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New hydroxypyridinone iron-chelators as potential anti-neurodegenerative drugs

Daniela Arduino[1], Daniel Silva[2], Sandra M. Cardoso,[1,3] Silvia Chaves[2] Catarina R. Oliveira[1,4], M. Amelia Santos[2]

[1]Centro de Neurociencias e Biologia Celular, Universidade de Coimbra, 3030 Coimbra, Portugal, [2]Centro Quimica Estrutural, Instituto Superior Tecnico-UTL, Av. Rovisco Pais, 1049-001 Lisboa, Portugal, [3]Instituto de Biologia, Faculdade de Medicina, Universidade de Coimbra, 3030 Coimbra, Portugal, [4]Instituto de Bioquimica, Faculdade de Medicina, Universidade de Coimbra, 3030 Coimbra, Portugal

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1. ABSTRACT

The neuroprotective action of a set of new hydroxypyridinone-based (3,4-HP) compounds (A, B and C), which are iron chelators extra-functionalized with a propargylamino group for potential MAO-B inhibition, was evaluated after cell treatment with MPP^+ (an *in vivo* inducer of parkinsonism) and $Abeta_{1-40}$ and/or $Abeta_{1-42}$ peptides. Our results show that all these compounds improved cell viability in cells treated with MPP^+ and $Abeta_{1-40}$ peptide or $Abeta_{1-42}$ peptide. In order to evaluate the cellular mechanisms underlying the activity of these compounds, we studied their protective role in caspase activation All compounds tested were able to prevent MPP^+ and Brefeldin A induced caspase-2 activation. They also sowed quite effective in the inhibition of caspase-4 and caspase-3 activity, an effector caspase in the apoptotic process. Finally, detection of apoptotic-like cell death after cell exposure to MPP^+ was also performed by TUNEL assay. Our results demonstrated that all tested compounds prevented DNA fragmentation by decreasing TUNEL positive cells. A, B and C were more effective than DFP (a 3,4-HP iron-chelating agent in clinical use) in MPP^+ induced cell death. Therefore, these results evidenced a neuroprotective and antiapoptotic role for the compounds studied.

2. INTRODUCTION

One third of the diseases in Europe correspond to brain disorders and most of them are of neurodegenerative nature, like Alzheimer`s disease (AD) and Parkinson`s disease (PD). AD is the leading cause of dementia in western countries and the most prevalent neurodegenerative disease. The clinical hallmarks of AD are progressive impairment in memory, cognition, orientation to physical surroundings and language. AD is a progressive neurological disease that results in irreversible loss of neurons, particularly in the hippocampus and cortex. Other pathological hallmarks are extracellular senile plaques containing the beta-amyloid peptide (Abeta), and intracellular neurofibrillary tangles (NFT) (1).

Parkinson's disease is the most common neurodegenerative movement disorder affecting more than 1% of the population above 60 years of age and for which there is no effective treatment (2). Clinically, PD is characterized by rigidity, tremor, slowness and balance problems. PD pathology is characterized by selective neuronal loss of dopaminergic neurons in the substantia nigra pars compacta, and formation of Lewy bodies (LB), which are intracytoplasmic inclusions, mainly composed of α-synuclein and other cytoskeletal proteins (3).

The fact that age is a key risk factor in AD and PD sporadic forms provides considerable support for the free radical hypothesis, because effects of free radicals can accumulate over the years (4, 5). Furthermore, a considerable number of oxidative stress markers are found in the brain of AD patients associated with senile plaques and NFT (6, 7). Increased concentrations of copper, iron and zinc are detected in affected brain regions within amyloid plaques (8). Metal ions were shown are able to bind to Abeta and to accelerate its aggregation and enhance metal-catalysed oxidative stress associated with amyloid plaque formation (9). The accumulation of the redox-active metals, iron and copper, may be a major source of ROS, which are in turn responsible for oxidative stress observed in AD.

Moreover, there are several potential sources of oxidative damage in PD and a number of substantial data from *post-mortem* studies support an increased oxidative damage in this disease. A consistent increase in lipid peroxidation and an increase in protein carbonyl groups have been reported in PD substantia nigra (10, 11). In addition, a decrease in catalase and glutathione peroxidase activities was reported, although superoxide dismutase activity in substantia nigra of PD patients was shown to be increased (12, 13) Also this is relevant to the fact that iron levels, which in a non-pathological situation are significantly higher in substantia nigra than in the other brain regions, further increase in substantia nigra of PD patients (13). Nigral dopaminergic neurons are particularly exposed to oxidative stress, since dopamine can easily auto-oxidize into toxic dopamine-quinone species, superoxide radicals and hydrogen peroxide (14). Moreover, dopamine can be metabolized *via* enzymatic deamination by monoamine oxidase (MAO), with the production of hydrogen peroxide (15), leading to oxidative damage and subsequently to neuronal cell death.

Increasing evidences propose that apoptosis may be one of the mechanisms leading to neuronal death in AD and PD disorders. For instance, lymphocytes bearing genetic or sporadic risk factors of AD show an increased susceptibility to apoptotic cell death, suggesting that apoptosis is a common feature shared by both sporadic and familiar forms of AD (16). Apoptotic features have been also shown in AD and PD patients by the demonstration of DNA fragmentation and caspase activation (16-19). Moreover, it has been described that pro-apoptotic genes are up-regulated in substantia nigra of PD patients, and five fold higher caspase-3 positive neurons were found in patients compared to controls (18). These evidences, together with other findings from different groups suggest that, in pathological states of the brain associated with induction of apoptosis, endoplasmic reticulum (ER) dysfunction may be in an upstream process (5). A growing number of reports recognize ER as key player in the sensing and execution of apoptotic signals in familiar and sporadic forms of AD and PD (20-23).

Thus, neuroprotective strategies employing antioxidant, iron-chelating or free radical-scavenging abilities is one approach to protect neurons and to restrict the progression of these disorders. In fact, iron chelators, antioxidants or monoamine oxidase B (MAO-B) inhibitors have been shown to possess neuroprotective activity. MAO-B inhibitors are amongst the drug candidates which are claimed to have protective effects on both vascular and neuronal tissues. There is, thus, an imperative need to search for neuroprotective compounds with novel mechanisms to assess their potential benefit in such disorders.

In the present study, a number of novel bitargeting ligands, based on 3-hydroxy-4-pyridinones (3,4-HP), were developed and investigated in their protective action, as potential anti-neurodegenerative drugs. In particular, three hydroxypyridinone agents A, B and C, which are iron chelators similar to deferriprone (DFP), a chelator clinically used for iron removal form iron-overload patients (24, 25), have been bifunctionalized with an propargylamine group to account for their potential role on the MAO-B inhibition (26, 27). After preparation and characterization of the lipophilic character, we investigated the neuroprotective efficacy of these novel compounds in MPP^+ (an *in vivo* inducer of parkinsonism) and $Abeta_{1-40}$ and/or $Abeta_{1-42}$ induced cell death.

3. MATERIALS AND METHODS

3.1. Materials

Optimum medium and fetal calf serum were purchased from Gibco BRL, Life Technologies (Scotland, UK). 1-methyl-4-phenylpyridinium (MPP^+), dantrolene, Brefeldin A, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the substrate for caspase-2 (*N*-acetyl-Val-Asp-Val-Ala-Asp-*p*-nitroanilide (Ac-VDVAD-*p*NA)) were obtained from Sigma (St. Louis, MO, USA). The synthetic $Abeta_{1-40}$ and $Abeta_{1-42}$ peptides were from Bachem (Bubendorf, Switzerland). The colorimetric substrate for caspase-3 (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA)) was from Calbiochem, Merck KGaA (Darmstadt, Germany) whereas the caspase-4 substrate (*N*-acetyl -Leu-Glu-Val-Asp-*p*-nitroanilide (Ac-LEVD-*p*NA)) was obtained from MBL International Corporation (Woburn, MA, USA). *In situ* cell death detection kit was purchased from Roche Diagnostics GmbH (Penzberg, Germany). Bio - Rad protein dye assay was from Bio-Rad (Hercules, CA, USA). All other bench reagents were of the highest grade of purity and were commercially available.

