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Protective role of new nitrogen compounds on ROS/RNS-mediated damage to PC12 cells

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Abstract
Reactive oxygen (ROS) and nitrogen (RNS) species are known to be involved in many degenerative diseases. This study reports four new nitrogen compounds from organic synthesis, identified as FMA4, FMA7, FMA762 and FMA796, which differ mainly by the number of hydroxyl groups within their phenolic unit. Their potential role as antioxidants was evaluated in PC12 cells by assessing their protection against oxidative and nitrosative insults. The four compounds, and particularly FMA762 and FMA796, were able to protect cells against lipid peroxidation and intracellular ROS/RNS formation to a great extent. Their protective effects were likely mediated by their free radicals scavenging ability, as they appeared to be involved neither in the induction of natural antioxidant enzymes like GSH-PX and SOD, nor in the inhibition of NOS. Nevertheless, these results suggest a promising potential for these compounds as ROS/RNS scavengers in pathologies where oxidative/nitrosative stress are involved.

Keywords: *Nitrogen compounds, antioxidants, lipid peroxidation, oxidative stress, nitrosative stress*

Abbreviations: *DCF, 2′-7′-dichlorofluorescein; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase; SNP, sodium nitroprusside; TBARS, thiobarbituric acid-reactive substances;* t-*BHP,* tert-*butyl-hydroperoxide*

Introduction

Reactive oxygen (ROS) and nitrogen (RNS) species are produced during normal cellular function and in response to various stimuli [1,2]. Vital beneficial physiological cellular use of ROS, which include superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) has been demonstrated in different functions including intracellular signalling, host defence and redox regulation [3–6]. Moreover, nitric oxide ($^{\cdot}NO$), a RNS, can act as an important neurotransmitter or neuromodulator in the central and peripheral nervous systems [7,8].

Endogenous and exogenous factors may trigger the over-production of ROS/RNS [9]. To withstand these sudden stressful changes, organisms have developed a variety of enzymatic (e.g. superoxide dismutase, catalase and glutathione-related enzymes) and non-enzymatic (e.g. glutathione and vitamin E) antioxidant systems that can either prevent the formation of ROS/RNS or convert them to inactive derivatives [10–12]. Nevertheless, when an imbalance occurs between oxidants and antioxidants in favour of the oxidants, we come to a situation defined as oxidative stress, which leads to cellular damage responsible for degenerative conditions like those that occur in Alzheimer's, Parkinson's and atherosclerosis, among others [13–17].

In an effort to prevent or diminish ROS/RNS-induced damage, many investigators have been

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focused on the evaluation of either natural or synthetic compounds that can act beyond and/or potentiate the cells natural defence mechanisms [18–21].

In this study, we used new nitrogen compounds from organic synthesis, which are nitrogenated structures composed of an amidine unit and a phenol ring (Figure 1). The choice of these molecules was based both on the knowledge that the hydroxyl groups of the phenol ring are usually responsible for the antioxidant properties and on the knowledge that nitrogen compounds (in particular, nitrogen heterocycles incorporating an imidazole or an amidine unit) can easily interact with active centres responsible for different functions in living organisms [22]. The association of these two moieties in the same molecule was expected to result in new structures capable of acting as antioxidants in living systems. Molecules incorporating conjugated systems with nitrogen atoms are also known to stabilize free radicals [23] and this combination was also expected to enhance the antioxidant activity of the phenolic unit [24].

We previously reported [25] a good antioxidant potential for two of these new nitrogen compounds, named FMA4 and FMA7. Furthermore, in a parallel study involving the synthesis of nitrogen structures [22], two other compounds, FMA762 and FMA796, emerged as promising in which concerns their antioxidant capacities. That study suggested that the presence of three hydroxyl groups in C-3, -4 and -5 positions of the aromatic substituent, as is the case of these two structures, improved the anti-radical activity of the compounds.

In the present study, we intended to further characterize these four new nitrogen structures (FMA4, FMA7, FMA762 and FMA796) with respect to ROS/RNS-mediated damage induced in a neuronal cell model. PC12 cells were used since they have some advantages over primary cultured neuronal cells, including the homogeneity of the cell population [26,27].

Materials and methods

Compounds

The new compounds used in this study (Figure 1) were prepared in the group of Organic Synthesis, Chemistry Department, University of Minho, from the reaction of an appropriate phenolic aldehyde with a substituted amidine, as described in Areias [22]. The experimental procedure was adapted from previous work [28] carried out on a selection of monosubstituted aldehydes. All of these structures present a linear chain, differing mainly by the presence of one (FMA4 and FMA7) or three (FMA762 and FMA796) hydroxyl groups within the phenol ring.

The compounds were provided as a yellowish powder, which was reconstituted in DMSO, aliquoted and maintained frozen at $-80^{\circ}C$ until utilization. Each aliquot was thawed only once.

Foetal bovine serum (FBS) was from BioChrom KG (Berlin, Germany); horse serum donor herd was purchased from Gibco (Paisley, UK). 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was purchased from Molecular Probes (Eugene, OR, USA). NitroBlue Tetrazolium was purchased from Diagnostica Merck (Darmstadt, Germany). 6-

