, **P < 0.01, and ***P < 0.001 vs R24h by one-way analysis of variance followed by Holm–Sidak’s post-test, when analysis of variance was significant. Statistical significances above the R24h value are not shown.

Very interestingly, the most potent neuroprotective nitrone 4f is one of the most potent antioxidant nitrones in the ex vivo analysis, especially at a high, 1 mM, concentration, showing ROS (%) reduced values higher than those observed for PBN, NAC, and CCh (Figure 4B).

We observed that, among DEOPNs 4a−e, nitrones 4d and 4e are the most potent antioxidants. The incorporation of benzyl or t-butyl motifs, instead of the methyl group, seems to increase the antioxidant capacity of these nitrones, and the α-substitution on nitrone 4b by a methyl group significantly increases its antioxidant capacity, more strongly in the resulting (R)-enantiomer 4c than in the (S)-enantiomer 4d. However, both nitrones 4b and the (S)-enantiomer 4d show less antioxidant activity at 1 mM than at 0.1 mM concentrations, which is possibly due to the fact that at high concentrations both nitrones are more toxic than (R)-enantiomer nitrone 4c. By comparing DEOPNs 4a and 4b with the corresponding DBOPNs 4f and 4g, we conclude that nitrone 4f is 2-fold more potent than nitrone 4a (R = Me) (P < 0.01) but that nitrone 4b has a neuroprotective potency very similar to that of nitrone 4g (R = Bn) (nonstatistically significant).

Very interestingly, the most potent neuroprotective nitrone 4e shows high values for the inhibition of the lipid peroxidation (LP) (62%), inhibition of the 5-lipoxygenase (LOX) (45%), and lipophilicity (ClogP = 2.04) (Table 2). These results are in good agreement with its capacity to inhibit superoxide production shown in the ex vivo experiments (about 100% at 1 mM; Figure 4A,B). Thus, the antioxidant...
3. CONCLUSIONS

We report herein the synthesis and neuroprotective properties of some N-substituted C-dialkoxyphosphorylated nitrones 4, easily available in good yields from commercial starting materials. Regarding the neuroprotection analysis, DEOPNs t-butyl nitrone 4b and benzyl nitrone 4e have been identified as good neuroprotective nitrones, although in the case of nitrone 4b, no clear trends have been observed to correlate these results with its antioxidant capacity. The replacement of a methyl group with a t-butyl or benzyl group improves the neuroprotective power of the corresponding nitrone. Similarly, the replacement of the benzyl with a methyl group reduces the neuroprotective capacity, resulting in less-potent nitrones 4c and 4d, where we have observed that the (S)-enantiomer is 4.2-fold more potent than the (R)-enantiomer. To sum up, DEOPN 4e has been identified as the most potent neuroprotective, antinecrotic agent investigated here, showing the capacity to inhibit LP and decrease superoxide levels in neuronal cultures, and consequently deserves further investigation. The etiology and origin of stroke, as a complex and multifactorial disease, is unknown. However, as the oxidative stress is thought to play a key role, among other factors, we have tried to explain the observed neuroprotective effect by nitrone 4e on these grounds but without excluding other factors and mechanisms being involved.

4. EXPERIMENTAL SECTION

4.1. Chemistry: General Methods. 1H NMR spectra were recorded in CDCl3 on the following spectrometers: Varian Mercury-300 and Bruker Avance III (600 MHz) with tetramethylsilane as the internal standard. 13C NMR spectra were recorded for CDCl3 solution on the Varian Mercur-300 machine at 75.5 MHz, whereas for DMSO solution on Bruker Avance III at 151.0 MHz. IR spectra were recorded on an Infinity MI-60 FT-IR spectrometer. Melting points were determined on Boetius apparatus and were uncorrected. Elemental analyses were carried out on a PerkinElmer PE 2400 CHNS analyzer. The following adsorbents were used: column chromatography, Merck silica gel 60 (70–230 mesh); analytical TLC, Merck TLC plastic sheets silica gel 60 F254.

4.2. General Method for the Synthesis of Nitrones. To a stirred solution of oxalyl chloride (0.210 mL, 2.48 mmol) in CH2Cl2 (1.5 mL) was added a solution of the acid chloride in CH2Cl2 (5 mL) under an argon atmosphere. DMSO (0.360 mL, 5.15 mmol) dissolved in CH2Cl2 (1.5 mL) was added dropwise at −60 °C. After 30 min, a solution of the appropriate dialkoxy hydroxymethylphosphonate 5 or 6 (2.0 mmol) in CH2Cl2 (2 mL) was added followed by triethylamine (0.850 mL, 5.00 mmol) and the respective alkylhydroxylamine (0.850 mL, 5.00 mmol). The cooling bath was removed, and saturated aqueous NaHCO3 (10 mL) was added when the temperature of the reaction mixture reached 0 °C. The aqueous layer was extracted with CH2Cl2 (3 × 5 mL), and the organic phases combined were washed with brine, dried (MgSO4), and concentrated to yield the crude nitrone 4, which was purified by column chromatography using a chloroform–MeOH mixture (50:1). More details about these methods are briefly described in the Supporting Information.