3.2. Synthesis of the compounds A-C

Analytical grade reagents were purchased from Aldrich, Sigma and Fluka and they were used as supplied. Solvents were dried according to standard methods (28). The chemical reactions were monitored by TLC using alumina plates coated with silica gel 60 F_{254} (Merck). Column flash chromatography separations were performed on silica gel Merck 230-400 mesh ASTM. Melting points were measured with a Leica Galen III hot stage apparatus and are uncorrected. The ^1H NMR spectra were recorded on a Varian Unity 300 spectrometer at 25 ^oC. Chemical shifts are reported in ppm (δ) with tetramethylsilane (TMS) as internal reference, in organic solvents and sodium 3-

(trimethylsilyl)- (2,2,3,3-d4)-propionate (DSS) in D2O solutions. The following abbreviations are used: s = singlet; d = duplet; t = triplet; q = quintuplet; m = multiplet; br = broad. Mass spectra (FAB) were performed in a VG TRIO-2000 GC/MS instrument. The High Resolution Mass Spectra (HRMS) were obtained with a High Resolution Fourier transform ion cyclotron resonance (FTICR) instrument, Finnigan FT/MS 2001-DT, equipped with a 3.0-T superconducting magnet, by electron impact (EI), typically with 15-eV electron beam energies, 5-micro emission currents and 150°C sample temperatures. Elemental analyses were performed on a Fisons EA1108 CHNF/O instrument.

1- (3´- (di (prop-2-ynyl)amino)propyl)-3-hydroxy-2-methyl.pyridin-4 (1H)-one (A). To a solution of 1- (3´-aminopropyl)-3-hydroxy-2-methyl-pyridin-4 (1H)-one (29) (1.50 g; 8.23 mmol) in anhydrous DMF (30 mL), potassium carbonate (2.28 g; 16.5 mmol) was added and the mixture was stirred for *ca.* 15 min. A solution of propargyl bromide (0.71 mL; 8.23 mmol) in anhydrous DMF (15 mL) was added dropwise to the reaction mixture which was under nitrogen atmosphere and at 0ºC. The reaction mixture was stirred for 24 h. After the end of the reaction the solvent was evaporated to dryness and worked up by adding water (70 mL) which was extracted with ethyl acetate (8 × 30 mL). The organic layer was dried with anhydrous sodium sulphate. The solvent was evaporated to dryness and 1- (3- (di (prop-2-ynyl)amino)propyl)-3-hydroxy-2-methylpyridin-4 (1H)-one was recrystallized from ethanol/acetonitrile affording a dark yellow solid (0.75 g) with 35% yield. M.p.: 138-140ºC. ^{1}H-NMR (CD_3OD; δ/ppm**)**: 7.65 (1 H, d, *J* = 7.5 Hz, 6-H pyridinone), 6.39 (1 H, d, *J* = 6.6 Hz, 5-H pyridinone), 4.12 (2 H, t, *J* = 7.5 Hz, CH_2 Pi), 3.45 (4 H, s, CH_2-C≡C), 2.59 (2 H, t, *J* = 7.5 Hz, CH_2NH_2), 2.45 (3 H, s, CH_3), 1.93 (2 H, t, *J* = 7.2 Hz, $CH_2\mathbf{C}\mathbf{H}_2CH_2$). ^{13}C-NMR ($CD_3OD$; δ/ppm)**:** 170.6 (C=O), 139.1 (C-6), 132.9 (C-3), 112.6 (C-5), 112.0 (C-2), 79.2 ($CH_2\boldsymbol{C}$≡CH), 74.8 (CH_2C≡$\boldsymbol{C}$H), 52.8 ($\boldsymbol{C}H_2$N-CH_2C≡CH), 48.1 (N-$\boldsymbol{C}H_2CH_2CH_2$), 42.8 (N-$\boldsymbol{C}H_2$C≡CH), 28.8 ($CH_2\boldsymbol{C}H_2CH_2$), 11.9 ($CH_3$ Pi). FAB-MS (m/z): 297 $(M+K)^+$, 259 $(M+1)^+$, 219 (M- CH_2C≡$\mathbf{C}$H)$^+$. Elemental analysis: calculated for $C_{15}H_{18}N_2O_2$: C, 69.74; H, 7.02; N, 10.84; found: C, 69.76; H, 7.30; N, 11.01%.

3-Benzyloxy-2-methyl-1- (2- (piperazin-1'-yl)ethyl)pyridin-4-one. To a solution of 1- (2-aminoethyl)piperazine (3.83 mL; 29 mmol) in water (40 mL) at pH 13 was added 3-benzyloxy-2-methylpiran-4-one (29) (6,26 g; 29 mmol) dissolved in methanol (40 mL). The mixture was refluxed overnight. The reaction mixture was concentrated to a ¼ of initial volume, the pH adjusted to 4 and the aqueous solution was washed with diethyl ether (3×70 mL). The aqueous layer was basified to pH 8 and extracted with dichloromethane (3×70 mL). The organic layer was dried with anhydrous sodium sulphate. The solvent was evaporated to dryness and the pure compound (2.45 g; 7.5 mmol) was obtained (26% yield). ^{1}H-NMR (D_2O; δ/ppm): 7.71 (1 H, d, *J* = 7.2 Hz, 6-H Pi), 6.56 (1 H, d, *J* = 7.2 Hz, 5-H Pi), 5.06 (2 H, s, CH_2Ph), 4.09 (2 H, t, *J* = 6.9 Hz, CH_2NH_2), 3.21 (4 H, t, *J* =5.1 Hz, 3,4-H piperazine), 2.6 (6 H, m, 2,6-H piperazine + CH_2-N piperazine), 2.05 (3 H, s, CH_3). FAB-MS (m/z): 328 $(M+1)^+$.

3-Hydroxy-2-methyl-1- (2- (piperazin-1-yl)ethyl)pyridin-4-one. 3-benzyloxy-2-methyl-1- (2'-(piperazin-1'-yl)ethyl)-pyridin-4-one (2.0 g; 5.53 mmol) and Pd/C 10% (150 mg) were dissolved in methanol and the solution was stirred at a pressure of 2 bar for 4 h at room temperature. The inorganic material was filtered off, the solvent evaporated to dryness and the remaining solid was recrystallized from ethanol, affording a yellow solid (1.83 g; 4.35 mmol) with a yield of 79%. ^{1}H-NMR (D_2O; δ/ppm): 8.04 (1 H, d, *J* = 7.2 Hz, 6-H Pi), 7.09 (1 H, d, *J* = 7.2 Hz, 5-H Pi), 4.52 (2 H, t, *J* = 6.9 Hz, N-CH_2), 3.34 (4 H, t, *J* =5.1 Hz, 3,5-H piperazine), 3.08 (2 H, t, *J* = 6.9 Hz, CH_2-N piperazine), 2.99 (4 H, t, *J* = 5.1 Hz, 2,6-H piperazine), 2.60 (3 H, s, CH_3). FAB-MS (*m/z*): 238 $(M+1)^+$.

3-Hydroxy-2-methyl-1- (2- (4- (prop-2-inyl)piperazin-1-yl)ethyl)pyridin-4-one (B). To a solution of 3-hydroxy-2-methyl-1- (2- (piperazin-1-yl)ethyl)pyridin-4-one (0,79 g; 3,33 mmol) in anhydrous DMF (40 mL) was added potassium carbonate (0.92 g; 6.67 mmol) and the mixture was stirred for *ca.* 10 min. A solution of propargyl bromide (0.29 mL; 3.33 mmol) in anhydrous DMF (5 mL) was added dropwise to the reaction mixture which was under nitrogen atmosphere at 0ºC. The reaction mixture was stirred for 24 h. After the end of the reaction the solvent was evaporated to dryness and worked up by adding water (50 mL) and extracting with ethyl acetate (4 × 30 mL). The organic layer was dried with anhydrous sodium sulphate. The solvent was evaporated to dryness and the pure compound was recrystallized from ethanol, affording a light beige solid (0.24 g) with 26% yield. M.p.: 213-215ºC. ^{1}H-NMR (D_2O; δ/ppm)**:** 7.68 (1 H, d, *J* = 7.2 Hz, 6-H pyridinone), 6.52 (1 H, d, *J* = 7.2 Hz, 5-H pyridinone), 4.24 (2 H, t, *J* = 7.8 Hz, N-CH_2), 3.29 (2 H, s, CH_2-C≡C), 2.78 (2 H, t, *J* = 7.5 Hz, CH_2-N piperazine ring), 2.68 (8 H, m, H piperazine ring), 2.45 (3 H, s, CH_3). FAB-MS (*m/z*): 314 **(**$M+K)^+$, 276 $(M+H)^+$. HRMS *m/z* calculated for $C_{15}H_{21}N_3O_2$: 275.163377; found: 275.163328.