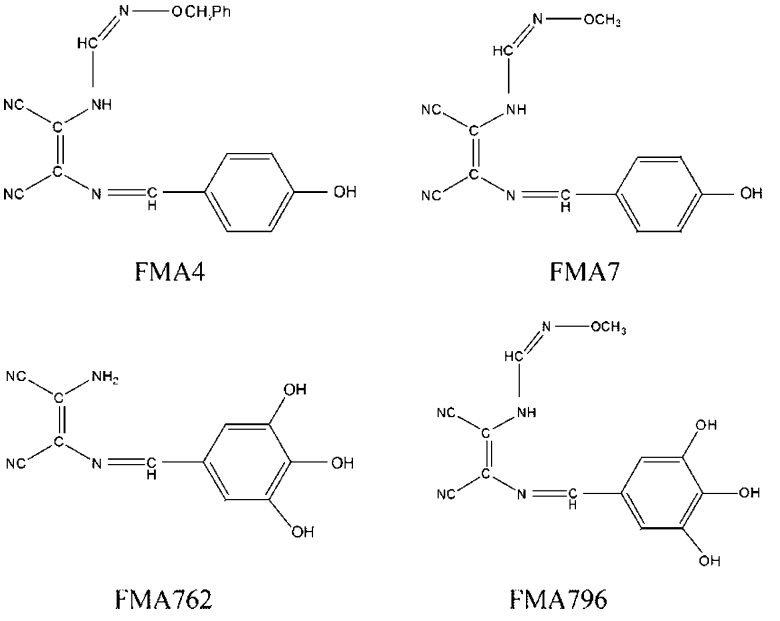

Figure 1. Schematic structure of the newly synthesized nitrogen compounds. Two phenolic units are linked to an amidine function through a linear chain containing a nitrogen atom in three carbon atoms. FMA762 and FMA796 differ mainly from FMA4 and FMA7 by the presence of three hydroxyl groups. The compounds were prepared by F. Areias, Group of Organic Chemistry, University of Minho [22].

hydroxi-2,5,7,8-tetrametilcromano-2-carboxylic acid 97% (trolox) was purchased from Sigma-Aldrich Chemie (Berlin, Germany). All other reagents, including those for cell culture, were purchased from Sigma Chemical Company (St Louis, MO, USA).

PC12 cell culture and treatment conditions

PC12, a neuronal cell line established from a rat adrenal pheochromocytoma [29] was used in this study. There are many reports on the use of this cell line as a model for the study of neurodegenerative disorders in which oxidative stress is involved, such as Parkinson's or Alzheimer's disease [7,13,30–32].

Cells were cultured in suspension in 75 cm^2 flasks, in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated foetal bovine serum and 1% (v/v) of an antibiotic/antimycotic solution. Cultures were maintained in a humidified incubator containing 95% air and 5% CO_2 and passed twice a week. Before each assay, the cell aggregates were carefully disrupted by gently pipetting and the separated cells plated in poly-D-lysine-coated multiwells, at a density of 2.5×10^5 cells/cm^2 for the MTT assay and at a density of 1.6×10^5 cells/cm^2 for other studies. After plating, cells were left for adhesion overnight. The compounds in study were usually added to the cells 3 h prior to the addition of the deleterious stimuli.

Determination of the radical scavenging effect—DPPH assay

Free radical scavenging capacity of the compounds was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) discolouration method, according to the procedure previously described [25]. The reduction of this radical by an antioxidant compound results in a decrease in absorbance and is proportional to the number of electrons absorbed [33], indicating the anti-radical capacity of the substances in study.

Briefly, the discolouration rate of a 0.002% ethanolic solution of DPPH was followed at 517 nm, along the time, in a SpectraMax 340PC microplate reader, vs a control containing ethanol instead of the compound in study. The inhibition of discolouration was expressed as a percentage, towards the control, and the IC_{50} were then obtained from the inhibition curve. The absorbance stabilization time was also determined. Antiradical Efficiency (AE) was determined according to the formula

$$AE = 1/(IC_{50} \times T_{IC50}) \quad (1)$$

where IC_{50} is the concentration needed to reduce the DPPH discolouration by 50% and T_{IC50} is the time needed to reach the discolouration steady state at IC_{50} concentration [34].

Measurement of 2-deoxy-D-ribose degradation

The compounds scavenging activity against the hydroxyl radical was evaluated by the 2-deoxy-D-ribose degradation assay. Hydroxyl radicals formed through a mixture of ascorbic acid, H_2O_2 and EDTA-Fe^{3+} (Haber-Weiss reaction) degrade deoxyribose in a series of fragments, which react on heating with thiobarbituric acid to give a pink chromogen. If a compound is added to the reaction mixture, that can scavenge the hydroxyl radical more efficiently than deoxyribose, then its degradation will be slower, as well as the chromogen formation [35].

A reaction mixture was prepared in 10 mM KH_2PO_4-KOH, pH 7.4, containing 2.8 mM deoxyribose, 20 μM $FeCl_3$ (dissolved in 2 mM Na_2EDTA), 1.42 mM H_2O_2, 50 μM ascorbic acid, in the presence or absence of the compounds in study at their IC_{50} concentrations (obtained by the DPPH discolouration method). After 1 h incubation at 37°C, the reaction was stopped by adding 1% thiobarbituric acid in 50 mM NaOH and 2.8% trichloroacetic acid. The reaction mixture was then heated at 100°C for 15 min. After cooling, absorbance values were determined at 535 nm in a microplate reader (SpectraMax 340PC).

Evaluation of the degree of hydrophobicity

The drugs hydrophobicity was determined by measuring the partition coefficients (PC), in an *n*-octanol/HEPES system. The nitrogen compounds were dissolved in *n*-octanol at a concentration of 20 μM and 1 ml of each solution was shaken with 20 ml HEPES (20 mM, pH 7.4) for ~10 min, at room temperature. The two different phases formed were then separated by centrifugation. The absorbance peaks of each drug, needed to assess the concentrations in each solution, were determined as 380 nm for FMA762 and 415 nm for FMA796. PC values were then calculated using the formula:

$$PC = \log(C_o/C_H) \quad (2)$$

where C_O and C_H are the concentrations of the drugs in *n*-octanol and in HEPES, respectively. The C_H values were indirectly determined by calculating the difference between the initial and the final concentrations of the drug in octanol.

Analysis of cell survival

Cell viability in the presence of the compounds was evaluated by the MTT reduction test, as previously described [25]. Briefly, 0.5 ml MTT (final concentration 0.5 mg/ml, in Krebs medium, pH = 7.4), prepared just before usage and maintained in the dark, was added to the PC12 cells. The plate, wrapped in aluminium foil, was left incubating for 2 h. Hydrogen chloride 0.04 M in isopropanol was then

added, followed by 2 h more of orbital shaking, in the dark, to dissolve the formazan crystals. The survival of PC12 cells was expressed as the percentage of OD towards control cells, containing the same amount of the drug solvent, DMSO.

