RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Journal Name

RSCPublishing

ARTICLE

Biology-oriented development of novel lipophilic antioxidants with neuroprotective activity

Cite this: DOI: 10.1039/x0xx00000x

T. Silva[a], J. Bravo[b], T. Summavielle[b], F. Remião[c], C. Pérez[d], C. Gil[d,e], A. Martínez[d,e] and F. Borges[a†]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

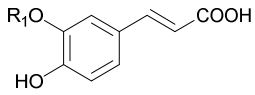
www.rsc.org/

Hydroxycinnamic derivatives based on ferulic and caffeic acids were designed to meet the pharmacokinetic requirements to cross the blood-brain barrier and to display neuroprotective activity within the central nervous system. Biological screening included the assessment of acetylcholinesterase and glycogen synthase kinase 3β inhibition, iron chelation properties, *in vitro* blood-brain barrier permeability, evaluation of cytotoxicity and neuroprotection against 6-hydroxydopamine induced damage in SH-SY5Y cells. Although the chemical modifications did not significantly alter the *in vitro* activity of the parent compounds, the results of the PAMPA-BBB assay show that some derivatives have higher diffusion rates and may reach the brain. The majority of the synthesized compounds did not display cytotoxicity and successfully prevent 6-hydroxydopamine damage. In this series, compound **14** stands out as a promising neuroprotective agent combining a number of key features: iron chelation, neuroprotection against oxidative damage, mild acetylcholinesterase activity and ability to permeate the blood-brain barrier. This biology-oriented approach provides new tools for the generation of new chemical entities to tackle the oxidative damage associated with neurodegenerative disorders.

1 Introduction

Natural products exert their biological function by binding to multiple targets in the course of their biosynthesis, therefore defining privileged and biologically relevant molecular frameworks. Consequently, compound libraries inspired in natural products are expected to yield relevant modulators of multiple biological processes.[1-3] Thus, the synthesis of natural product inspired compounds is a highly promising approach driving drug discovery programs.[4]

Biology-oriented synthesis (BIOS) employs biological relevance and scaffold validation as key criteria for the development of focused libraries. The underlying scaffolds of natural product classes define the areas of the chemical space explored by nature in evolution. In this context, naturally occurring hydroxycinnamic acids (HCAs) can be viewed as privileged structures for the development of bioactive compounds with therapeutic potential. HCAs like ferulic acid and caffeic acid (compounds **1** and **2**, Figure **1**) are phenolic acids widely distributed in plants, fungi and algae and recognized for their antioxidant activity.[5] Arising as secondary metabolites of L-tyrosine and L-phenylalanine, HCAs bear a phenylpropanoid scaffold that can be found in a variety of compounds present in human diet.[5,6]



1, R = CH_3, Ferulic acid
2, R = H, Caffeic acid

Figure 1. Chemical structures of ferulic (**1**) and caffeic acid (**2**).

The antioxidant activity of HCAs is mediated by a combination of mechanisms namely (a) direct scavenging of reactive species (RS) by hydrogen donation and/or electron transfer, [7,8] (b) chelation of pro-oxidant transition metals (*e.g.* Cu and Fe), [9] (c) induction of cytoprotective signalling pathways (*e.g.* Nrf2-ARE), [10,11] and (d) regeneration of the reduced forms of endogenous antioxidants. [12]

Dietary HCAs have demonstrated a significant impact on human health, with potential benefits for several disorders associated with oxidative stress. 13-15 This observation is

particularly relevant for neurodegenerative diseases (ND), like Alzheimer's disease (AD) and Parkinson's disease (PD), since the human brain has the highest rate of oxygen consumption [16] and is therefore greatly susceptible to oxidative damage. This susceptibility is further aggravated by the brain's high content in oxidizable unsaturated fatty acids and in the pro-oxidant iron and in a noticeable low level of endogenous antioxidants. [17-19] Antioxidant therapy has received considerable attention as an approach to delay or prevent the events that lead to neurodegeneration, with many antioxidants undergoing clinical trials over the past years. [20-25] However, the results of these studies remain controversial, as the majority of the tested compounds lack any therapeutic advantage. There is indeed a significant mismatch between the results obtained in pre-clinical studies and the outcome of clinical trials. This gap may be related not only by the design of the clinical trials (*e.g.* posology, duration of treatment, age and disease stage of the patients enrolled) but also by the pharmacokinetics of the antioxidants under evaluation. In fact, some of them have a noticeable hydrophilicity that restricts their distribution through complex biological systems, constituting a major pharmacokinetic drawback. [26] The inability to successfully diffuse through biological barriers, in particular the blood-brain barrier (BBB), hinders their activity at the target sites within the central nervous system (CNS). This makes them promising agents *in* vitro but rather obsolete *in vivo*. [27]

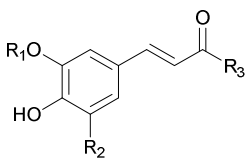
In this context, the development of innovative HCA derivatives able to overcome these pharmacokinetic constrains is a rational strategy for the discovery of centrally-active antioxidants with neuroprotective activity. The introduction of minor structural variations on the original scaffold allows the modulation of lipophilicity while maintaining or improving the neuroprotective potential of the parent compounds. [28-34] Several factors regulate the ability of a molecule to penetrate the BBB, namely lipophilicity, molecular weight, number of hydrogen bond donors and acceptors, polar surface area and molecular flexibility [35,36]. Lipophilicity is directly attained by the partition coefficient (cLogP) and can be directly modulated via structural refinement to improve the odds of obtaining effective CNS drugs. On the other hand, high lipophilicity frequently leads to compounds with poor solubility, high metabolic turnover and poor absorption. Theoretical studies found that optimal BBB penetration occurs when the cLogP values are in the range of 1.5-2.7. Indeed the mean value for the cLogP of marketed CNS drugs is 2.5, which provides a valuable guide for CNS drug design.[35,37]

Herein, we report the synthesis of a set of lipophilic HCA derivatives (Scheme **1**) using the dietary HCAs **1** and **2** (Figure **1**) as templates. Moreover, the biological performance of HCA derivatives towards ND-related targets (cholinesterases and glycogen synthase kinase-3β, GSK-3β), iron chelation capacity, BBB permeability and cell-based studies in a human neuroblastoma cell line (SH-SY5Y cells) was evaluated to check their potential application in ND.

2 Results and discussion

2.1 Chemistry

To obtain lipophilic HCA derivatives without compromising solubility and the antioxidant profile of the parent compounds, two simple modifications on the parent scaffold were performed: introduction of an unsubstituted aromatic ring at C5 and/or formation of an *N*-hexylamide moiety. The selection of the *N*-hexylamide moiety is based on previous studies from our group [33] and from literature data, which indicates that this is the optimal length of a saturated carbon homologous chain without compromising solubility and pharmacological activity[38]. Simultaneously, the change of the aromatic substitution pattern (4-methoxy-3-hydroxy, 3,4-dihydroxy and 3,4,5-trihydroxy) enabled further insights on the influence of this parameter on the overall activity of the synthesized derivatives.