3-Hydroxy-2-methyl-1- (3-(methylamino)propyl)pyridin-4-one. To a solution of 1- (3-aminopropyl)-3-hydroxy-2-methylpiridin-4-one (2.0 g; 11.0 mmol) in anhydrous ethanol (50 cm^3) was added paraformaldehyde (1.65 g; 5.5 mmol) and sodium cyanoborohydride (0.83 g; 13.2 mmol). The mixture was refluxed overnight. After the end of reaction the solvent was evaporated to dryness. The residue was redissolved in anhydrous methanol and the inorganic material was filtered off. The solvent was evaporated to dryness and the residue was recrystallized from ethanol/acetonitrile affording a light yellow solid (1.05 g; 5.35 mmol) with 49% yield. M.p. 197-200ºC. ^{1}H-NMR (D_2O; δ/ppm): 8.07 (1 H, d, *J* = 6.6 Hz, 6-H Pi), 7.07 (1 H, d, *J* = 6.6 Hz, 5-H Pi), 4.42 (2 H, t, *J* = 6.6 Hz, CH_2N), 3.31 (2 H, t, *J* = 6.6 Hz, CH_2NH_2), 2.91 (3 H, s, $NHCH_3$), 2.57 (3 H, s, CH_3), 2.30 (2 H, t, $CH_2CH_2CH_2$). FAB-MS (*m/z*): 197 $(M+1)^+$.

3-Hydroxy-2-methyl-1- (3-methyl-3- (prop-2'-inylamino))pyridin-4-one (C). To a solution of 3-hydroxy-2-methyl-1- (3´- (methylamino)propyl)piridin-4-one (1.0 g; 5.10 mmol) in anhydrous DMF (50 mL) was added potassium carbonate (1.41 g; 10.2 mmol) and the mixture was stirred for *ca.* 10 min. A solution of propargyl bromide (0.66 mL; 6.12 mmol) in anhydrous acetonitrile (20 mL) was added dropwise to the reaction mixture which was under nitrogen atmosphere and at 0ºC. The reaction mixture was stirred for 80 h. After the end of the reaction the solvent was evaporated to dryness and redissolved in anhydrous methanol to remove any inorganic material by precipitation. The solvent was evaporated to dryness and the compound was recrystallized from methanol/ethyl acetate, affording a highly hygroscopic yellow solid (0.57 g) with 48% yield. ^{1}H-NMR (D_2O; δ/ppm): 8.18 (1 H, d, *J* = 7.2 Hz, 6-H pyridinone), 7.09 (1 H, d, *J* = 7.2 Hz, 5-H pyridinone), 4.42 (2 H, t, *J* = 7.8 Hz, CH_2N pyridinone), 3.67 (2 H, t, *J* = 6.9 Hz, CH_2N), 4.21 (2 H, s, CH_2-C≡C), 3.60 (3 H, s, $\mathbf{CH_2}NHCH_3$), 3.20 (3 H, s, N-$\mathbf{CH_3}$), 2.60 (3 H, s, CH_3), 2.41 (2 H, t, *J* = 7.5 Hz, $CH_2\mathbf{CH_2}CH_2$). ^{13}C-NMR (CD_3OD; □/ppm): 153.0 (C-6), 145.0 (C-3), 144.7 (C-5), 115.2 (C-2), 84.3 ($CH_2\boldsymbol{C}$≡CH), 79.8 (CH_2C≡$\boldsymbol{C}$H), 56.7 ($\boldsymbol{C}H_2N$-CH_2C≡CH), 54.9 (N-$\boldsymbol{C}H_2CH_2CH_2$), 52.3 (N-$\boldsymbol{C}H_2C$≡CH), 25.8 ($CH_2\boldsymbol{C}H_2CH_2$), 15.3 ($CH_3$ Pi). FAB-MS (m/z): 235 $(M+1)^+$. HRMS *m/z* calculated for $C_{13}H_{18}N_2O_2$: 234.1368279; found: 234.136796.

3.3. Partition coefficients

The octanol-water partition coefficients (log *P*) were calculated at 25ºC, based on the concentration ratio of each compound between 1-octanol and a buffered aqueous phase (*Tris*, pH = 7.4). The two phases were previously pre-saturated with respect to each other. The "shakeflask" method was used, as previously described (29, 30), and the species concentrations were evaluated by UV-Vis spectrophotometry, using the hydroxypyridinone benzenoid bands at ca 280-290 nm.

3.4. Cell culture and treatments

Human teratocarcinoma NT2 cells were purchased from Stratagene (La Jolla, CA, USA) and were cultured as described previously (31). Briefly, the cells were grown in Optimen medium with 10% heat inactivated fetal calf serum, containing 50 U/mL penicillin, and 50 µg/mL streptomycin, under a humidified atmosphere of 95% air- 5% CO_2 at 37ºC. Cells were plated at 0.1×10^6 /mL for cell viability assays, 0.5×10^6 /mL for measurement of caspase activity and 0.75×10^6 /mL for *in situ* cell death assay. Medium was changed after 24 h and immediately before treatments. MPP^+ was prepared as 10 mM stocks (in DMSO) immediately before use and added to the medium to 1 mM final concentration. $Abeta_{1-40}$ (5 µM) and $Abeta_{1-42}$ (1 µM) peptides were added from a 231.5 µM and 221.5 µM stock PBS solutions, respectively. Previously, the peptides were dissolved in sterile distilled water at a concentration of 6 mg/mL and diluted to 1 mg/mL (231.5 M or 221.5 µM, respectively) with PBS and then incubated for 5-7 days to induce fibril formation. Brefeldin A (2 µM) was added from 5 mM stock ethanol solution.

The tested compounds (A, B, C, DFP) were dissolved in DMSO at a concentration of 2 mM and aliquots were stored at -20ºC. These compounds were added to the medium to 5 µM final concentration. In parallel experiments and before the addition of stress inducers, cells were also preincubated for 1 h with dantrolene (10 µM), an inhibitor of ryanodine receptors of ER, which was used as a control for neuroprotective action. For combinatorial treatments, both simultaneous and sequential treatment approaches were tested and the selected compounds concentrations shown to exert the maximal protective action and had no toxic effects to cells. The final concentration of ethanol/DMSO in culture media did not exceed 0.05% (v/v) and no alterations on cells were observed. For all conditions tested, control experiments were performed in which the compounds tested or the stress agents was not added.

Cell reduction ability as a surrogate of cell viability was measured by using a quantitative colorimetric assay with MTT according to the method of Mosmann (1983) (32). In brief, cells were incubated with MTT solution (in sodium medium) for 3 h at 37ºC. The medium was then discarded, the stained cells were dissolved with isopropanol/HCl, and thereafter the absorbance at 570 nm was measured using a Spectramax Plus 384 spectrophotometer (Molecular Devices). MTT reduction ability was expressed as a percentage of the control value obtained for untreated cells.