Measurement of the extent of lipid peroxidation—TBARS assay

Lipid peroxidation was evaluated by measuring the levels of Thiobarbituric Acid-Reactive Substances (TBARS), which are expressed in terms of malondialdehyde (MDA) equivalents that react with thiobarbituric acid (TBA) [36]. TBARS formation was induced by the oxidant pair ascorbate/iron for 1 h at 37°C, as previously described [25]. Absorbance was read at 530 nm in a multiplate reader (Spectramax 340PC). The amount of TBARS produced was calculated using the molar absorption coefficient of 1.56×10^5 M^{-1} cm^{-1}, corrected for the total protein content [37] and expressed as nmol TBARS/mg protein.

The antioxidant capacity of the drugs was evaluated determining the percentage of protection offered by each drug against the lipid peroxidation induced by the oxidant pair, by using the normalization proposed by Singh et al. [38]:

$$\% \text{ Protection} = 1 - [(D - C)/OP] \times 100 \quad (3)$$

where D is the amount of TBARS in the presence of the drug, C is the basal lipid peroxidation (negative control) and OP is the amount of TBARS in the presence of the oxidant pair.

Measurement of intracellular reactive oxygen and nitrogen species

Levels of intracellular ROS were measured by flow cytometry as the fluorescence of DCF, which is the oxidation product of $DCFH_2$-DA that is an ester that freely permeates the cells membranes. After entering the cells, $DCFH_2$-DA loses its diacetate group (becoming $DCFH_2$), by esterase action and can then be oxidized to highly fluorescent DCF. ROS and RNS like H_2O_2, NO and its reaction product with $O_2^-\cdot$, which is the highly reactive peroxynitrite ($ONOO^-$), can oxidize $DCFH_2$ [39,40].

PC12 cells were incubated with 10 μM $DCFH_2$-DA for 45 min, in the dark and at 37°C, after being subjected to the oxidative insult (2 mM ascorbate/100 μM Fe^{2+}) for 1 h or with the NO donor Sodium Nitroprusside (SNP) for 20 h. After two washes with PBS, cells were scrapped and collected for flow cytometry analysis in an Epics® XL-MCL™ (Beckman Coulter) flow cytometer equipped with an argon-ion laser emitting a 488-nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass. At least 20 000 cells were analysed per sample at a low flow rate. Data were analysed by WinMDI 2.8 software.

Nitrite assay

Accumulation of extracellular nitrites (NO_2^-), an indicator of NO synthase activity, was measured in the culture medium by the Griess reaction. This assay relies on a simple colourimetric reaction between nitrite and Griess reagent (0.1% N-(naphthyl)ethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid) to produce a pink azo product [41,42]. Concentrations of nitrite, the end-product of NO production, were quantified 20 h after addition of the compounds and/or SNP to the cells. The Griess reagent (100 μl) was then added to 100 μl of a sample of the cell culture medium, mixed and incubated for 20 min at room temperature. The optical density (OD) was measured at 540 nm in a microplate reader (SpectraMax 340PC). Nitrites concentrations were determined from a calibration curve of the absorbances obtained for a set of standards of sodium nitrite prepared in culture medium.

Determination of GSH/GSSG content

The glutathione (GSH) content of PC12 cells, incubated for 2.5 h in the presence of 1 mM *tert*-butylhydroperoxide (*t*-BHP), was assessed by the DTNB-GSSG reductase recycling assay, based in the methodology of Anderson [43], with some slight modifications. Briefly, 360 μl of culture medium was added to 40 μl of 50% (w/v) 5-sulphosalicylic acid (SSA) for protein precipitation and centrifuged for 1 min at 10 000 *g*. These samples allowed the quantification of GSH and oxidized glutathione (GSSG) in the extracellular medium. The cells that remained attached to the wells were scrapped in PBS and 360 μl of this cell suspension were added to 40 μl of 50% (w/v) SSA and then centrifuged. This procedure allowed for quantification of intracellular GSH and GSSG. Total glutathione levels were measured in the supernatants, by following the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) oxidation, at 415 nm, and comparing it to a GSH standard curve. For GSSG measurement, 100 μl of supernatant was derivatized with 2 μl of 2-vinylpyridine and 10 μl of 50% (v/v) triethanolamine and continuously mixed for 1 h. GSSG levels were then quantified in the same way as previously described for total glutathione, but using a GSSG standard curve instead. GSH content was calculated by subtracting GSSG content from the total glutathione level.

Determination of antioxidant enzymes activity

The activity of antioxidant enzymes was measured after a pre-incubation period of 3h, with the

compounds. PC12 cells were then subjected to 1 mM *t*-BHP for 2.5 h more. After that, cells were washed and suspended in ice-cold PBS (pH 7.4) and, after a short-pulse sonication to promote cell lysis, centrifuged at 13 000 *g* for 1 min. All enzyme activities were measured in the supernatants. Determination of glutathione peroxidase (GSH-Px) activity is based on the oxidation of reduced glutathione by GSH-Px, using *t*-BHP as a substrate, coupled to the disappearance of NADPH by glutathione reductase [44,45]. This reaction was monitored by following the decrease in absorbance at 340 nm, on a microplate reader (SpectraMax 340PC). One milli-unit (mU) of GSH-Px was defined as the amount of enzyme that catalyses the oxidation of 1 nmol of NADPH per minute. Superoxide dismutase (SOD) activity was determined spectrophotometrically at 546 nm by following the reduction of NitroBlue Tetrazolium (NBT) by the xanthine/xanthine oxidase system [46,47]. One unit (U) of SOD was defined as the amount of enzyme needed to inhibit the reduction rate of NBT in 50%. This assay measures the activities of the three most discussed types of SOD, namely Cu/Zn-SOD, Mn-SOD and Fe-SOD. All the enzymes activities were expressed relatively to the amount of protein in the cell extracts.

Total protein quantification

Protein content was measured with the Bradford Reagent purchased from Sigma (St. Louis, Missouri) using a bovine serum albumin standard.

Statistical analysis

Data are expressed as the mean $\pm$ SEM of the indicated number of experiments. The significance of the differences between the means observed was evaluated using the unpaired two-tailed Student's *t*-test. A difference of $p \leq 0.05$ was considered significant.