7-14

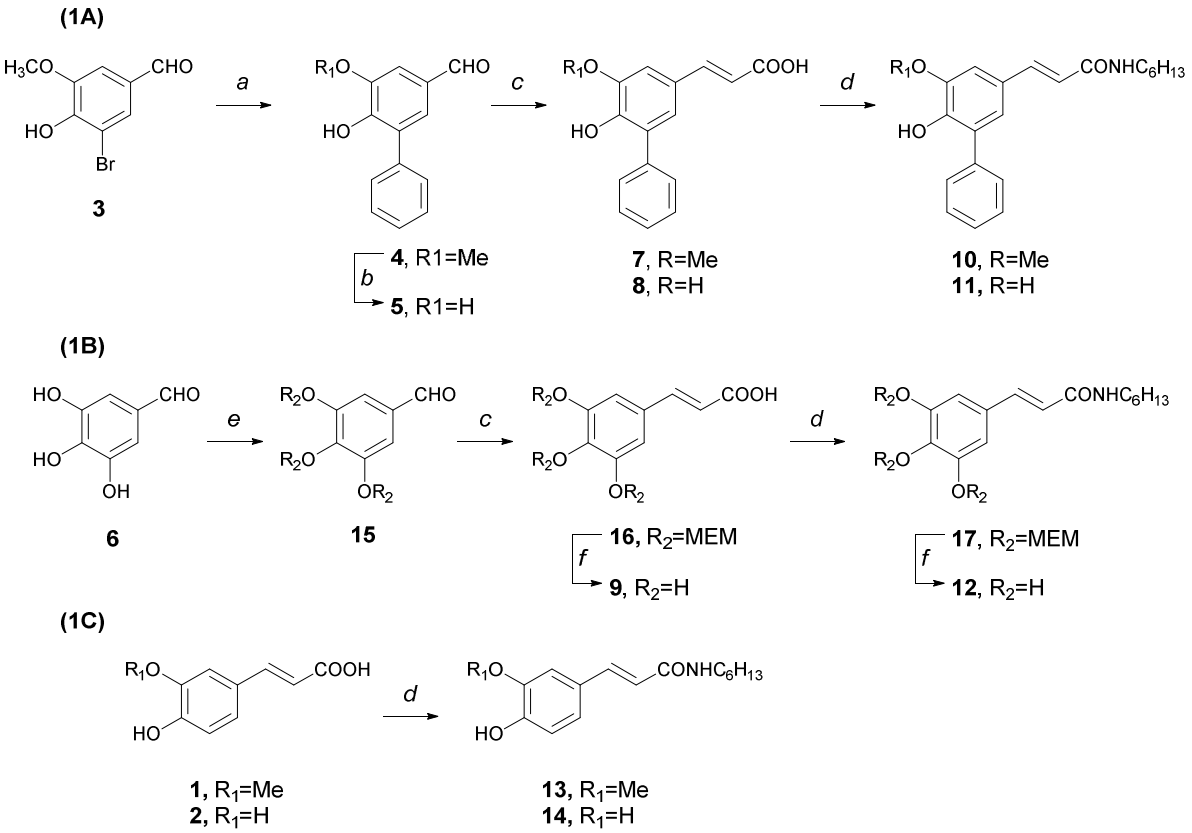
7, R_1=Me, R_2=Ph, R_3=OH
8, R_1=H, R_2=Ph, R_3=OH
9, R_1=R_3=H, R_2=OH
10, R_1=Me, R_2=Ph, R_3=NHC_6H_{13}
11, R_1=H, R_2=Ph, R_3=NHC_6H_{13}
12, R_1=H, R_2=OH, R_3=NHC_6H_{13}
13, R_1=Me, R_2=H, R_3=NHC_6H_{13}
14, R_1=R_2=H, R_3=NHC_6H_{13}

Figure 2. Chemical structures of HCA derivatives **7**-**14.**

The synthetic strategy pursued to obtain HCA derivatives **7**-**14** (Figure **2**) is depicted on Scheme **1**. The ferulic (4-methoxy-3-hydroxy aromatic pattern) and caffeic derivatives (3,4-dihydroxy aromatic pattern) derivatives were obtained as depicted on Scheme **1A**. Firstly, 5-bromovanilin (**3**) was treated with phenyl boronic acid in the presence of $Pd(OAc)_2$ under microwave (MW) irradiation, yielding the 5-aryl derivative **4**. This compound was then *O*-demethylated with boron tribromide to render the 3,4-dihydroxy precursor **5**. Aldehydes **4** and **5** were then submitted to a Knoevenagel-Doebner condensation with malonic acid in pyridine and piperidine, yielding the corresponding cinnamic derivatives **7** and **8** in moderate to good yields (54-72%). The final step involved the formation of the *N*-hexylcarboxamide **10** and **11** through activation with a phosphonium coupling agent (PyBOP) and subsequent reaction with hexylamine. To obtain the 3,4,5-trihydroxycinnamic derivatives an additional step using a phenol protecting agent had to be incorporated in the synthetic strategy, to overpass difficulties arising in the purification steps and formation of by-products (Scheme **1B**). Pyrogallol protection was successfully achieved using methoxyethoxymethoxy (MEM) ether, by treatment of the benzaldehyde precursor **6** with methoxyethoxymethyl chloride (MEMCl) in diisopropylethylamine (DIPEA) and dichloromethane, yielding the protected benzaldehyde **15**. This compound was then treated as previously described for compounds **4** and **5**, yielding the cinnamic derivative **16** and the *N*-hexylcarboxamide **17** that were then deprotected using an acidic resin (Amberlyst 15®) in methanol/water (95:5) at 50ºC. This step enabled the obtention of the 3,4,5-trihydroxy acid (**9**) and *N*-hexylcarboxamide derivative **12**. In spite of the moderate yield of the final step (~50%), the reaction work-up was straightforward and the final products were easily purified by column chromatography, which presents a major advantage over the poor yields obtained when working with a free pyragallol moiety. The ferulic and caffeic *N*-hexylcarboxamide

derivatives **13** and **14** were also synthesized as previously described (Scheme **1C**).[38]

The identity of the synthetic intermediates and the final products was determined by NMR spectroscopy (^{1}H, ^{13}C and DEPT135) and mass spectrometry (ESI-MS).



Scheme 1. Synthesis of HCAs derivatives **7-11**. *a* $PhB(OH)_2$, $Pd(OAc)_2$, TBAB, H_2O; *b* BBr_3, anhydrous CH_2Cl_2; *c* malonic acid, pyridine, piperidine; *d* PyBOP, DIPEA, hexylamine, CH_2Cl_2, DMF; *e* MEMCl, DIPEA, TBAI, CH_2Cl_2; *f* Amberlyst 15®, MeOH/H_2O (95:5).

2.2 Cholinesterase inhibitory activity

The cholinergic network of the brain undergoes extensive neurodegeneration in AD causing acetylcholine (ACh) depletion. [39] The inhibition of acetylcholinesterase (AChE) to restore the synaptic levels remains a symptomatic therapeutic approach towards AD. The use of AChE inhibitors is also used in the palliative treatment of mild to moderately moderate severe dementia in patients with idiopathic PD. Additionally, AChE is known to co-localize and directly promote the assembly of the cytotoxic β-amyloid. [40,41] The data regarding the effect of natural HCAs on cholinesterase' activity is scarce and heterogeneous. For instance, caffeic acid (**2**) has been reported to induce AChE activity in different rat brain regions while inhibiting muscular AChE, [42] and *in vitro* screening assays showed inconsistent IC_{50} values ranging from 23.3 μM [43] to over 100 μM. [44,45] Nevertheless, HCAs have been used as templates for the development of multitarget directed agents for ND and promising results have been recently reported.[46-48] In this context, the AChE and butyrrylcholinesterase (BChE) inhibitory activity of the natural HCAs **1** and **2** and the derivatives **7-14** was evaluated following the method of Ellman. [49] From the results depicted in Table **1** one can conclude that all compounds exhibited higher activity than parent compounds **1** and **2** and that some derivatives (compounds **8-10**, **13** and **14**) displayed mild inhibitory activity towards human AChE at the low micromolar range. None of the tested compounds display a noticeable activity towards BChE at 10 μM (data not shown).