Caspase activation assays were performed by using the method described by Cregan *et al.* (1999) (33), with slight modifications**.** After described treatments**,** cells were washed once in PBS and harvested on ice with a lysis buffer containing 25 mM HEPES, 1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$, and protease inhibitors (0.1 M PMSF, 2 mM DTT, and a 1:1000 dilution of a protease inhibitor cocktail). The cellular suspension was frozen/defrosted three times on liquid nitrogen and sonicated twice for 30 sec. Then, the lysate was centrifuged for 10 min at 20,200 × *g* and at 4ºC. The resulting supernatant was collected and assayed for protein concentration using Bio-Rad protein dye assay reagent. To evaluate caspase activation, cell extracts containing 50 µg or 100 µg of protein were incubated at 37ºC for 2 h in 25 mM HEPES, pH 7.5 containing 0.1% (w/v) CHAPS, 10% (w/v) sucrose, 2 mM DTT with 100 □M Ac-VDVAD-*p*NA, 100 µM Ac-DEVD-*p*NA and 50 µM Ac-LEVD-*p*NA, colorimetric substrates for caspase-2, 3 and 4, respectively. Substrate cleavage was detected at 405 nm using a Spectramax Plus 384 spectrophotometer (Molecular Devices) and the results were expressed as the increase above control value obtained for untreated cells.

Detection of apoptotic cell death after cell exposure to MPP^+ was performed by using an *in situ* cell death detection kit, based on labelling of DNA strand breaks (TUNEL technology), according to the procedure provided by the manufacturer. Briefly, cells in coverslips were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 1 h. Then, cells were washed twice with PBS and immersed in a permeabilisation solution (0.1% Triton X-100 in 0.1%

sodium citrate) for 2 min on ice. After washing twice with PBS, cells were incubated with the nucleotide mixture and terminal deoxynucleotidyl-transferase in 50 µL of equilibration buffer, in a humidified atmosphere at 37°C for 1 h in the dark, to allow that the end labelling reactions occur. The reaction was stopped by washing three times in PBS. Ultimately, the cells were treated with DakoCytomation Fluorescent mounting solution on a microscope slide and analyzed in an Axiovert 200 fluorescence microscope (Zeiss, Germany). The photographs were taken at 40× magnification.

All data were expressed as mean ±S.E.M. of at least three independent experiments performed in duplicates. Statistical analyses were performed using one-way ANOVA followed by Bonferroni Multiple-Comparisons Procedure as post -hoc test. A P - value< 0.05 was considered statistically significant.

4. RESULTS

4.1 Design and Chemistry

The option for the hydroxypyridinone-based derivatives (3,4-HPs) in this study was based on the fact that, besides their recognized excellent iron-chelating properties, other biologically relevant properties can be combined by the easy introduction of a wide variety of functional groups, according to the desired roles of the final molecular entity. 3,4-HPs are heterocyclic *O*,*O*-chelators with high affinity for iron (compare to deferriprone, which is clinically used as iron scavenger (25)); they are very stable, non-toxic and bio-available at any physiological pH conditions; they are easily made and ring-substituted to tune a diversity of physico-chemical or biological properties, including lipo-hydrophilicity and permeability to the BBB and the interaction with specific relevant bio-sites or proteins (34, 35); they can form neutral metal-complexes which can easily efflux from brain (36).

This set of new compounds includes a free 3-hydroxy-4-pyridinone group (3,4-HP) to chelate the iron (III) and at least one propargylamine group to interact and inhibit the MAO-B activity. These two pharmacophore units are separated by spacers of slightly different size and type to account for some differentiation in the lipophilicity and membrane crossing abilities and also for interaction with biological targets. The piperazine segment may also be expected to have some interaction with Cu (II), which is involved in the binding of amyloid plaque precursor proteins. An additional benefit of application of these compounds is that they are cheap and easily made. The synthetic route for the preparation of the new A-C compounds involves always a previous preparation of the corresponding 3-hydroxy-2-methyl-4-pyridinone backbone, possessing an alkylamine spacer as the ring *N*-substituent. This terminal amine group can be either primary or secondary amine group, and it works as the actual point of attachment between the propargylic and the 3,4-HP units. The preparation of the amino derivatives of 3,4-HP follows standard strategies previously described (28, 34). That coupling reaction involves a nucleophilic substitution of propargyl bromide, under anhydrous DMF conditions and in presence of base (potassium carbonate). Noteworthy to mention that for this last reaction, the phenolic group of hydroxyyridinone cannot be protected with benzyl group, because its final deprotection *via* hydrogenolysis would lead back to the disconnection of the propargyl group. In case of compound B, the *O*-benzylation was used to simplify the working up of the products, but the *O*-deprotection was required before the amine propargylation.

The results of the lipophilicity studies (log P, octanol/water) evidenced that A and C are more lipophilic than DFP, a benefit for their bioavailability and BBB requirements as potential drugs, although the lipophilicity charater of B and DFP are quite similar. Therefore, while in A and C the hydrophilic character of the neutral amine group is compensated by the lipophilic *N*-substituent alkyl groups, in case of B one the amine groups of the piperazine unit may be protonated at neutral pH (26-a), thus contributing to some extra-hydrophilic character. Although no solution studies were performed to assess iron-chelating efficacy of this set compounds, it is anticipated that it should be close to the reported for DFP and other bifunctional hydroxypyridonate derivatives (37), because the spacer group is long enough to diminish the effect of different substituent. In case of compound B, the amine groups of the piperazine moiety (boat conformation) have some affinity for Cu (II) and so, besides its high affinity for Fe (III) due to the hydroxypyridinone moiety, some extra-interaction with soft double-charged metal ions can also occur.

4.2. Hydroxypyridinone based compounds prevent cell death in PD and AD cellular models

4.2.1. *In vitro* neuroprotective activity

To evaluate the potential therapeutic action of hydroxypyridinone- based compounds, NT2 cells were treated with MPP^+ and $Abeta_{1-40}$ or $Abeta_{1-42}$ peptides, which constitute good *ex vivo* cellular models for studying neurodegeneration associated to PD and AD disorders. Figure 1 shows a significant decrease in cell viability after a 24 h treatment with 1 mM MPP^+ and 1 µM $Abeta_{1-42}$ peptides. Hydroxypyridinone- based compounds by itself did not affect cell viability at the same end point (data not shown). Furthermore, all these compounds (A, B, C and DFP) improved cell viability in cells treated either with MPP^+, $Abeta_{1-40}$ and $Abeta_{1-42}$ peptides.

4.2.2. Hydroxypyridinone based compounds prevent caspase activation

Apoptosis is mediated by a family of cysteinyl-containing, aspartate - specific proteases (caspases) that, in turn, activated the caspase cascade resulting in cell death (38). Different caspases mediate cell death in response to different apoptotic stimuli. Caspase-2 plays an important function in initiation of ER stress apoptosis and nuclear stress signalling. Moreover, the role of this caspase in neuronal cells has been of particular interest because of its essential role in neuronal death elicited by growth factor deprivation and amyloid-beta toxicity (39). Recent studies show that caspase-4 in humans and caspase-12 in mouse mediate ER stress induced apoptosis (40, 41).