Results

Determination of the radical scavenging effect

It is well established that the antioxidant activity of a drug depends on its ability to scavenge free radicals [48]. The DPPH discolouration assay, a method frequently used to evaluate the radical scavenging activity of the compounds by themselves [49,50], was used as a first approach. The first step was to determine the stabilization time for the discolouration of a DPPH ethanolic solution, as previously reported [25]. We considered 40 min as a relative steady state discolouration time for all the compounds. With the absorbance results obtained at this timepoint we calculated the percentages of inhibition of discolouration for each drug concentration. For the anti-radical efficiency calculations we considered two parameters: the IC_{50} obtained for each drug (directly taken from the dose-response curves) and the time required by each compound to reach the steady state of DPPH discolouration at this IC_{50} concentration. This time of stabilization at the IC_{50} concentration was found to be 15 and 35 min for FMA762 and FMA796, respectively. Results in Table I show that FMA762 and FMA796 have higher anti-radical efficiency values than the antioxidant trolox, used as a reference, and than FMA4 and FMA7 [25]. This is the first indication of their improved ability to scavenge free radicals.

The scavenging effect of the compounds on hydroxyl radicals was assayed by measuring the effect on the 2-deoxy-D-ribose degradation produced by the reaction of Fe^{3+} with ascorbic acid, in the presence of EDTA. Table I shows that FMA762 and FMA796, at their IC_{50} concentrations, attenuated the iron plus ascorbic acid mediated deoxyribose degradation by

Table I. Free radical scavenging parameters of the different compounds tested. Different concentrations of each drug were added to the ethanolic solution of DPPH and the discolouration measured spectrophotometrically, at 517 nm, after 40 min. Results were expressed as the percentage of DPPH discolouration towards a positive control containing only the DPPH solution. The anti-radical efficiency was calculated as described in the Materials and methods section. Degradation of 2-deoxy-D-ribose was assessed by adding the compounds, at the IC_{50} concentrations, to a reaction mixture as described in the Materials and methods section. Results were expressed as the percentage, relative to the control (containing DMSO instead of the compound), of 2-deoxy-D-ribose degradation occurring in the presence of Fe^{3+} and ascorbic acid.

	DPPH discolouration assay			
Compounds	IC_{50} (μM)	T_{IC50} (min)	Antiradical efficiency ($\times 10^{-3}$)	2-Deoxy-D-ribose degradation (%)
Control	–	–	–	100.0 $\pm$ 4.5
FMA762	3.7 $\pm$ 0.7	15	18.02	37.9 $\pm$ 2.3***
FMA796	3.4 $\pm$ 0.3	35	8.40	38.7 $\pm$ 2.1***
FMA4[a]	19.8 $\pm$ 0.1	40	1.26	–
FMA7[a]	20.4 $\pm$ 0.2	40	1.23	–
Trolox	9.0 $\pm$ 0.2	20	5.56	76.6 $\pm$ 2.6**

Data represent mean $\pm$ SEM for at least three different experiments. ** $p \leq 0.01$, *** $p \leq 0.001$, compared to control. [a] FMA4 and FMA7 results in [25].

62.1 ± 2.3 and 61.3 ± 2.1%, respectively. This attenuation was higher than the one observed for trolox (23.4 ± 2.6%), indicating the compounds greater ability to scavenge hydroxyl radicals when compared with the traditional antioxidant, trolox. These results indicate that FMA762 and FMA796 are good scavengers of hydroxyl radicals and validate the ones obtained for the DPPH discolouration assay.

Determination of intracellular reactive oxygen species

An effective antioxidant should be able to scavenge intracellular reactive oxygen/nitrogen species (ROS/RNS) in order to stop radical chain reactions or to inhibit the reactive oxidants from being formed in the first place [4,51].

The effects of the new compounds on intracellular ROS content were assessed with the fluorescent probe DCF, by flow cytometry analysis. The pair ascorbate/Fe^{2+}, which has been described as adequate to induce oxidative stress in neuronal models such as retinal cells [24,52] and was also used in our previous studies [25], was used as a free radical generator. In this case, the ferrous iron (Fe^{2+}) outside the cells is easily oxidized to ferric iron (Fe^{3+}). Ascorbate acts as a pro-oxidant agent, reducing Fe^{3+} and originating Fe^{2+}. The interaction of this Fe^{2+} with the hydrogen peroxide inside the cells originates hydroxyl radicals through the Fenton reaction, which are susceptible to induce the oxidative stress cascade of events [30,53].

Our data in Figure 2 shows the increase in fluorescence originated by the reaction of DCF with

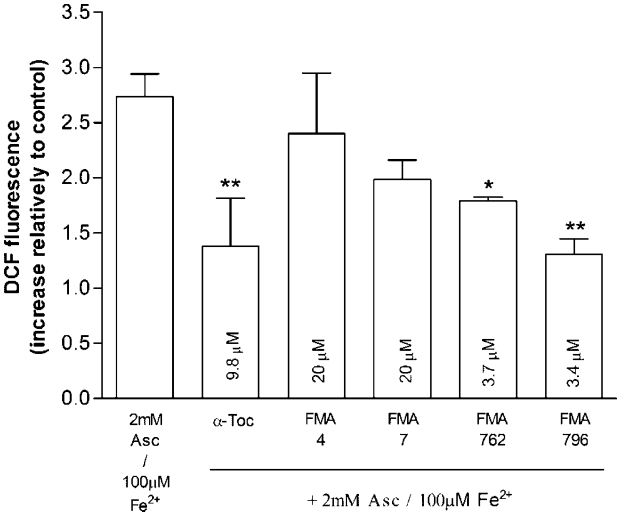

Figure 2. Inhibitory effect of the selected compounds on intracellular ROS formation. Compounds were added to the cells 3 h prior to the incubation with the oxidant pair 2 mM ascorbate/100 µM Fe^{2+} for 1 h. ROS formation was assayed by flow cytometry after 45 min incubation with 10 µM DCFH-DA. α-Tocopherol (α-Toc) was used as a positive control. Results were expressed as the increase in fluorescence relatively to control. For each bar is represented the mean ± SEM for at least three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, compared to oxidant pair.