2.3 GSK-3β inhibitory activity

GSK-3β is a serine/threonine protease known to play an important role within the CNS, modulating several aspects that range from neurogenesis, neuronal and synaptic plasticity to neuronal survival and death.[50] Deregulation of GSK-3β-associated signalling pathways and GSK-3β activity is related with the pathogenesis of several neurological and psychiatric disorders, particularly ND. In fact, GSK-3β is significantly involved in key pathological events, namely Tau hyperphosphorylation, amyloidogenesis, inflammatory response and ACh deficit. [51] Data obtained from ND patients corroborates the importance of this enzyme, [52] supporting the

rationale for GSK-3β as a promising target for ND. Recent studies report the ability of compound **1** and its natural phenethyl ester derivative (CAPE) to modulate the Akt/GSK-3β signalling pathway and GSK-3β activity. [53,54] Moreover, a synthetic hydrocinnamic derivative (DH9) has also been reported to modulate GSK-3β activity by inducing its phosphorylation.[55] As the modulation of GSK-3β activity was also mediated by a diversity of polyphenolic systems[56-58] it was decided to screen HCAs and derivatives (compounds **1**, **2** and **7-14)** towards GSK-3β, at a fixed concentration of 10 μM, using a previously described luminescent technique.[59] From the data one can concluded that none of the tested compound displayed noteworthy activity at the established concentration (Table **1**). Only compounds **8**, **9** and **12** exhibited mild inhibitory activity at 10 μM. In summary, no direct inhibition of GSK-3β was observed under the assay conditions. Therefore, we can speculate that the reported effects of HCAs on GSK-3β may be due to the modulation of events upstream in the signalling pathways that regulate the enzyme's activity, which cannot be observed in a direct enzymatic inhibition assay and require further studies.

2.4 Iron chelation capacity

Iron is a redox active metal that plays a part in the production of RS and in diseases in which oxidative damage is recognised as a pathological stimulus. In particular, unbound iron can undergo Fenton reaction with hydrogen peroxide and generate hydroxyl radicals ($HO^{\cdot}$), that undergo further reactions with severe implications for human health and disease.[60-63] iron overload is particularly critical for CNS disorders as brain is particularly susceptible to oxidative damage. In this context, iron chelation can be viewed as a co-adjuvant property that can boost brain total antioxidant capacity.[64,65] The chelation capacity of HCAs towards ferrous (Fe^{2+}) cations was determined using the ferrozine method, [66] a spectrophotometric assay based on the formation of a complex between ferrous cations and ferrozine [3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt]. Compounds with iron chelation capacity hinder the formation of such complex, which is translated into a significant decrease in the absorbance when compared to the control. Ethylenediaminetetracetic acid (EDTA) was usually used as a standard. Each compound was tested at 100 μM and results are expressed as % of iron chelation ± standard deviation (n = 3) (Table **1**). Compounds bearing the catechol (**2**, **8**, **11** and **14**) and pyrogallol (**9** and **12**) moieties displayed noteworthy chelation properties, comparable to that of EDTA. Ferulic acid (**1**) and its derivatives (**7**, **10** and **13**), bearing only one hydroxyl group showed, in the experimental assay conditions, absence or low chelation ability. These results are in accordance with previous reports that describe for other type catechol and pyrogallol systems, such as coumarins, [67,68] flavonoids, [69,70] and for other polyphenols[72] remarkable iron chelation properties. Indeed, ferrous ions can assume an octahedral geometry and efficiently coordinate catechol or pyrogallol groups present in a diversity of ligands.[72]

Table 1. Data from enzymatic inhibition (hAChE and hGSK-3β), iron chelation, BBB permeability and cytotoxicity for HCA parent compounds (**1** and **2**) and derivatives (**7-14**). [a] AChE from human erythrocytes; [b] Data expressed as mean ± S.D (n =3).; [c] Iron chelation at 100 μM. Results expressed as mean % chelation ± S.D (n =3).; [d] Permeability coefficient in PBS:ethanol (7:3). Results are expressed as mean permeability ± S.D. (n = 4); [e] Cytotoxicity in SH-SY5Y cells incubated with two different concentrations of the compounds. Results expressed as mean % of cell viability ± S.D. (n = 3).

Compound	*hAChE[a] IC_{50} (μM)[b]*	*hGSK-3β % inhibition*	*Iron chelation % chelation[c]*	*PAMPA-BBB*		*% Cell viability[e]*	
				Pe (10^{-6}/cm)[d]	*Prediction*	*1 μM*	*10 μM*
1	n.a.	n.a.	n.a.	2.15 ± 0.04	CNS-	113 ± 7.11	121 ± 9.34*
2	7.05 ± 0.36	n.a.	89.43 ± 2.98	1.66 ± 0.15	CNS-	96.4 ± 6.62	99.2 ± 0.72
7	n.a.	n.a.	n.a.	3.71 ± 0.22	CNS+/-	94.5 ± 10.8	91.6 ± 6.40
8	3.50 ± 0.72	28%	78.98 ± 6.27	2.95 ± 0.16	CNS-	116 ± 16.5	88.3 ± 17.7
9	5.68 ± 0.63	22%	89.51 ± 0.37	1.94 ± 0.05	CNS-	106 ± 8.99	104 ± 8.13
10	3.24 ± 0.34	n.a.	26.32 ± 9.59	10.6 ± 0.25	CNS+	96.8 ± 5.80	105 ± 9.47
11	n.a.	n.a.	90.83 ± 5.32	11.61 ± 0.23	CNS+	126 ± 11.4*	116 ± 6.34
12	n.a.	29%	99.76 ± 0.27	3.41 ± 0.10	CNS+/-	108 ± 9.91	109 ± 9.77
13	4.93 ± 0.31	n.a.	n.a.	and	CNS+	101 ± 4.33	102 ± 3.10
14	4.61 ± 0.44	n.a.	99.50 ± 0.71	6.85 ± 0.15	CNS+	94.4 ± 11.9	94.3 ± 8.34
EDTA	-	-	99.33 ± 0.39	-		-	

n.a. not active; * $p < 0.05$

2.5 *In vitro* blood-brain barrier permeability

Promising CNS agents need to present the ability to cross the BBB and reach the therapeutic targets. So, the permeability properties of HCAs were evaluated using artificial membrane permeation assay (PAMPA-BBB) following the method previously described by Di *et al.*[73] The method is simple and rapid and has been widely used in the evaluation of BBB permeability as it successfully predicts BBB passive diffusion, [73-75] which is an essential requirement for the development of effective CNS drugs. The *in vitro* permeability coefficients (*Pe*)

of the parent compounds **1** and **2**, HCA derivatives **7-14** and 11 commercial drugs through a lipid extract of porcine brain (PBL) were determined (Table **1**). Assay validation was achieved by plotting the experimental permeability (*Pe* (exp)) *vs* the reported values for commercial drugs (*Pe* (bibl)), which showed a good correlation (*Pe* (exp) = 1.0161 *Pe* (bibl) – 1.0622, R^2 = 0.9049). From this equation, and considering the limits established by Di *et al.* for BBB permeability, [73] the ranges of permeability were established as (a) compounds with high BBB permeation (CNS+): *Pe* (10^{-6} cm.s^{-1}) > 5.13, (b) compounds with low BBB permeation (CNS-): *Pe* (10^{-6} cm.s^{-1}) < 3.09 and (c) compounds of uncertain BBB permeation (CNS+/-): 3.09 < *Pe* < 5.13. The data (Table **2**), allow concluding that the majority of the HCAs present *Pe* values over the threshold for BBB permeability suggesting that these compounds can cross the BBB by passive diffusion. Parent compounds **1** and **2** showed *Pe* values below the lower permeability limit (CNS-), which is in accordance with the literature. [5,14] With the exception of compounds **8** and **9**, displaying low permeability (CNS-) and compounds **7** and **12** (in the limit of prediction, CNS+/-), the majority of the HCA derivatives (compounds **10**, **11**, **13** and **14**) were able to successfully diffuse through the lipidic membrane, in the present experimental conditions.