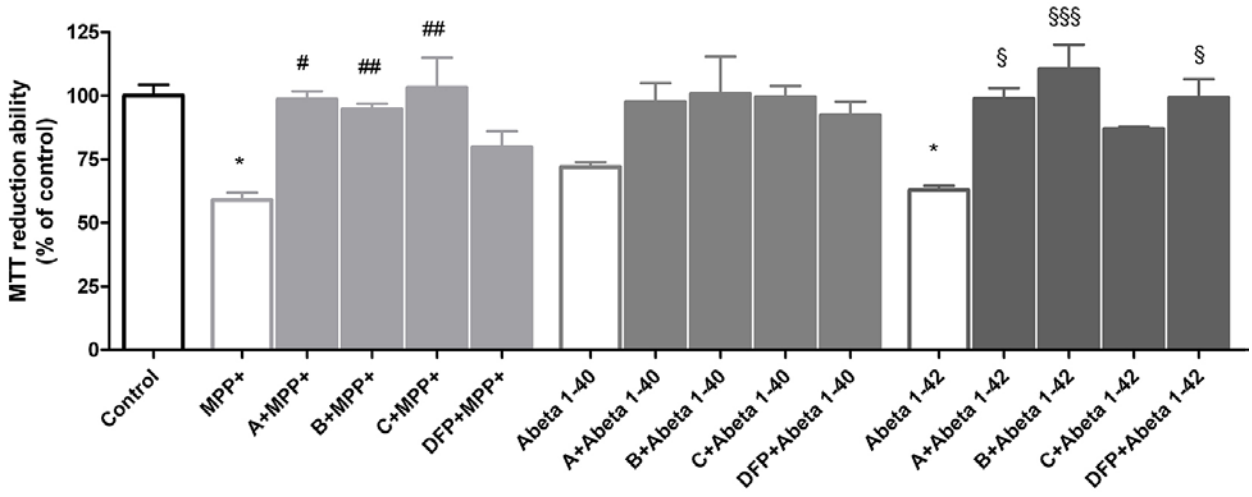


Figure 1. Effect of A, B, C and DFP on MPP^+ and $Abeta_{1-40}$ or $Abeta_{1-42}$ peptides toxicity in NT2 cells. NT2 cells were treated with MPP^+ (1 mM) and $Abeta_{1-40}$ (5 μM) or $Abeta_{1-42}$ (1 μM) peptides, for 24h, in the absence or the presence of either 5 μM of compounds A, B, C or DFP. Evaluation of cell viability was performed by using MTT reduction test. Results are expressed as the percentage of NT2 untreated cells, with the mean ± S.E.M. derived from 6 different experiments. $^*p < 0.05$, significantly different when compared with NT2 untreated cells; $^{§}p < 0,05$; $^{§§}p < 0,01$; $^{§§§}p < 0,001$, significantly different when compared with $Abeta_{1-42}$ treated cells; $^{\#}p < 0,05$; $^{\#\#}p < 0,01$, significantly different when compared with MPP^+ treated cells.

We observed that an increase in VDVAD (*N*-acetyl-Val-Asp-Val-Ala-Asp-*p*-nitroanilide) cleavage (caspase-2 activation) occurred after cells treatment with MPP^+ or Brefeldin A (a classical ER stress agent, acting by ER - Golgi protein trafficking inhibition) for 24 h. Nevertheless, it was also evident that the co-treatment with either of these hydroxypyridinone compounds restrained this increase. So, it means that all these compounds are able to protect cells against MPP^+ and Brefeldin A induced caspase-2 activation. Similar results were observed relatively to caspase-4 activation (Figure 2B). Cell exposure to MPP^+, but not to $Abeta_{1-42}$ peptide, for 24 h, resulted in a significant increase in LEVD cleavage, which was remarkably reduced in combinatorial treatments with A or DFP, most effective compounds in prevention of caspase-4 activation. In previous work from our laboratory, it was demonstrated that MPP^+ and $Abeta_{1-40}$ induced mitochondrial dependent apoptosis *via* mitochondrial cytochrome c release and caspase activation in these cellular models (unpublished work and 42). Additionally, since caspase-3 is an effector caspase in the apoptotic process, and to better characterize the neuroprotection mechanisms mediated by these compounds, we analysed the protective effect of these compounds against ER and mitochondria stressors (Brefeldin A, and MPP^+, respectively) on caspase-3 activation. As shown in Figure 2C, when cells were co-treated with A and C compounds, the caspase-3 activation was decreased.

Since caspases were shown to be activated, we intended to further analyse DNA fragmentation in cells upon MPP^+ exposure and to evaluate the effectiveness of these compounds on rescue cells from MPP^+ mediated cell death. After 24 h exposure to MPP^+, apoptotic cells were observed both by phase contrast and fluorescence microscopy (Figure 3). All tested compounds prevented DNA fragmentation by decreasing TUNEL positive cells, A, B and C being the most successful compounds in prevention of MPP^+ induced cell death.

5. DISCUSSION

The major mechanisms underlying the neurodegenerative processes in Parkinson's and Alzheimer's diseases are not yet known. Nonetheless, many biochemical evidences provided support to a cascade of events, including oxidative stress, iron deposition at the site of neuronal lesion, inflammatory processes, excitotoxicity, and apoptosis. Taking into account that these events involve multifactorial conditions, the development of drugs that could modulate multiple targets simultaneously with eventual synergistic side effects seems the most wised strategy for improving drug efficacy. In fact, the design of compounds as dual target drugs has been object of recent research but mostly based on the task of extra-functionalization of high cost mono-target drugs (43,44). Recently, 8-hydroxyoxyquinoline-bearing derivatives have also been developed to combine the iron-chelating and MAO-B inhibitory properties (45).

In the present study a set of bitargeting ligands, based on 3-hydroxy-4-pyridinones were designed and their protective action, as potential anti-neurodegenerative drugs, was investigated. Firstly, our results demonstrated that all the selected compounds provided substantial protection from cell death induced by MPP^+, $Abeta_{1-40}$ and $Abeta_{1-42}$ treatment of cultured human teratocarcinoma NT2 cells, a cell line that has been shown to exhibit a neuronal phenotype and has several neurochemical markers (46). MPP^+, $Abeta_{1-40}$ and $Abeta_{1-42}$ treatments in NT2 cells constitute a reliable model for screening potential neuroprotective compounds because they mimic some