ROS. In the presence of the oxidant pair 2 mM ascorbate/100 µM Fe^{2+} the cells display an increase in fluorescence (2.73 ± 0.21, $p \leq 0.001$) relative to the control (cells alone). Pre-treatment with FMA762 and FMA796 for 3 h resulted in a statistically significant reduction in fluorescence intensity, when compared to the cells treated with the oxidant pair. This decrease in intracellular ROS formation was similar to the one observed for α-tocopherol, the liposoluble form of vitamin E, used as a positive control due to its known ROS scavenging properties [27]. FMA7 and FMA4 were not able to reduce intracellular ROS levels induced by the oxidant pair.

Thus, we can say that among the new compounds tested, FMA762 and FMA796 are the most efficient ones, showing a high protective effect on ascorbate/iron-induced intracellular ROS formation.

Effect of the compounds on ascorbate/iron-induced lipid peroxidation

It is known that metal-induced generation of oxygen radicals results in the attack of different cellular components, including polyunsaturated fatty acid residues of phospholipids which are, because of their multiple double bonds, excellent targets for free radical attacks [3,6].

The extent of protection on lipid peroxidation presented by the compounds in study is shown in Figure 3. The new compounds were tested at their IC_{50} concentrations and at a concentration 5-fold their IC_{50}. For the lower concentrations, the protective effect was of ~ 40–50%. Furthermore, for the higher ones (17 µM and 18.5 µM), FMA762 and FMA796 protection (64.9 ± 5.7% and 50.5 ± 2.0%, respectively) is higher than the one of trolox (37.5 ± 3.1%) and, in the case of FMA762, is even similar to the one of α-tocopherol (68.3 ± 4.7%).

In comparison to our results previously obtained with FMA4 and FMA7 [25], FMA762 and FMA796, which differ mainly by the presence of three hydroxyl groups within their phenol ring, show an improved ability to protect cells from the ascorbate/iron-induced lipid peroxidation, which is in agreement with their greater capacity to prevent intracellular ROS formation (Figure 2).

Measurement of the compounds liposolubility

The partition coefficient is a physico-chemical property of a compound that can be linked to its biological behaviour [54,55]. Therefore, the degree of hydrophobicity of the new compounds was measured by determining this parameter in an *n*-octanol/HEPES system. Partition coefficients for FMA762 and FMA796 were 1.30 ± 0.02 and 1.91 ± 0.12, respectively. These results are indicative of the relative liposolubility of the new compounds in study, which is intermediate between the hydrosoluble form of

vitamin E, trolox and the liposoluble one, α-tocopherol [25]. This fact could explain the intermediate protection (between these two forms of vitamin E) previously observed on lipid peroxidation (Figure 3).

Among the tested compounds, FMA796 presents a higher affinity for the octanol phase (highest PC value). This is even higher than the PCs previously obtained for FMA4 and FMA7 [25], which again confirms its increased ability to act intracellularly.

Effect of the compounds on SNP-induced toxicity

Nitric oxide production is commonly associated with degenerative conditions such as Alzheimer's and Parkinson's diseases [41,56]. Therefore, an antioxidant compound would be of greater value if it could also decrease the levels of nitrosative stress.

In this context, Sodium Nitroprusside (SNP) was used as a NO generator inside the cells. However, it has been previously reported that toxicity induced by SNP is associated with a decrease in cell viability, as a consequence of the stimulation of reactive oxygen and nitrogen species related to the release of NO and subsequent generation of even more reactive molecules, like peroxynitrite [7,57].

Indeed, treatment of PC12 cells with 400 μM SNP resulted in a decrease in cell viability of 51.5% (MTT assay), as it is shown in Figure 4A. Both FMA762 and FMA796 led to a significant increase in cell survival, in the presence of SNP, revealing a good ability to attenuate the NO-induced cytotoxicity. Their beneficial effect was even better than the one observed for the vitamin E hydrosoluble analogue, trolox.

As expected, the DCF assay indicated that 400 μM SNP caused a significant increase in ROS/RNS accumulation (2.4 ± 0.2 above control values, $p \leq 0.001$) 20 h after its addition to the culture medium (Figure 4B). SNP-induced ROS/RNS production was significantly attenuated by FMA762 (1.7 ± 0.2, $p \leq 0.05$) and FMA796 (1.6 ± 0.3, $p \leq 0.05$). On the other hand, both FMA4 and FMA7 were unable to attenuate the effect of SNP on ROS/RNS intracellular accumulation. The inhibitory effects of the former against toxic events initiated by SNP were shared by 45 μM trolox (used as control), which is in accordance with other published papers [58]. So, FMA762 and FMA796 seem to be effective also as RNS scavengers.

The fact that these new compounds are nitrogenated has raised a question regarding whether these structures *per se* could, directly or indirectly, lead to nitric oxide production inside the cells, which could somehow influence the results.

As previously reported [57], an exposure to SNP leads to an increase in nitrites in the culture medium. For this reason, the increase in nitrites concentration induced in the presence or absence of SNP was

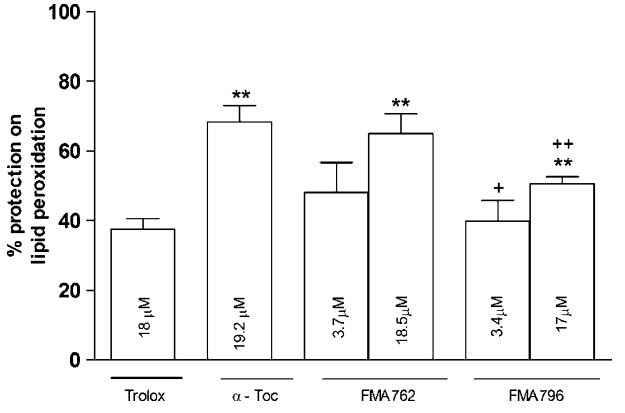

Figure 3. Relative drug protection on lipid peroxidation, in PC12 cell model. The oxidant pair concentration used was 2 mM ascorbate/100 μM Fe^{2+}. Cells, at a density of 1.6×10^5 cells/cm^2, were pre-incubated with the drugs for 3 h, prior to the addition of the oxidant pair, which was left for 1 h more. Each column represents the mean ± SEM, considering the results obtained for at least three different experiments. **$p \leq 0.01$, compared to trolox. $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, compared to α-tocopherol.