2.6 Cytotoxicity and cellular viability

The cytotoxicity of the HCA derivatives **7-14** was evaluated by the determination of the cellular viability (MTT method) of SH-SY5Y cells after a 24h exposure to the compounds, in two different concentrations (1 μM and 10 μM). The parent compounds **1** and **2** were also screened under the same experimental conditions. Control experiments were performed by adding vehicle (1 μL) instead of the compound solution. The results are depicted in Table **1**. Overall, the cellular viability did not suffer any significant decrease when compared to the control groups. In fact, an opposite outcome was frequently observed (cell viability > 100%), and for compounds **1** at 10 μM (121 ± 9.34%) and **11** at 1 μM (126 ± 11.40%) this increase was statistically significant ($p < 0.05$). These results indicate that the HCA derivatives under study do not display significant toxicity and exhibit a wide safety window.

2.7 Neuroprotection against 6-hydroxydopamine induced damage

Finally, the effects of the parent compounds **1** and **2** and their derivatives **7-14** against 6-hydroxydopamine (6-OHDA)-induced oxidative damage were evaluated in SH-SY5Y cells. The stress inducer is a dopaminergic neurotoxin that is widely used in ND model studies. Under physiological conditions, 6-OHDA rapidly undergoes oxidation by molecular oxygen to form hydrogen peroxide, inducing the production of ROS and causing oxidative damage.[76] Compounds able to prevent 6-OHDA-induced damage may provide neuroprotection against the oxidative damage promoted by the neurotoxin in neurological disorders. Briefly, the cells (10^5/well) were pre-incubated with each compound at 1 and 10 μM for 1h and then 6-OHDA (30 μM) was added. The plates were incubated for 24h at 37°C and 5% CO_2 and cell viability was then determined by the MTT method.

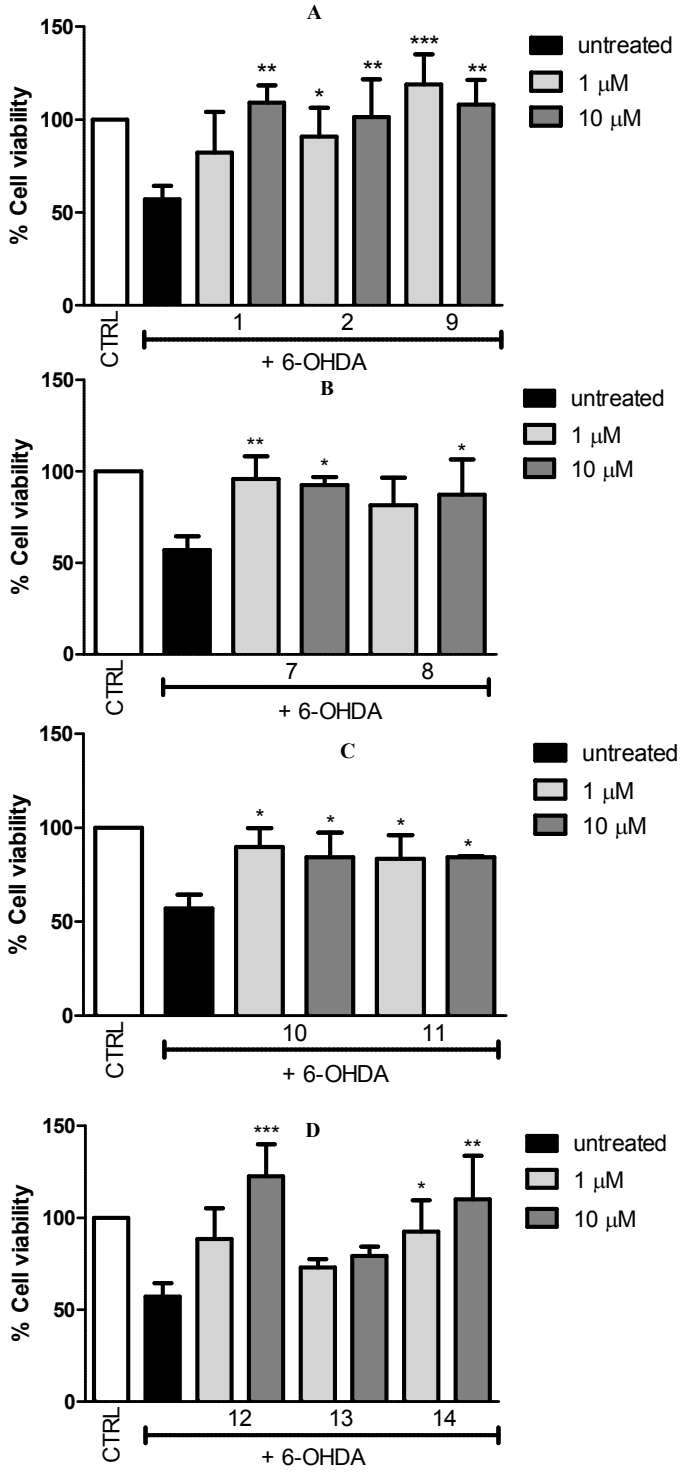


Figure 3. Neuroprotection against 6-OHDA-induced damage in SH-SY5Y cells for **(A)** hydroxycinnamic acids (**1**, **2** and **9**), **(B)** 5-phenylcinnamic acids (**7** and **8**), **(C)** 5-phenyl-*N*-

hexylcarboxmides (**10** and **11**) and **(D)** *N*-hexylcarboxamides (**12-14**). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To determine the statistical significance of the effects the data was compared with the % viability of the cells in the presence of 6-OHDA. The results are depicted in Figure **3**. 6-OHDA caused a significant loss of cell viability when compared to the control (59.06 ± 7.35%, $p < 0.01$). In general, the HCA derivatives under study display a remarkable outline. The best results were observed for the di- and trihydroxylated compounds (compounds **2**, **9**, **12** and **14**, Figures **3A** and **3D**). Noteworthy, that a simple structural modification of HCA, by the introduction of an *N*-hexylcarboxamide, yielded derivatives with significant neuroprotective activity (compounds **12** and **14**, Figure **3D**). The introduction of an aromatic ring in HCA core also provided compounds, in this cellular model, with interesting neuroprotective activity (Figures **3B** and **3C**), although issues with their solubility were detected. Parent ferulic and caffeic acids (compounds **1**, **2**, Figure **3A**) were also protective against the inflicted damage, which is in accordance with previous reports.[32,77-80] These results indicate that simple structural modifications on natural bioactive templates can lead to neuroprotective derivatives that retain the activity of the parent compounds.

2.8 Evaluation of drug-like properties

Although the structural modifications performed in HCAs were designed to produce an increment on lipophilicity, while retaining or improving the activity of the parent compounds, t as found important to evaluate their drug-like properties. Besides lipophilicity, other factors that regulate the ability of a molecule to penetrate the BBB, namely molecular weight, number of hydrogen bond donors (n-OHNH) and acceptors (n-ON), polar surface area and molecular flexibility (by determination of the number of rotable bonds, n-ROTB) were assessed.[35,36] The optimal values for these parameters to obtain orally active drugs are established in the "Lipinski's rule of five". The theoretical evaluation of some drug-like properties of the compounds under study was performed using Molinspiration Cheminformatics Software ®. The results are depicted on Table **2**.

As expected, the introduction of an aromatic ring and a *N*-hexylcarboxamide moiety led to a significant increase in lipophilicity, as shown by the increase in the cLog*P* values (compounds **1-2** *vs* compounds **7-8**/**10-11**/**13-14**). However, the increase observed for compounds **10** and **11** goes beyond the limits established by the Lipinski's "rule of five", presenting the only violation of the current HCAs derivatives. Additionally, the data correlates well with the results obtained in the PAMPA-BBB assay ($R^2 = 0.8395$, Figure **4**), since the CNS+ compounds are the ones with higher cLog*P* values (compounds **10-11**, **13-14**). Neutral molecules (compounds **10-14**) are more permeable than their acidic counterparts (compounds **1**, **2** and **7-9**). These findings are in accordance with the general requirements for drug BBB penetration.[81] Regarding the other calculated parameters, no clear relation with the partition coefficient and BBB permeability was observed.