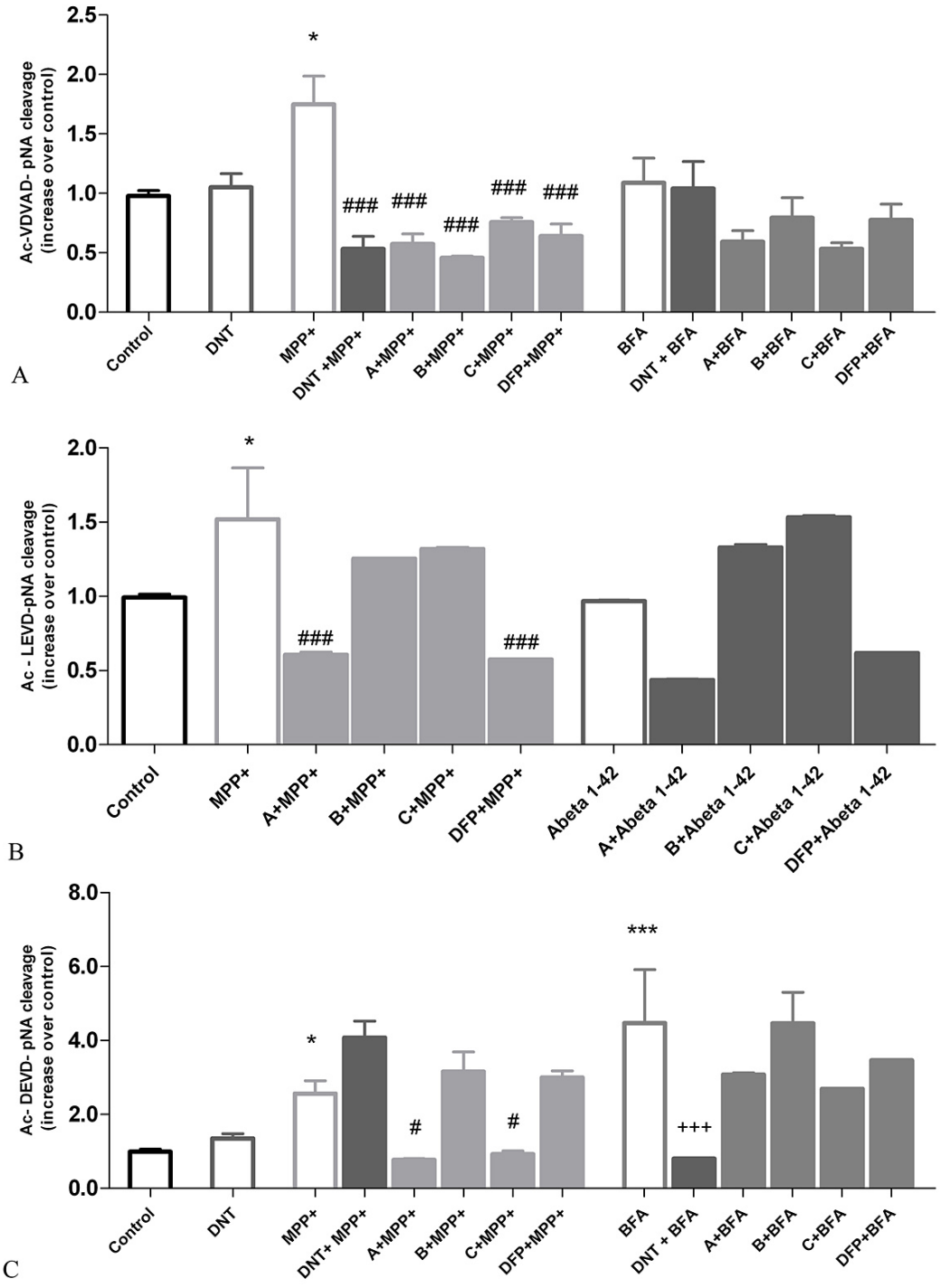


Figure 2. Caspase activation in NT2 cells induced by MPP^+ and $Abeta_{1-42}$ or Brefeldin A (BFA). (A) Effect of compounds A, B, C, DFP and dantrolene (DNT) on caspase 2-like activity (Ac-VDVAD-pNA cleavage) increase; (B) Effect of compounds A, B, C and DFP on caspase 4-like activity (Ac-LEVD-pNA cleavage) increase; (C) Effect of compounds A, B, C, DFP and dantrolene on caspase 3-like activity (Ac-DEVD-pNA cleavage) increase. Caspase activation was evaluated spectrophotometrically at 405 nm as described in Materials and methods. Data are expressed relative to the basal activity observed in the untreated NT2 cells, with the mean ± S.E.M. derived from 4 different experiments. . *$p < 0,05$, significantly different when compared with NT2 untreated cells; $^{\#}p < 0,05$; $^{\#\#\#}p < 0,001$, significantly different when compared with MPP^+ treated cells; $^{+++}p < 0,001$, significantly different when compared with BFA treated cells.

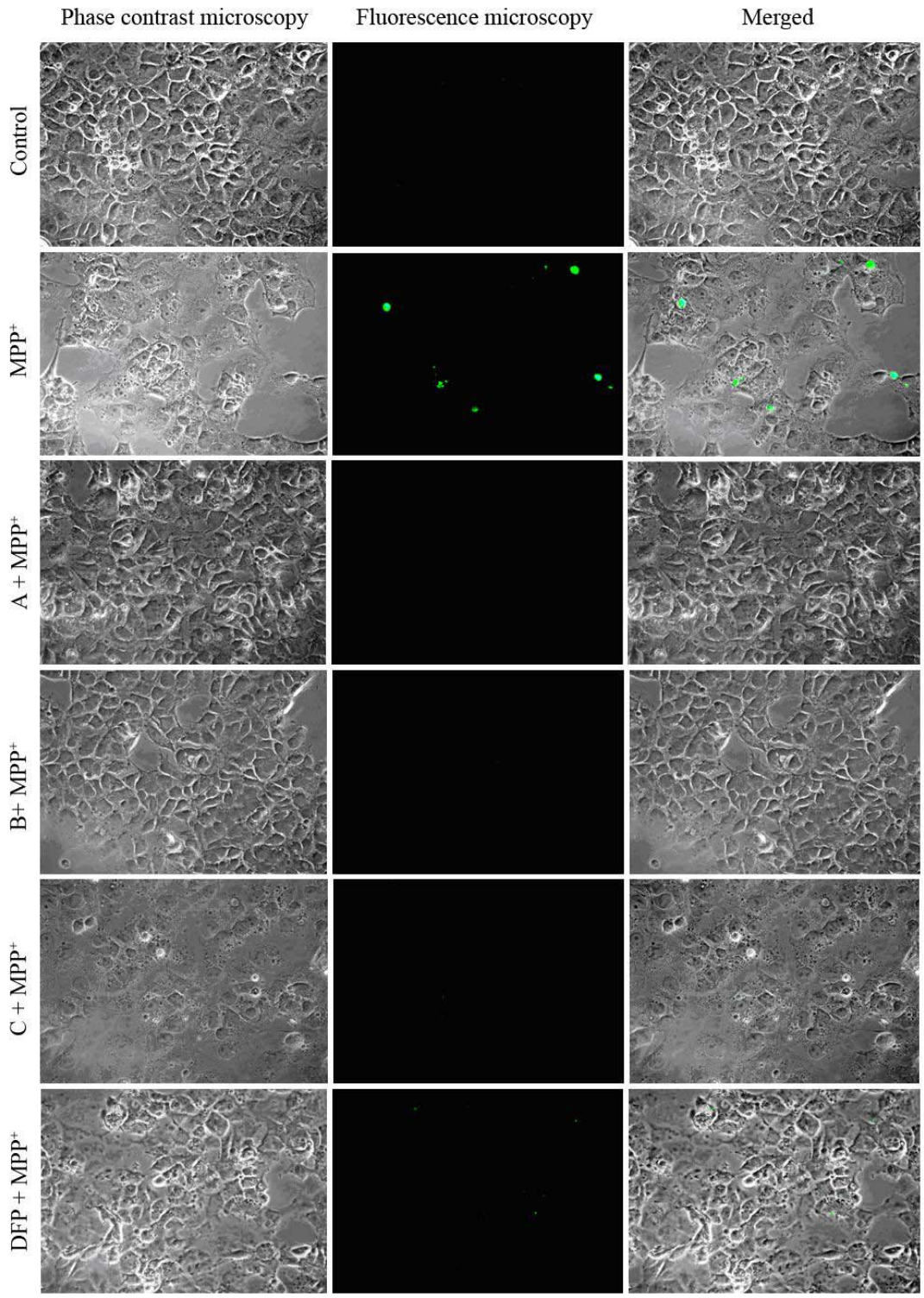
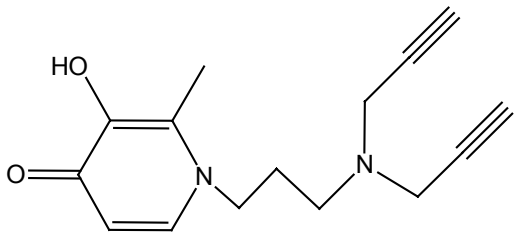
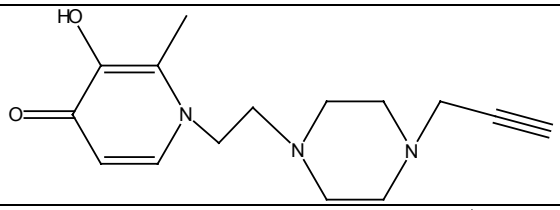
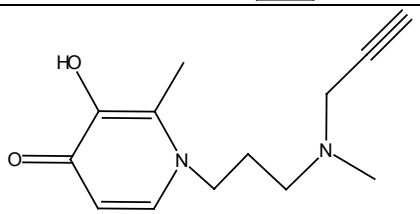
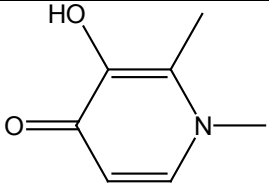


Figure 3. Effect of A, B, C and DFP on MPP^+ induced apoptotic cell death in NT2 cells. NT2 cells were treated with MPP^+ (1 mM) alone or in the presence of compound A, B, C or DFP (5 μM), for 24 h. Apoptotic cell death was determined by TUNEL assay and was analysed by phase contrast and fluorescence microscopy. Merged images illustrate that all tested compounds prevented DNA fragmentation by decreasing TUNEL positive cells, being A, B and C most effective compounds in prevention of MPP^+ induced cell death.

Table 1. Structural formula and log *P* of the compounds used in study

Compound name	Molecular Structure	Log *P*
A		-0.13
B		-0.94
C		-0.32
DFP		-1.03

aspects of neurodegeneration underlying the Parkinson's and Alzheimer's diseases physiopathology, respectively. In addition, it was also evident that, in both cellular models, A, B and C compounds were more effective than DFP, a chelator clinically used for iron removal (Figure 1).