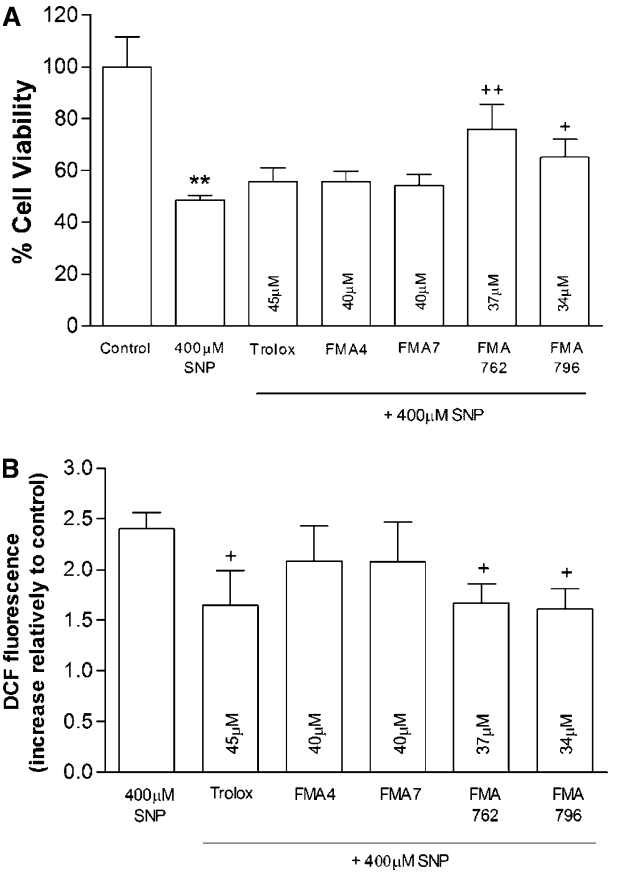

Figure 4. Effects of the tested compounds on SNP-induced toxicity and intracellular ROS/RNS formation. Cells, at a density of 1.6×10^5 cells/cm^2, were incubated in the presence of 400 μM SNP together with the compounds for 20 h. (A) Cell viability assessed by the MTT assay. (B) Intracellular ROS/RNS formation, assessed by flow cytometry, using the fluorescent probe DCF. For each bar is represented the mean ± SEM for at least three independent experiments. ** $p \leq 0.01$, compared to control cells; $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, compared to 400 μM SNP.

evaluated for each experimental condition. So, first we determined if the compounds led to an increase in the accumulation of nitrites, which are the end-products of NO production. SNP was used as a positive control, leading to an increase from 0.7 to 21.6 μM in nitrites concentration, as shown in Figure 5A. Results in this figure clearly show that the new compounds in study do not act as substrates for nitric oxide synthase and do not contribute, *per se*, to the increase of intracellular nitrite levels. Moreover, there are no differences in nitrites production between them.

Additionally, none of the compounds tested decreased the SNP-induced accumulation of nitrites, indicating that they were unable to modulate the activity of nitric oxide synthase elicited by SNP (Figure 5B).

Effects of the nitrogen compounds on cellular total glutathione levels and on the activities of GSH-Px and SOD

Besides the effect of the compounds as ROS/RNS scavenging agents we wanted to investigate their role on enzymatic and non-enzymatic intracellular defences against oxidative stress.

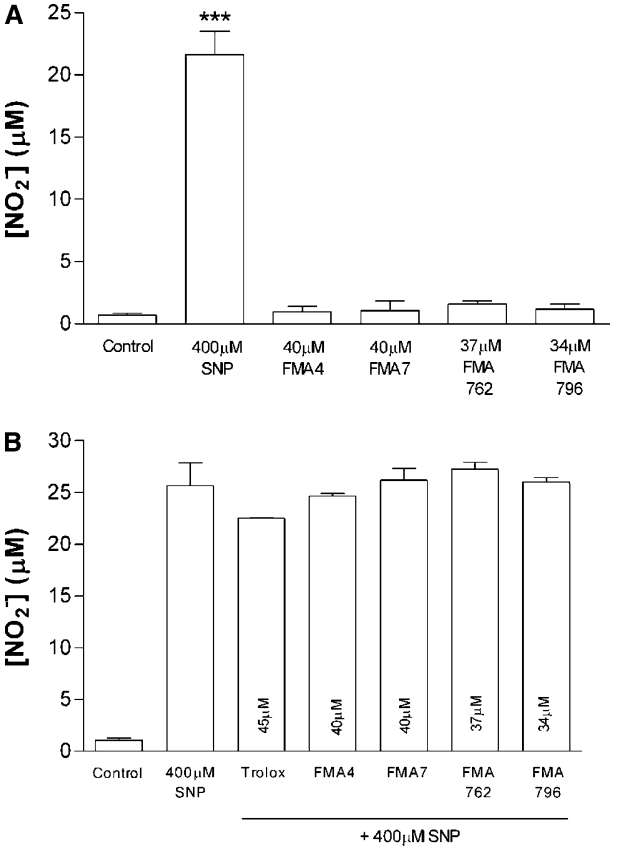

Figure 5. Effects of the compounds on nitrite accumulation in the culture medium. Cells were incubated in the presence of 400 μM SNP and/or the compounds for 20 h. (A) Effect of the compounds alone on nitrite levels. In this case, 400 μM SNP was used as a positive control. (B) Effects of the compounds on nitric oxide synthase activity induced by SNP. For each bar is represented the mean ± SEM for at least three independent experiments. *** $p \leq 0.001$, compared to control cells.

So, as an indicator of the intracellular non-enzymatic antioxidant defences, the GSH content was measured in cells treated with 1 mM *t*-BHP for 2.5 h. The thiol-oxidizing agent, *t*-BHP, has been widely used as an inducer of oxidative stress [59], being its use reported to study changes in the levels of antioxidant defences [60,61].

Our results show that in the presence of *t*-BHP there was a decrease in cytoplasmic GSH content (Table II). A 3 h pre-incubation with the compounds prior to the addition of *t*-BHP was not able to revert this *t*-BHP-induced decrease in GSH content, since no significant changes were observed between the compounds and *t*-BHP. There were neither significant changes in oxidized glutathione (GSSG) content (Table II) produced by pre-incubation of the cells with the nitrogen compounds.