Table 2. Drug-like properties. of the HCAs and derivatives[a] Partition coefficient; [b] number of rotable bonds; [c] number of hydrogen bond acceptors; [d] number of hydrogen bond donnors.

Compound	*cLogP*[a]	*n-ROTB*[b]	*n-ON acceptors*[c]	*n-OHNH donnors*[d]
1	1.25	3	4	2
2	0.94	2	4	3
7	3.20	4	4	2
8	2.89	3	4	3
9	0.65	2	5	4
10	5.51	9	4	2
11	5.20	8	4	3
12	2.96	7	5	4
13	3.56	8	4	2
14	3.25	7	4	3

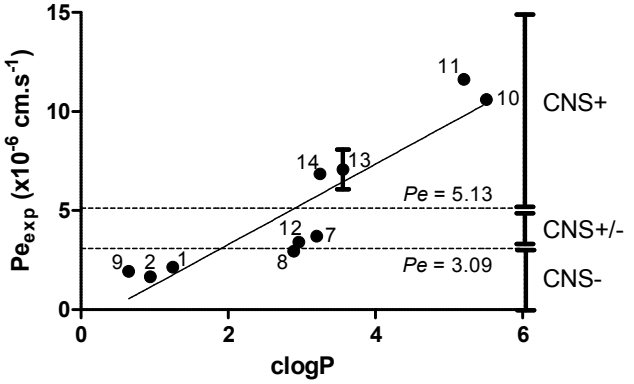


Figure 4. Correlation between the experimental permeability values determined in the PAMPA-BBB assay and the theoretical cLogP values. CNS+: compounds with high BBB permeation; CNS-: compounds with low BBB permeation; CNS+/-: compounds of uncertain BBB permeation.

3. Experimental

3.1. Chemistry

3.1.1. Reagents. All reagents were purchased from Sigma-Aldrich Química S.A. (Sintra, Portugal). Deionised water (conductivity < 0.1 μScm-1) was used for all experiments. All solvents were pro analysis grade and were acquired from Merck (Lisbon, Portugal). Thin layer chromatography (TLC) was performed on precoated silica gel 60 F254 acquired from Merck (Darmstad, Germany) and spots were detected using a UV lamp at 254 nm. Reaction progress was monitored by TLC. Following the extraction step, subsequent work up of the organic layers included drying over anhydrous sodium sulphate, filtration and elimination of solvents under reduced pressure. Column chromatography was carried out with silica gel 60A

acquired from Carlo-Erba Reactifs (SDS, France). The crude products were purified by flash column chromatography and/or recrystallization. The fractions containing the desired product were gathered, concentrated and the crude product was recrystallized. The elution systems used for analytical TLC control and flash chromatography and the recrystallization solvents are specified for each compound.

3.1.2. Apparatus. Solvents were evaporated with a Buchi Rotavapor. ^{1}H and ^{13}C NMR data were acquired at room temperature on a Brüker AMX 300 spectrometer operating at 400 and 100 MHz, respectively. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference and coupling constants (*J*) are given in Hz. Electrospray ionization mass spectra (ESI-MS) were obtained on a VG AutoSpec instrument. The data are reported as *m/z* (% of relative intensity of the most important fragments). Microwave-assisted synthesis was performed in a Biotage® Initiator Microwave Synthesizer

3.1.3. General synthetic procedures

Microwave assisted Pd-catalyzed Suzuki cross coupling reaction. In a 20 mL microwave glass vial the aldehyde with the appropriate aromatic substitution pattern (1 mmol), phenylboronic acid (1 mmol), tetrabutylammonium bromide (TBAB) (1 mmol), potassium carbonate (K_2CO_3) (3 mmol), $Pd(OAc)_2$ (0.04% mmol) and water (10-20 mL) were mixed. The vial was sealed and the reaction mixture was placed under microwave irradiation for 20 minutes at 150˚C. Upon completion, the reaction mixture was filtered with celite, to discard the palladium residues, and rinsed with ethyl acetate. The combined organic layers were washed with HCl 1M (3×10mL) and brine (10 mL). The crude residue was purified by flash column chromatography. The procedure was adapted from Leadbeater et al. (2003).[82]

***O*-Demethylation reaction.** The *O*-methylated compound (1 mmol) was dissolved in anhydrous dichloromethane (5 mL) in a 250 mL round bottom flask under argon atmosphere. The mixture was cooled at -70˚C and boron tribromide (1 mL of a 1M solution in anhydrous dichlorometane) was added. The mixture was stirred under inert atmosphere and allowed to reach room temperature for a period of 12 hours. The demethylating agent was quenched with water (25 mL) and the final product was isolated by filtration and purified by recrystallization. The procedure was performed as reported by Milhazes *et al.* (2006).[83]

Knoevenagel-Doebner condensation reaction. In a 100 mL round bottom flask the aldehyde with the appropriate aromatic substitution pattern (1 mmol), malonic acid (2.2 mmol), anhydrous pyridine (9.6 mmol) and piperidine (4 drops) were mixed. The mixture was protected from light and heated at 50˚C. It was stirred until maximum aldehyde consumption (a variable period for each compound). Upon completion, the mixture was poured over ice water, neutralized with HCl 1M and extracted with ethyl acetate (3×10 mL). The combined organic layers were washed with HCl 1M (3×10 mL), water (3×10 mL) and brine (10 mL). The procedure was performed as reported by Teixeira et al. (2013).[84]

Amidation reaction. In a 100 mL round bottom flask the acid with the appropriate aromatic substitution (1 mmol), DIPEA (1 mmol) and DMF (2 mL) were mixed. The flask was placed on ice and PyBOP (1 mmol) in dichloromethane (2 mL) was added to the mixture, which was kept on ice and stirred for 30 minutes. Afterwards, hexylamine (1 mmol) was added. The mixture was allowed to reach room temperature and stirred for 6 hours. Dichloromethane (20 mL) was added and the mixture was washed with water (3×10 mL), HCl 1M (3×10 mL) and NaHCO3 5% (3×10 mL). The procedure was adapted from Gaspar et al. (2011).[85]

Phenol protection procedure. To a cooled solution of the phenolic compound (1 mmol) and TBAI (0.05 eq.) in dichloromethane (5 mL) and DIPEA (6 eq.) was carefully added methoxyethoxymethyl chloride (MEMCl, 1.5 eq. per OH). The mixture was stirred at 0˚C for three hours. Upon completion, the reaction mixture was neutralized with HCl 1M. Dichloromethane (15 mL) was the added and the organic layer was washed with water (3 x 15 mL) and brine (10 mL). The procedure was adapted from the literature.[86]

Phenol deprotection procedure. To a solution of the MEM-protected product (1 mmol) in MeOH/H_2O (10 mL) Amberlyst 15 ® wet (500 mg) was added. The mixture was protected from light and stirred at 40˚C. Additional aliquots of Amberlyst 15 ® (500 mg) were periodically added over the following 48 hours until TLC analysis (dichloromethane/methanol/formic acid, 9:1:0.01) showed maximal substrate consumption. The presence of phenolic groups can be checked by revelation with solution of iron chloride. Upon completion, the mixture was filtered to remove the resin residue, the solvents were eliminated under reduced pressure and the crude product was purified by flash chromatography (cellulose and dichloromethane/methanol (9:1)). The procedure was adapted from the literature.[87]