This finding can be explained regarding the hypothesis that neuroprotection in neurodegenerative diseases requires a permeable drug combining antioxidant and iron chelating properties. Several lines of evidence propose that metal ion homeostasis is severely deregulated and may be related to oxidative damage in AD brain (47). Increased concentrations of copper, iron and zinc are detected in affected brain regions within amyloid plaques (48).These metals bind to Abeta and were shown to accelerate its aggregation and enhance metal-catalysed oxidative stress, associated with amyloid plaque formation (9). In addition, copper, iron and zinc have been reported to increase Abeta toxicity (8,49).

Thus, our findings demonstrating that compound A markedly decreased Abeta toxicity (Figure 1) and suppressed caspase-4 activation (Figure 2B) can be explained taking into account its antioxidant ability and the presence of a piperazine moiety which exhibits some affinity for Cu (II), preventing further Abeta aggregation. Therefore, the probable copper chelating action could prevent Abeta induced toxicity, by reducing the possibility of generation of toxic-amyloid peptides, which can disrupt intraneuronal Ca^{2+} levels, leading to ER perturbated Ca^{2+} homeostasis and, subsequently, to ER associated caspases activation.

In addition, accumulating evidences have shown that iron-dependent oxidative stress, increased levels of iron, increased MAO-B activity, and depletion of antioxidant enzymes in the brain may be the main pathogenic factors in PD (4). Besides, it is plausible that stress responses from ER involves transcriptional signalling, intracellular Ca^{2+} mobilization, activation of apical caspases like, caspases-4 or -2, that may proceed with or without the contribution of mitochondrial-dependent apoptotic pathway (50). In this case, the results of the present study show that compounds A and C significantly reduced MPP^+-induced caspases-2, -4 and -3 activation (Figure 2 A, B and C) in a similar way than dantrolene, an inhibitor of ryanodine receptors of ER. Dantrolene prevents Ca^{2+} release from ER lumen to cytosol, and was used as a control for neuroprotection activity (Figure 2 A). In addition, caspases-mediated apoptotic cell death inhibition was corroborated by the efficiency of these compounds on rescue cells from MPP^+ mediated cell death (Figure 3). Herein, modified compounds A, B and C were more successful than DFP in preventing MPP^+ induced cell death. Thus, we suggest that compounds A and C could act at the ER stress level that may proceed with mitochondrial-dependent caspases activation, by preventing the successive caspase-3 activation and, subsequently, DNA fragmentation.

Overall, we were able to prove that compound A was the most efficient product acting on several cellular targets. The high neuroprotective effect of this compound, in these cellular models, may be due to the combined potential actions of iron chelation, antioxidant, MAO B inhibition and good permeability. Among these compounds only DMHP was assessed for the iron (III) chelating and other anti-oxidant properties, although all of them are expected to present similar behaviours. In fact, the affinity of the 3-hydroxy-4-pyridinones for iron (III) showed to be quite high (pFe = 19.4 for DMHP) and with minimal dependence on the type of *N*-substituent group (37). On the other hand, preliminary studies indicated other anti-oxidant properties for DMHP, namely those associated with the inhibition of deoxyribose degradation and of lipoperoxidation (IC_{50} *ca* 200 and 20 μM, respectively) (51).

The results in this study are consistent with the lipophilicity studies, which evidenced that A and C are more lipophilic than DFP, representing a benefit for their bioavailability and Blood Brain Barrier crossing facility. The particular properties of compound A show that it is a superior product comparing with the others, exerting its effects by inhibiting MAO dependent generation of hydrogen peroxide, and chelating iron-dependent generation of reactive hydroxyl radical (by Fenton reaction) from hydrogen peroxide generated by other reactions. Taking together, the several targets and various biochemical properties of compound A make of it a potential drug in PD and AD treatment.

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7. REFERENCES

1. Selkoe, D.J.: Clearing the brain's amyloid cobwebs. *Neuron* 32, 177-80 (2001)
2. Roman, M.J., D.C. Delis, J.V. Filoteo, T.L. Demadura, J. Paulsen, N.R. Swerdlow, M.R. Swenson, D. Salmon, N. Butters & C. Shults: Is there a "subcortical" profile of attentional dysfunction? A comparison of patients with Huntington's and Parkinson's diseases on a global-local focused attention task. *J Clin Exp Neuropsychol* 20, 873-84 (1998)
3. Forno, L.S.: Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol* 55, 259-72 (1996)
4. Cardoso, S.M., P.I. Moreira, P. Agostinho, C. Pereira & C.R. Oliveira: Neurodegenerative pathways in Parkinson's disease: therapeutic strategies. *Curr Drug Targets CNS Neurol Disord* 4, 405-19 (2005)
5. Pereira, C., P. Agostinho, P.I. Moreira, S.M. Cardoso & C.R.Oliveira: Alzheimer's disease-associated neurotoxic mechanisms and neuroprotective strategies. *Curr Drug Targets CNS Neurol Disord* 4, 383-403 (2005)
6. Butterfield, D.A., J. Drake, C. Pocernich & A. Castegna: Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. *Trends Mol Med.* 7, 548-54 (2001)
7. Pratico, D., C.M. Clark, V.M. Lee, J.Q. Trojanowski, J. Rokach & G.A. FitzGerald: Increased 8,12-iso-iPF2alpha-VI in Alzheimer's disease: correlation of a noninvasive index of lipid peroxidation with disease severity. *Ann Neurol* 48, 809-12 (2000)
8. Crichton, R.R. & R.J. Ward: Metal-based Neurodegeneration. John Wiley & Sons Lda, Chichester, pp 62-87 (2006).
9. Smith, M.A., P.L. Harris, L.M. Sayre & G. Perry: Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc Natl Acad Sci USA* 94, 9866-9868 (1997)
10. Dexter, D.T., C.J. Carter, F.R. Wells, F. Javoy-Agid, Y. Agid, A. Lees, P. Jenner & C.D. Marsden: Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. *J Neurochem* 52, 381-9 (1989)
11. Floor, E., M.G. Wetzel: Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. *J Neurochem* 70, 268-75 (1998)
12. Saggu, H., J. Cooksey, D. Dexter, F.R. Wells, A. Lees, P. Jenner & C.D. Marsden: A selective increase in particulate superoxide dismutase activity in parkinsonian substantia nigra. *J Neurochem* 53, 692-7 (1989)
13. Ben-Shachar, D., P. Riederer & M.B. Youdim: Iron-melanin interaction and lipid peroxidation: implications for Parkinson's disease. *J Neurochem* 57, 1609-14 (1991)
14. Graham, D.G.: Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol Pharmacol* 14, 633-43 (1978)
15. Maker, H.S., C. Weiss, D.J. Silides & G. Cohen: Coupling of dopamine oxidation (monoamine oxidase activity) to glutathione oxidation via the generation of hydrogen peroxide in rat brain homogenates. *J Neurochem* 36, 589-93 (1981)
16. Schindowski, K., T. Kratzsch, J. Peters, B. Steiner, S. Leutner, N. Touchet, K. Maurer, C. Czech, L. Pradier, L. Frölich, W.E. Müller & A. Eckert: Impact of aging: sporadic and genetic risk factors on vulnerability to apoptosis in Alzheimer's disease. *Neuromolecular Med* 4, 161-78 (2003)
17. Tatton, N.A., A. Maclean-Fraser, W.G. Tatton, D.P. Perl & C.W. Olanow: A fluorescent double-labelling method to detect and confirm apoptotic nuclei in Parkinson's disease. *Ann Neurol* 44, S142-8 (1998)
18. Hartmann, A., S. Hunot, P.P. Michel, M.P. Muriel, S. Vyas, B.A. Faucheux, A. Mouatt-Prigent, H. Turmel, A. Srinivasan, M. Ruberg, G.I. Evan, Y. Agid & E.C. Hirsch: Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. Proc Natl Acad Sci USA 14; 97, 2875-80 (2000)
19. Tatton, N.A.: Increased caspase 3 and Bax immunoreactivity accompany nuclear GAPDH translocation and neuronal apoptosis in Parkinson's disease. *Exp Neuro* 166, 29-43 (2000)