In order to study the effects of the nitrogen compounds on the antioxidant defence enzymes in our model, the activities of GSH-Px and SOD were determined after incubation with 1 mM *t*-BHP for 2.5 h.

Results in Table II show that 1 mM *t*-BHP induced a statistically significant decrease in enzymes activities (~35% and 42% for GSH-Px and SOD, respectively) as expected. However, this decrease was not attenuated by pre-incubating cells with the nitrogen compounds. This indicates that the compounds antioxidant effects are not mainly due to a modulation of the studied natural antioxidant enzymes.

Discussion

In a previous study, a broad range of nitrogen compounds were synthesized and chemically characterized regarding their antioxidant potential [22]. From that study, which also tried to determine the ideal structures an antioxidant should have in order to be effective, two compounds emerged as promising in which concerns their antioxidant potential. The present work intended to continue that study, by focusing on four structurally related new nitrogen compounds with slight structural differences, FMA4, FMA7, FMA762 and FMA796. Our first results with FMA4 and FMA7 had shown an ability to scavenge free radicals [25]. However, modifications made to their structures resulted in more promising compounds in terms of antioxidant activity (FMA762 and FMA796), as the results obtained with chemical approaches like the DPPH discolouration and 2-deoxy-D-ribose degradation assays demonstrate (Table I). Essentially, FMA762 and FMA796 differ from FMA4 and FMA7 by the presence of three hydroxyl groups within the phenol ring, instead of just one, which seems very important since the

Table II. Effect of the compounds on glutathione levels and antioxidant enzymes activities. Cells were pre-incubated in the presence of the compounds for 3 h prior to the addition of 1 mM *t*-BHP for 2.5 h. Glutathione content was determined by the DTNB-GSSG reductase recycling assay. Enzymatic activities were performed as described in the Materials and methods section.

Compound	1 mM *t*-BHP, 2.5 h	GSH (nmol/mg protein)	GSSG (nmol GSH equiv/mg protein)	GSH-Px (mU/mg protein)	SOD (U/mg protein)
–	–	24.3±4.4	0.7±0.4	14.3±0.5	2.4±0.5
–	+	5.8±1.8**	9.0±1.9**	9.3±0.2**	1.4±0.2*
40 μM FMA4	+	6.1±0.7	6.3±0.9	10.2±0.1	1.2±0.2
40 μM FMA7	+	5.9±1.4	9.3±0.6	10.5±1.9	1.4±0.3
37 μM FMA762	+	9.3±2.5	10.5±2.6	10.1±0.7	1.3±0.1
34 μM FMA796	+	7.7±2.2	12.1±2.3	10.5±0.7	1.2±0.1

For each condition is represented the mean±SEM for at least three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, compared to control cells.

substituents present in the molecule, and particularly their positions, contribute to the anti-radical activity differences seen between compounds [62–64]. In addition, the compounds in study differ by the presence or absence of functional groups in the amidine unit which have the intent to make them more liposoluble and thus permeate biological membranes more easily. Previous studies with flavonoids indicated that the more hydroxyl substitutions in a molecule, the stronger the antioxidant activities observed [65]. Therefore, the presence of three hydroxyl groups in FMA762 and FMA796 is expected to improve their free radical scavenging ability, when compared to FMA4 and FMA7 (see Figure 1).

The extent of oxidative stress induced by ascorbate/iron (II) was determined by following the changes in DCF fluorescence, which is a measure of intracellular ROS formation. As the oxidant stimulus, we used the pair ascorbate/Fe^{2+}, which has been shown to significantly increase the formation of intracellular free radicals in PC12 [66] and retinal [24] cells. It is described that iron is present in very high concentrations in different regions of the brain and that an increase in free iron concentration occurs in many neuropathological situations, like Parkinson's and Alzheimer's diseases, as a result of a disruption of iron homeostasis [35,67,68]. Furthermore, free iron can be reduced by ascorbate that exists at a high (millimolar) concentration in the nervous tissue [69,70], generating hydroxyl radicals by the Fenton reaction. This makes the pair adequate to our cell model. As expected from the results regarding their higher ability to scavenge the DPPH radical and to inhibit 2-deoxy-D-ribose degradation, FMA762 and FMA796, as opposite to FMA4 and FMA7, were effective in decreasing ascorbate/iron-induced intracellular ROS formation, at their IC_{50} concentrations (previously determined by the DPPH discolouration method). This decrease was similar to the one observed for α-tocopherol, commonly used as an antioxidant. These results are of great relevance since the pair ascorbate/iron, used in this study as the free radical generator, as previously stated, is known to induce an excessive production of free radicals in several pathological conditions where it is involved in an alteration of the intracellular iron homeostasis.

There seems to be a relationship between the compounds structure, their anti-radical activity and their ability to protect cells from intracellular ROS formation. When compared with FMA4 and FMA7, which have only one hydroxyl group in their structures, the presence of three hydroxyl groups in the phenol ring of FMA762 and FMA796 provides them a higher ability to scavenge free radicals, as determined by the chemical approaches (DPPH discolouration and 2-deoxy-D-ribose degradation assays). This was also observed in our biological model, by assessing their greater ability to prevent the formation of ROS inside PC12 cells. The protection profile of the compounds in study against increased ROS formation induced by the oxidant pair ascorbate/iron seems to correlate with the protective effect observed against lipid peroxidation.

FMA762 and FMA796 also presented an ameliorated ability, in comparison to FMA4 and FMA7, to prevent lipid peroxidation induced by ascorbate/iron. The amount of protection observed for FMA762 and FMA796 was higher when compared to trolox, the hydrosoluble form of vitamin E, and FMA762 was similar to the one of α-tocopherol. It should be noted that α-tocopherol is the most active form of vitamin E in humans and has been considered the major membrane bound antioxidant employed by the cell, whose main function is to protect cells against lipid peroxidation [6]. Therefore, the ability of the compounds to match α-tocopherol's protection on lipid peroxidation, as well as to prevent the intracellular ROS formation, could be indicative of a superior antioxidant potential of the compounds in study.