4-hydroxy-3-methoxy-5-phenylbenzaldehyde (4) Compound **4** was obtained by a Suzuki cross coupling reaction between compound **3** and phenylboronic acid in the following conditions: compound **3** (1.0 g, 4.3 mmol), phenylboronic acid (0.53 g, 4.3 mmol), K_2CO_3 (1.8 g, 13 mmol), TBAB (1.4 g, 4.3 mmol), $Pd(OAc)_2$ (0.001 g, 0.0044 mmol) and water (18 mL). Eluent systems were dichloromethane/methanol (9:1) for TLC analysis and dichloromethane for flash chromatography. Compound **4** was recrystallized from ethyl acetate/n-hexane. Yield (%): 59.2. ^{1}H NMR (DMSO-*d6*) δ 3.94 (s, 3H, OCH_3), 7.36 (m, 1H, H(4')), 7.44 (m, 3H, H(2), H(3'), H(5')), 7.54 (d, *J* = 1.9 Hz, 1H, (H(5)), 7.57 (d, *J* = 1.2 Hz, H(2')), 1H, 7.59 (d, *J* = 1.4, 1H, H(6')), 9.85 (s, 1H, CHO), 9.90 (s, 1H, OH). ^{13}C

NMR (DMSO- *d6*) δ 56.61, 109.25, 127.70, 127.83, 128.40, 128.59 (2 x C(Ar)), 128.77, 129.56 (2 x C(Ar)), 137.59, 148.86, 150.15, 191.79.

3,4-dihydroxy-5-phenylbenzaldehyde (5) Compound **5** was obtained by *O*-demethylation of compound **4** with boron tribromide in the following conditions: compound **4** (0.84 g, 3.7 mmol), anhydrous dichloromethane (10 mL), BBr_3 (4 mL), TBAB (1.4 g, 4.3 mmol), $Pd(OAc)_2$ (0.001 g, 0.0044 mmol) and water (18 mL). TLC analysis was performed with dichloromethane/methanol (9:1). Compound **5** was recrystallized from ethyl acetate/n-hexane. Yield (%): 60.7. ^{1}H NMR (DMSO-*d6*) δ 7.29 (d, *J* = 2.0 Hz, 1H, H(2)), 7.34 (m, 1H, H(4')), 7.40 (d, *J* = 2.0 Hz, 1H, H(6)), 7.44 (m, 2H, H(3'), H(5')), 7.58 (m, 2H, H(2'), H(6')), 9.59 (s, 1H, OH), 9.78 (s, 1H, CHO), 10.28 (s, 1H, OH). ^{13}C NMR (DMSO- *d6*) δ 113.41, 127.41, 128.45, 129.43 (2 x C(Ar)), 129.51, 129.71, 130.41 (2 x C(Ar)), 138.82, 147.44, 150.61, 192.77.

***(E)*-5-phenylferulic acid (7)** Compound 7 was obtained by a Knoevenagel-Doebner condensation between the aldehyde **4** and malonic acid in the following conditions: compound 4 (0.8 g, 3.3 mmol), malonic acid (0.8 g, 7.7 mmol), anhydrous pyridine (3.8 mL, 47.2 mmol) and piperidine (4 drops), stirred protected from light at 50˚C for 7 days. TLC analysis and flash chromatography were performed with dichloromethane/methanol (9:1). Compound **7** was recrystallized ethyl acetate/diethyl ether/n-hexane. Yield (%): 72.7. ^{1}H NMR (DMSO-*d6*) δ 3.91 (s, 3H, OCH_3), 6.46 (d, *J* = 15.9 Hz, 1H, H(α)), 7.18 (d, *J* = 1.9 Hz, 1H, H(2)), 7.33 (m, 1H, H(β)), 7.35 (d, *J* = 2.0 Hz, 1H, H(6)), 7.41 (m, 2H, H(3'), H(5')), 7.54 (m, 2H, H(2'), H(6')), 7.57 (m, 1H, H(4')), 9.20 (s, 1H, OH), 12.14 (s, 1H, COOH). ^{13}C (DMSO) δ 57.54, 110.71, 117.84, 125.41, 126.97, 128.24, 129.25, 129.31 (2 x C(Ar)), 130.53 (2 x C(Ar)), 139.01, 145.67, 147.12, 149.54, 169.37. ESI-MS: *m/z* 270 $[M + H]^+$. mp: [171 - 174]˚C.

***(E)*-5-phenylcaffeic acid (8)** Compound **8** was obtained by a Knoevenagel-Doebner condensation between the aldehyde **5** and malonic acid in the following conditions: compound **5** (1 g, 4.7 mmol), malonic acid (1.0 g, 9.61 mmol), anhydrous pyridine (5.0 mL, 61.8 mmol) and piperidine (4 drops), stirred protected from light at 50˚C for 7 days. TLC analysis and flash chromatography were performed with dichloromethane/methanol (9:1). Compound **8** was recristallized from ethyl acetate/n-hexane. Yield (%): 54.2. ^{1}H NMR (DMSO-*d6*) δ 6.23 (d, *J* = 15.9 Hz, 1H, H(α)); 7.07 (2H, s, H(2), H(6)), 7.31 (m, 1H, H(4')), 7.41 (m, 2H, H(3'), H(5')), 7.48 (d, *J* = 15.9 Hz, 1H, H(β)), 7.56 (m, 2H, H(2'), H(6')), 8.97 (s, 1H, OH), 9.89 (s, 1H, OH), 12.17 (s, 1H, COOH). ^{13}C NMR (DMSO-*d6*) δ 114.10, 116.97, 123.82, 126.70, 128.16, 129.29 (2 x C(Ar)), 129.79, 130.48 (2 x C(Ar)), 139.28, 145.91, 146.72, 147.16, 169.20. ESI-MS: *m/z* 257 $[M + H]^+$. mp: [194 - 197]˚C.

***(E)*-3,4,5-trihydroxycinnamic acid (9)** Compound **9** was obtained by Amberlyst 15® mediated deprotection of compound **16** in the following conditions: compound **16** (0.250 g, 0.543 mmol), wet Amberlyst 15 ® (500 mg) and $MeOH/H_2O$ 95:5 (15 mL). Flash chromatography was perfomed in cellulose with dichloromethane/methanol (9:1). TLC analysis and flash chromatography were performed with dichloromethane/methanol (9:1) and (8:2), respectively. Yield (%): 43. The spectroscopic data obtained is in accordance with the published data.[84]

***(E)*-*N*-hexyl-5-phenylferuloylamide (10)** Compound **10** was obtained by a PyBOP-assisted amidation of compound **7** with hexylamine in the following conditions: compound **7** (0.4 g, 1.47 mmol), hexylamine (0.194 mL, 1.47 mmol), PyBOP (0.770 g, 1.47 mmol), DIPEA (0.250 mL, 1.47 mmol), DMF (5 mL) and dichloromethane (5 mL). TLC analysis and flash chromatography were performed with dichloromethane/methanol (9:1). Yield: 39.01 %. ^{1}H NMR (DMSO-*d6*) δ 0.88 (s, 3H, CH_3), 1.27 (s, 6H, $(CH_2)_3$), 1.44 (s, 2H, $NHCH_2CH_2$), 3.16 (s, 2H, $NHCH_2$), 3.90 (s, 3H, OCH_3), 6.54 (d, *J* = 15.68 Hz, 1H, H(α)), 7.10 (s, 1H, H(2)), 7.18 (s, 1H, H(6)), 7.45 (m, 6H, 5xH(Ar), H(β)), 7.93 (s, 1H, NH), 9.15 (s, 1H, OH). ^{13}C NMR (DMSO-*d6*) δ 15.30, 23.45, 27.52, 30.51, 32.39, 40.03, 57.35, 110.62, 121.03, 123.81, 127.59, 128.26, 129.34, 129.39, 130.46, 139.20, 140.05, 146.30, 149.62, 166.62. EI-MS: *m/z* 354 $[M + H]^+$.