20. Paschen, W.: Endoplasmic reticulum: a primary target in various acute disorders and degenerative diseases of the brain. Cell *Calcium* 34, 365-83 (2003)
21. Ryu, E.J., H.P. Harding, J.M. Angelastro, O.V. Vitolo, D. Ron & L.A. Greene: Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *J Neurosc* 22, 10690-8 (2002)
22. Yamamuro, A., Y. Yoshioka, K. Ogita & S. Maeda: Involvement of endoplasmic reticulum stress on the cell death induced by 6-hydroxydopamine in human neuroblastoma SH-SY5Y cells. *Neurochem Res* 31, 657-64 (2006)
23. Hoozemans, J.J., E.S. van Haastert, P. Eikelenboom, R.A. de Vos, J. M. Rozemuller & W. Scheper: Activation of the unfolded protein response in Parkinson's disease. *Biochem Biophys Res Commun* 354, 707-11 (2007)
24. Hider, R.C., G.J. Kontoghiorghes & J. Silver: Pharmaceutical compositions. UK PATENT gb2118176, 1983
25. Kontoghiorghes, G.J., M.A. Aldouri, L. Sheppard & A.V. Hoffbrand: 1,2-dimethyl-3-hydroxypyrid-4-one, an orally active chelator for treatment of iron overload. *Lancet* 1 (8545), 1294-1295 (1987)
26. Hubálek, F., C. Binda, M. Li, J. Sterling, D.E. Edmondson & A. Mattevi: Inactivation of purified human recombinant monoamine oxidases A and B by rasagiline and its analogues. *J Med Chem* 47, 1767-1774 (2004)
27. Youdim, M.B.H. & Y.S. Bakhle: Monoamine oxidase: isoforms and inhibitors in Parkinson's disease and depressive illness. *British J Pharmacol* 147, 287-296 (2006)
28. Armarego, W.L.F. & D.D. Perring (eds) Purification of Laboratory Chemicals 4rd ed., Butterworth-Heinemann Press, Oxford (1999).
29. Santos, M.A., R. Grazina, A.Q. Neto, G. Cantinho, L. Gano & L. Patricio: Synthesis, chelating properties towards gallium and biological evaluation of two N-substituted 3-Hydroxy-4-Pyridinones. *J Inorg Biochem* 78, 303-311 (2000)
30. Leo, A., C. Hansch & D. Elkins: Partition coefficients and their uses. *Chem Rev* 71, 526-616 (1971).
31. Cardoso, S.M. & C.R. Oliveira: The role of calcineurin in amyloid-beta-peptides-mediated cell death. *Brain Res* 1050, 1-7 (2005)
32. Mosmann, T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65, 55-63 (1983)
33. Cregan, S.P., J.G. MacLaurin, C.G. Craig, G.S. Robertso, D.W. Nicholson, D.S. Park & R.S. Slack: Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. *J Neurosc* 19, 7860-9 (1999)
34. a) Santos, M.A.: Hydroxypyridinone complexes with aluminium. *In vitro* studies and *in vivo* perspectives. *Coord Chem Rev* 228, 187-203 (2002); b) Santos, M.A.: Recent developments on 3-hydroxy-4-pyridinones in view of clinical applications. Mono and combined ligand approaches. *Coord Chem Rev* (2008)
35. Hider, R.C. & Z.D. Liu: Emerging understanding of the advantage of small molecules such as hydroxypyridinones in the treatment of iron overload. *Curr Med Chem* 10, 1051-1064 (2003)
36. Florence, A.L., A. Gauthier, R.J. Ward & R.R. Crichton. Influence of hydroxypyridones and desferrioxamine on the mobilization of aluminium from tissues of aluminium-loaded rats. *Neurodegeneration* 4, 449-455 (1995)
37. Santos, M.A., M. Gil, L. Gano, S. Chaves: Bifunctional 3-hydroxy-4-pyridinone derivatives as potential pharmaceuticals. Synthesis, complexation with Fe (III), Al (III) and Ga (III), and *in vivo* evaluation with 67Ga. *J Bio Inorg Chem* 10, 564-580 (2005)
38. Chang, H.Y. & X. Yang: Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev* 64, 821–846 (2000)
39. Troy, C.M. & M.L. Shelanski: Caspase-2 redux. *Cell Death Differ* 10, 101-7 (2003)
40. Nakagawa, T., H. Zhu, N. Morishima, E. Li, J. Xu, B.A. Yankner & J. Yuan: Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 403, 98-103 (2000)
41. Hitomi, J., T. Katayama, Y. Eguchi, T. Kudo, M. Taniguchi, Y. Koyama, T. Manabe, S. Yamagishi, Y. Bando, K. Imaizumi, Y. Tsujimoto & M. Tohyama: Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J Cell Biol* 165, 347-56 (2004)
42. Cardoso, S.M., R.H. Swerdlow & C.R. Oliveira: Induction of cytochrome c-mediated apoptosis by amyloid beta 25-35 requires functional mitochondria. *Brain Res* 931, 117-25 (2002)
43. Youdim, M.B., M. Fridkin & H. Zheng: Novel bifunctional drugs targeting monoamine oxidase inhibition and iron chelation as an approach to neuroprotection in Parkinson's disease and other neurodegenerative diseases. *J Neural Transm* 111, 1455-1471 (2004)
44. Sterling, J., Y. Herzig, T. Goren, N. Finkelstein, D. Lerner, W. Goldenberg, I. Miskolczi, S. Molnar, F. Rantal, T. Tamas, G. Toth, A. Zagyva, A. Zekany, J. Finberg, G. Lavian, A. Gross, R. Friedman, M. Razin, W. Huang, B. Krais, M. Chorev, M. B. Youdim & M. Weinstock,: Novel dual inhibitors of AChE and MAO derived from hydroxy aminoindan and phenethylamine as potential treatment for Alzheimer's disease. *J Med Chem* 45, 5260-5279 (2002)
45. Zheng, H., L.M. Weiner, O. Bar-Am, S. Epsztejn, Z.I. Cabantchik, A. Warshawsky, M.B. Youdim & M. Fridkin: Design, synthesis, and evaluation of novel bifunctional iron-chelators as potential agents for neuroprotection in Alzheimer's, Parkinson's, and other neurodegenerative diseases. *Bioorg Med Chem* 13, 773-783 (2005)
46. Cardoso, S.M., S. Santos, R. H. Swerdlow & C. R. Oliveira: Functional mitochondria are required for amyloid beta-mediated neurotoxicity. *FASEB J* 15, 1439-1441 (2001)
47. Rogers J.T. & D. K. Lahiri: Metal and inflammatory targets for Alzheimer's disease. *Curr Drug Targets* 5, 535-551 (2004).
48. Lovell, M.A., J. D. Robertson, W.J. Teesdale, J.L. Campbell & W.R. Markesbery: Copper, iron and zinc in Alzheimer's disease senile plaques. *J Neurol Sci* 158, 47-52 (1998)
49. Moreira, P., C. Pereira, M.S. Santos & C. Oliveira: Effect of zinc ions on the cytotoxicity induced by the

amyloid beta-peptide. *Antioxi Redox Signal* 2, 317-325 (2000)
50. Hicks, S.W. & C.E. Machamer: Golgi structure in stress sensing and apoptosis. *Biochim Biophys Acta* 1744, 406-14 (2005)
51. S. Canário, S. Chaves, M.L. Mira & M.A. Santos: Anti-oxidant properties of *O*,*O*- *and O*,*S*-Donor Ligands with pyrone and pyridinone molecular scaffolds. *In Press*

Abbreviations: AchE: acetylcholinesterase; AD: Alzheimer`s disease; ER: endoplasmic reticulum; MAO: Monoamine oxidase; MPP^+: 1-methyl-4-phenylpyridinium; MTT: 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFT: Neurofibrillary tangles; PD: Parkinson´s disease; ROS: reactive oxygen species; TUNEL: Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling

Key Words: Alzheimer`s disease, Parkinson`s Disease, Neurodegeneration, Apoptosis; Mpp^+, Hydroxypyridinone, Chelation, Therapy; Iron Chelator

Send correspondence to: M. Amelia Santos, Centro de Quimica Estrutural, Instituto Superior Tecnico (UTL), Av Rovisco Pais, 1, 1049-001 Lisboa, Tel:00351-218419273, Fax: 00351-218464455, E-mail: masantos@ist.utl.pt

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