Since lipid peroxidation occurs mainly due to the free radicals formed intracellularly it is important that the antioxidant compounds have the ability to cross the lipid bilayer and prevent those radicals from inducing the peroxidation of membrane lipids [71]. The partition coefficients obtained for the new compounds are indicative of their relative liposolubility, which enables them to permeate cells, further contributing to their intracellular action. Their lipophilic profiles were

similar to some common natural antioxidant compounds [24,72]. It seems difficult to establish a correlation between the nitrogen compounds structures and their liposolubility profiles, since the presence of more polar groups (e.g. hydroxyl) does not, by itself, contribute to a lower liposolubility. For example, FMA796, despite presenting an added number of hydroxyl groups, shows an increased liposolubility when compared to FMA7 (see Figure 1). However, the hydroxyl groups in the phenolic unit of FMA796 are engaged in a highly favourable intramolecular H-bonding. Consequently, the interaction with water molecules (HEPES solution) will be reduced and the solubility in the non-polar solvent *n*-octanol is favoured, compared to what is observed for compound FMA7, where the hydroxyl group interacts exclusively with the water molecules. Nevertheless, the liposolubility results can at least partially explain the good performance of the compounds in scavenging ascorbate/iron-induced intracellular ROS and in preventing lipid peroxidation. In fact, all the compounds, which presented higher PCs than trolox (0.49 ± 0.02) and lower than α-tocopherol (6.76 ± 0.39) [25], were more effective on the prevention of lipid peroxidation than the water-soluble analogue of vitamin E, but were not able to surpass the protection offered by its lipophilic form (α-tocopherol). The liposoluble form of vitamin E, α-tocopherol, in contrast to the more hydrosoluble one, trolox, is known to bind the cells plasma membrane, where it exerts its main function of protecting cells against lipid peroxidation [6]. Therefore, the ability of the compounds to match α-tocopherol's protection on lipid peroxidation, associated to their action on ROS scavenging at the intracellular level (Figure 2), makes them promising antioxidants with a broader action than α-tocopherol and suggests their ability to act intracellularly.

Besides ROS, reactive nitrogen species (RNS), like nitric oxide (NO) and peroxinitrite, can also exert harmful effects to cells [6,73]. NO is a short-lived radical that is generated during the conversion of L-arginine to citrulline by nitric oxide synthase. In the first step of the reaction, arginine is hydroxylated to *N*-hydroxy-arginine, which is then oxidized to citrulline and NO [74,75]. Although at physiological concentrations NO acts as an important second messenger, when present at concentrations higher than physiological ones, NO can initiate a toxic cascade that leads to cell death [76].

Among the nitrogen compounds herein studied, FMA762 and FMA796 showed the highest protective effect against SNP-induced toxicity as a result of NO generation (Figure 4), as they significantly reversed the decrease in cell viability induced by SNP (assessed by the MTT assay) and also significantly diminished the increase in intracellular RNS formation.

Since the new compounds are nitrogenated, a question arose regarding whether these structures *per se* could, directly or indirectly, lead to nitric oxide production inside the cells. In fact, with the exception of FMA762, all of them possess an amidine group in their structure, similar to the one of L-arginine, which could be oxidized inside the cells, originating nitric oxide. Depending on the concentration produced, this could be either harmful or beneficial to cells [6].

However, it was observed that in the presence of the NO donor none of the compounds was able to reduce the extracellular nitrite accumulation (see Figure 5). This is in accordance with results obtained for common natural compounds with antioxidant activity, which were also reported to exert no effect on NOS [57,58]. As can be observed in Figure 5, the concentration of nitrites did not increase in the presence of the compounds alone, indicating that they do not act as substrates for NO synthase and thus do not lead, by themselves, to an increased production of NO inside the cells. Altogether, these findings indicate that the protective effects of these compounds on nitrosative stress are not likely associated to the inhibition of nitric oxide synthase, but with their ability to scavenge reactive nitrogen species.

In addition to directly scavenge free radicals (ROS and RNS), another way by which an antioxidant can act is through the induction of expression of cells natural antioxidant defences [4]. It is well known that cells are well equipped with defence mechanisms against oxidative stress-induced cell damage [77]. These cellular antioxidant systems can be divided into two major groups: enzymatic (comprising SOD, catalase and GSH-related enzymes) and non-enzymatic (which include the low molecular-weight molecule GSH) [3,9]. Glutathione (GSH) is the major intracellular low-molecular-weight thiol that plays a critical role in the non-enzymatic cellular defence against oxidative stress in mammalian cells [78,79] and plays an important role in protecting brain tissue from oxidative stress [80,81]. Despite the cells ability to deal with mild oxidative stress through an upregulation of the antioxidant defence mechanisms, cell injury may occur when adaptation is not adequate for the build up of oxidation products, leading to oxidative damage to all types of biomolecules, including proteins [9]. In our case, the cells incubation with 1 mM *t*-BHP for 150 min caused a decrease in cell integrity of $\sim 50\%$ (data not shown), which may have induced the inactivation of the natural antioxidant enzymes and thus lead to a decrease in their activity. Such results have also been observed in o ther published papers using the same cell model [78,81–83].

We tested the effects of the new compounds on these antioxidant defence systems. No statistically

significant protective effect could be observed against *t*-BHP-induced decrease in GSH-Px and SOD activities. In the same way, no beneficial effect was observed on the decrease in intracellular GSH. Overall, these results indicate that the compounds protective effect against oxidative stress is not due to an involvement on the natural antioxidant defence systems but, again, rather to a direct scavenging of free radicals.

In conclusion, we have shown that the new nitrogenated compounds herein studied are capable of rescuing PC12 cells against oxidative and nitrosative stress. These effects are likely mediated by their ability to scavenge free radicals, as they proved to be quite effective in reducing lipid peroxidation, intracellular ROS and RNS formation in association with their ability to scavenge the DPPH radical and inhibit 2-deoxy-D-ribose degradation and do not appear to be involved neither in the induction of intracellular antioxidant enzymes such as GSH-Px and SOD nor in the inhibition of NOS. Nevertheless, our results suggest a real interest of these compounds as potent free radical scavengers and so with a possible pharmacological application against pathological situations in which oxidative/nitrosative stress is involved.

Further studies on basic structure modifications regarding other biological targets are under current investigation.

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