***(E)*-*N*-hexyl-5-phenylcaffeoylamide (11)** Compound **11** was obtained by a PyBOP-assisted amidation of compound **8** with hexylamine in the following conditions: compound **8** (0.24 g, 0.937 mmol), hexylamine (0.124 µL, 0.937 mmol), PyBOP (0.490 g, 0.937 mmol), DIPEA (0.160 mL, 0.937 mmol), DMF (3 mL) and dichloromethane (6 mL). TLC analysis and flash chromatography were performed with dichloromethane/methanol (9:1). Yield (%): 73.5. ^{1}H NMR ($CDCl_3$) δ (ppm): 0.87 (s, 3H, CH_3), 1.29 (s, 6H, $(CH_2)_3$), 1.44 (s, 2H, $NHCH_2CH_2$), 3.16 (s, 2H, $NHCH_2$), 3.90 (s, 3H, OCH_3), 6.54 (d, *J* = 15.68 Hz, 1H, H(α)), 7.10 (s, 1H, H(2)), 7.18 (s, 1H, H(6)), 7.45 (m, 6H, H(Ar), H(β)), 7.93 (s, 1H, NH), 9.15 (s, 1H, OH). ^{13}C NMR (DMSO-*d6*) δ (ppm): 15.60, 24.05, 27.50, 29.90, 33.30, 40.12, 114.13, 115.90, 123.83, 126.75, 128.20, 129.50 (2 x C(Ar)), 129.64, 130.47 (2 x C(Ar)), 139.7, 145.80, 146.71, 147.16, 166.56. ESI-MS: *m/z* 340 $[M + H]^+$.

***(E)*-*N*-hexyl-3,4,5-trihydroxycinnamoyl carboxamide (12)** Compound **12** was obtained after Amberlyst 15® mediated deprotection of compound **17** in the following conditions: compound **17** (0.576 g, 1.060 mmol), wet Amberlyst 15 ® (500 mg) and $MeOH/H_2O$ 95:5 (15 mL). Flash chromatography was perfomed in cellulose with dichloromethane/methanol (9:1). Yield (%): 48.02. ^{1}H NMR (DMSO-*d6*) δ 0.87 (t, *J* = 6.9 Hz, 3H, CH_3), 1.27 (m, 6H, $CONH(CH_2)_2(CH_2)_3$), 1.43 (m, 2H, $CONHCH_2CH_2$), 3.14 (m, 2H, $CONHCH_2$), 6.28 (d, *J* = 15.6 Hz, 1H, H(α)), 6.48 (s, 2H, H(2), H(6)), 7.13 (d, *J* = 15.6 Hz, 1H, H(β)), 7.94 (t, *J* = 5.6 Hz, 1H, CONH), 8.97 (s, H, 3xOH). ^{13}C NMR (DMSO-*d6*) δ 14.38, 22.52,

26.63, 29.65, 31.48, 39.09, 107.19, 119.20, 125.84, 135.60, 139.71, 146.58, 165.74. ESI-MS: *m/z* 280 $[M + H]^+$. mp (ºC): [96-100] ˚C.

***(E)-N*-hexylferuloylamide (13)** Compound **13** was obtained by a PyBOP-assisted amidation of compound **1** with hexylamine in the following conditions: compound **1** (1 g, 5.15 mmol), hexylamine (0.680 µL, 5.15 mmol), PyBOP (2.68 g, 5.15 mmol), DIPEA (0.882 mL, 5.15 mmol), DMF (10 mL) and dichloromethane (10 mL). TLC analysis and flash chromatography were performed with dichloromethane/methanol (9:1). Yield (%): 62.4. The spectroscopic data obtained is in accordance with the published data.[33]

***(E)-N*-hexylcaffeoylamide (14)** Compound **14** was obtained by a PyBOP-assisted amidation of compound **2** with hexylamine in the following conditions: compound **2** (1 g, 5.55 mmol), hexylamine (0,733 µL, 5.55 mmol), PyBOP (2,89 g, 5.55 mmol), DIPEA (0,951 mL, 5.55 mmol), DMF (10 mL) and dichloromethane (10 mL). TLC analysis and flash chromatography were performed with dichloromethane/methanol (9:1). Yield (%): 59%. The spectroscopic data obtained is in accordance with the published data.[33]

3,4,5-tri((2-methoxyethoxy)methoxy)benzaldehyde (15) Compound **15** was obtained following the general phenol protection protocol in the following conditions: compound **9** (1.067 g, 6.198 mmol), TBAI (0.343 g, 0.930 mmol), CH_2Cl_2 (15 mL), DIPEA (6.400 mL, 37.188 mmol) and MEMCl (3.185 mL, 27.891 mmol). TLC analysis and flash chromatography were performed dichloromethane/ethyl acetate (7:3) and dichloromethane until dichloromethane/ethyl acetate (1:4), respectively. Yield (%): 56.14. ^{1}H NMR ($CDCl_3$) δ 3.34 (m, 3H, OCH_3), 3.40 (m, 6H, 2x(OCH_3)), 3.56 (m, 6H, 3x(OCH_2CH_2O)), 3.85 (m, 4H, 2x(OCH_2CH_2O)), 4.97 (m, 2H, OCH_2CH_2O), 5.30 (s, 2H, OCH_2O), 5.34 (s, 4H, 2x(OCH_2O)), 7.42 (s, 2H, H(2), H(6)), 9.85 (s, 1H, CHO).^{13}C NMR ($CDCl_3$) δ 59.93, 59.96 (2xOCH_3), 69.07 (2x(OCH_2CH_2O)), 69.70, 72.41 (2x(OCH_2CH_2O)), 72.53, 95.27 (2x(OCH_2O)), 98.22, 112.50, 133.38, 142.78, 152.40, 191.76.

***(E)*-3,4,5-tri((2-methoxyethoxy)methoxy)cinnamic acid (16)** Compound **16** was obtained by a Knoevenagel-Doebner condensation between the aldehyde **15** and malonic acid in the following conditions: compound **15** (1.4553 g, 3.478 mmol), malonic acid (1.430 g, 13.636 mmol), anhydrous pyridine (5 mL, 59.501) and piperidine (5 drops) at 60˚C for 6 hours. TLC analytical control was performed using dichloromethane/methanol (9:1). The compound was used without further purification. Yield (%): 86.69. ^{1}H NMR (400 MHz, MeOD) δ 3.37 (s, 3H, OCH_3), 3.39 (s, 6H, 2x(OCH_3), 3.59 (m, 6H, 3x(OCH_2CH_2O)), 3.85 (m, 4H, 2x(OCH_2CH_2O), 3.98 (m, 2H, OCH_2CH_2O), 5.23 (s, 2H, OCH_2O), 5.29 (s, 4H, 2x(OCH_2O)), 6.34 (d, *J* = 15.9 Hz, 1H, H(α)), 7.10 (s, 2H, H(2), H(6)), 7.58 (d, *J* = 15.9 Hz, 1H, H(β)). ^{13}C NMR (101 MHz, MeOD) δ 58.84, 58.86 (2xOCH_3), 67.88 (2x(OCH_2CH_2O)), 68.61, 71.47 (2x(OCH_2CH_2O)), 71.59, 94.42 (2x (OCH_2O), 97.30, 110.51, 118.02, 130.73, 138.24, 144.84, 151.19, 169.00.