4.3. Antioxidant “in Vitro” Assays. In vitro inhibition of linoleic acid peroxidation and soybean-lipoxygenase was evaluated spectrophotometrically as reported previously. These methods are briefly described in the Supporting Information.
4.4. Neuroprotection Assays. 4.4.1. Neuroblastoma Cell Cultures. The human neuroblastoma cell line SHSY5Y was cultured in Dulbecco’s modified Eagle’s medium: Ham’s F12 medium as described in ref 16. 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 cells per well, respectively. When the SHSY5Y cells reached 80% confluence, the medium was replaced with fresh medium containing 0.01 μM to 10 mM compound concentrations or PBS in the controls, as indicated in each assay.

4.4.2. Neuroblastoma Cell Culture 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 and maintained in an anaerobic chamber containing a gas mixture of 95% N_2/5% CO_2 and humidified at 37 °C, as described in ref 17. Cells were exposed to OGD for a period of 3–4 h (OGD4h), as indicated. Then, the culture medium was replaced with oxygenated serum-free medium and cells were placed and maintained in the normoxic incubator for 24 h for recovery (R24h). Nitrones were analyzed independently three to five times with different batches of cultures, and each experiment was run in triplicate.

4.4.3. Assessment of Cell Viability. Measurements of cell viability in human SHSY5Y neuroblastoma cells (about (0.75–1) × 10^5 cells/well) were carried out in 96-well culture plates by the XTT methods, as described in ref 17,17

4.4.4. Measurement of LDH Activity. For these assays, cultured neuroblastoma cells grown in 96-well culture dishes at a density of 1.5 × 10^5 cells/well were used. LDH activity was measured as the rate of decrease of the absorbance at 340 nm, resulting from the oxidation of NADH to NAD^+ as described.17 Data are given as the percentage of LDH release with respect to the total LDH content (LDH in the culture medium and LDH inside the cells).

4.4.5. Analysis of Caspase-3 Activity. For these assays, cultured neuroblastoma cells grown in 48-well culture dishes, at a density of 2.5 × 10^5 cells/well, were used. After OGD treatment, cells were treated with different nitrones or indicated positive controls at 1–500 μM concentrations and subjected to 24 h reperfusion. Attached cells were lysed by 4 °C in a lysis medium containing 5 mM Tris/HCl (pH 8.0), 20 mM ethylenediaminetetraacetic acid, and 0.5% Triton X-100 and centrifuged at 13 000g for 10 min. The activity of caspase-3 was measured using the fluorogenic substrate peptide DEVD-amc (66081; BD Biosciences PharMingen), as described in refs 17, 18. Proteins were measured by the Bradford assay. Results were expressed as arbitrary fluorescence units [(AFU)/μg protein/h].

4.4.6. Measurement of ROS Formation. SHSY5Y human neuroblastoma cells (2 × 10^5 cells/well) were exposed to OGD for a period of 3 h (OGD3h). At the end of the OGD period, the culture medium was replaced with oxygenated Dulbecco’s modified Eagle’s medium containing glucose and 10% fetal calf serum. Cells were treated in the absence (controls) or presence of indicated concentrations of nitrones or different known neuroprotective agents and maintained at 37 °C in a normoxic incubator for 3 h for recovery. At the end of this period, 20 μM DHE (HEt; Molecular Probes) was added and fluorescence was recorded every 15–30 s during a 15 min period, using an excitation filter of 535 nm and an emission filter of 635 nm in a spectrophotometer (Bio-Tek FL 600) as previously described.18,19 Linear regression of fluorescence data [expressed as arbitrary fluorescence units (AFU)] was calculated for each condition, and the slopes (a) of the best fitting lines (y = ax) were considered as an index of O_2^- production. SNP was used as a positive control of superoxide production.19

4.5. Statistical Analysis. Data were expressed as mean ± SEM of results obtained from at least three independent experiments from different cultures, each of which was performed in triplicate. Statistical comparisons between the different experimental conditions were performed using one-way analysis of variance (ANOVA), followed by Holm–Sidák’s post-test when the analysis of variance was significant. A P-value <0.05 was considered statistically significant. Fit curves for EC_{so} determinations were performed according to the program of SigmaPlot v.11 (Systat Software INC., 2012).

■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00189.

Synthesis of nitrones 4a–g and antioxidant activity in vitro test methods; inhibition of linoleic acid peroxidation; in vitro inhibition of soybean-lipoxygenase; estimation of lipophilicity as Clog P (PDF)

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Notes
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