***(E)-N*-hexyl-3,4,5-tri((2-methoxyethoxy)methoxy)cinnamoylcarboxamide (17)**

Compound **17** was obtained by a PyBOP-assisted amidation of **16** with hexylamine in the following conditions: compound **16** (1.388g, 3.015 mmol), DMF (2.5 mL), DIPEA (0.400 mL, 3.028 mmol), PyBOP (1.58 g, 3.036 mmol), dichloromethane (8 mL) and hexylamine (0.520 mL, 3.035 mmol). TLC analysis and flash chromatography were performed in dichloromethane/methanol (9.5:0.5)). Yield (%): 47.69. ^{1}H NMR ($CDCl_3$) δ 0.90 (t, J = 6.9 Hz, 3H, $CONH(CH_2)_5CH_3$), 1.35 (m, 6H, $CONH(CH2)_2(CH_2)_3CH_3$), 1.57 (m, 2H, $CONHCH_2CH_2$), 3.47 (m, 11H, 3x(OCH3), $CONHCH_2$), 3.56 (m, 6H, 3x(OCH_2CH_2O)), 3.84 (m, 4H, 2x(OCH_2CH_2O)), 3.97 (m, 2H, OCH_2CH_2O), 5.22 (s, 2H, OCH_2O), 5.29 (s, 4H, 2x(OCH_2O)), 5.62 (t, *J* = 5.6 Hz, 1H, CONH), 6.30 (d, *J* = 15.5 Hz, 1H, H(α)), 7.06 (s, 2H, H(2), H(6)), 7.48 (d, J = 15.5 Hz, 1H, H(β)). ^{13}C NMR ($CDCl_3$) δ 14.02, 22.56, 26.63, 29.62, 31.51, 39.76, 67.91 (2xOCH_2CH_2O), 59.03 (3xOCH_3), 68.70, 71.54 (2xOCH_2CH_2O), 71.70, 94.39 (2xOCH_2O), 97.37, 109.92, 120.73, 131.27, 137.57, 140.25, 151.26, 165.72.

3.2 Enzymatic assays

3.2.1 AChE and BChE inhibition. The method of Ellman was followed.[49] The assay solution consisted of 0.1 M phosphate buffer pH 8, 400 µM 5,5'-dithiobis(2-nitrobenzoic acid), 0.05 U/mL AChE (AChE human recombinant, Sigma Aldrich), or 0.025 U/mL BChE (BChE from human serum, Sigma Aldrich) and 800 µM acetylthiocholine iodide or 500 µM butyrylthiocholine iodide as the substrate of the enzymatic reaction. The compounds were added to the assay solution and pre-incubated with the enzyme for 5 min at 30˚C. After that period, the substrate was added. The absorbance changes at 412 nm were recorded for 5 min with a UV microplate reader (Multiskan Spectrum, Thermo). The reaction rates were compared and the percentage of inhibition was calculated. The IC_{50} (*n* = 3) is defined as the concentration of each compound that corresponds to 50% of inhibition, relative to the control without inhibitor.

3.2.2 GSK-3β inhibition. Human recombinant GSK-3β and the prephosphorylated polypeptide substrate were purchased from Milipore (Milipore Iberica S.A.U.). Kinase-Glo Luminescent Kinase Assay was obtained from Promega (Promega Biotech Iberica, SL). All other reagents were purchased from Sigma Aldrich. The assay buffer contained 50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA and 15 mM magnesium acetate. The method of Baki *et al.*[88] was followed to attain the inhibition of GSK-3β. Kinase-Glo assays were performed in assay buffer using black 96-well plates. Briefly, 10 µL of test compound (10 µM) and 10 µL (20 ng) of enzyme were added to each well, followed by 20 µL of assay buffer containing 25 µM substrate and 1 µM ATP. The final DMSO concentration in the reaction mixture did not exceed 1%. After a 30 min incubation at 30˚C, the enzymatic reaction was stopped with 40 µL Kinase-Glo

reagent. Glow-type luminescence was recorded after 10 min using a FLUOstar Optima multimode reader (BMG Labtechnologies, GmbH, Offenburg, Germany). The activity is proportional to the difference of the total and consumed ATP. The inhibitory activities were calculated on the basis of maximal activities measure in the absence of inhibitor (n = 3).

3.3 Iron chelation capacity. The iron chelation capacity of the synthetized compounds was performed following the ferrozine method [66] using a BioTek Synergy HT plate reader to measure the absorbance of the $[Fe(Ferrozine)_3]^{2+}$ complex at 562 nm. All reagents were purchased from Sigma Aldrich. The assay was performed in ammonium acetate buffer (pH 6.7) using a 20 μM solution of ammonium iron (II) sulphate in ammonium acetate as the source of ferrous ions and EDTA as a standard chelator. All compounds were tested at 100 μM. In each well, a solution of the test compound (4 μL) and ammonium iron (II) sulphate in ammonium acetate (200 μL) were added, incubated for 10 min and the absorbance was read at 562 nm. Then an aqueous 5 mM solution of ferrozine was added to each well (4 μL, 96 μM final concentration), the plate was incubated at 37˚C for 10 min and the absorbance read at 562 nm. The final volume was 208 μL. Blank wells were run using DMSO instead of the test compounds. The absorbance of the first read was subtracted to the final values to discard any absorbance due to the test compounds. The results are expressed as mean % of iron chelation ± standard deviation (n = 3).

3.4 *In vitro* blood-brain barrier permeability. The parallel artificial membrane permeation assay (PAMPA) was performed following a previously described procedure. [73] Commercial drugs (testosterone, verapamil, imipramine, desipramine, promazine, corticosterone, piroxicam, hydrocortisone, caffeine, aldosterone and ofloxacin) and dodecane were purchased from Sigma Aldrich. Millex filter units (PVDF membrane, diameter 25 mm, pore size 0.45 μm) were acquired from Millipore. The porcine brain lipid (PBL) was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μm) and the acceptor 96-well microplate was an indented well, both from Millipore. The acceptor 96-well plate was filled with 180 μL (PBS/ethanol, 7:3) and the filter surface of the donor plate was impregnated with 4 μL of PBL in dodecane (20 mg/mL). The compounds were dissolved in PBS:ethanol (7:3) at 1mg/mL, filtered through a Millex filter and then added to the donor wells (180 μL). The donor filter plate was carefully placed over the acceptor plate to form a sandwich, which was left undisturbed for 4h at 25˚C. After the incubation, the donor plate was removed and the concentration of the compounds in the acceptor wells was determined by UV spectroscopy. Every sample was analysed at five wavelengths, in four wells and at three independent runs. The results are expressed as mean permeability coefficient ± standard deviation. In each experiment, 11 quality control standards of known BBB permeability were included to validate the analysis set.

3.5 Cell based studies. The human neuroblastoma cell line (SH-SY5Y) was obtained from the Amerucan Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dublecco's modified Eagle's medium (DMEM/F12) supplemented with fetal bovine serum (FBS, 10%), Glutamax ® (1%) and penicillin-streptomycin (1%) (reagents from Gibco) and grown at 37˚C in a humidified incubator with 5% CO_2. Cultures were seeded into 75 cm^2 flasks. Stock cultures were passed 1:20 once weekly. For cytotoxicity experiments of the test compounds alone, cells were seeded in 24-well plates (10^5cells/well) 24 hours prior to the additions and the incubated for another 24 hours with different concentrations of the compounds (1 μM and 10 μM) in FBS-free medium. For the neuroprotection studies, the cells were seeded in 24-well plates (10^5cells/well) 24 hours prior to the additions, pre-treated with the compounds (1 μM and 10 μM) in FBS-free medium for 1 hour and then incubated with 6-OHDA (30 μM, Sigma Aldrich) for 24 hours. Quantitative assessment of cellular viability was performed using the MTT assay, based on the ability of viable cells to reduce yellow MTT to purple formazan. The reduction of MTT is thought to mainly occur in the mitochondria through the action of succinate dehydrogenase, therefore providing a measure of mitrochondrial function.[89] After the final 24 hour incubations (compounds for cytotoxicity assay, compounds + neurotoxin for neuroprotection assay), the medium was discarded and each well was treated with MTT (0.5 mg/mL in PBS) and incubated for 2.5 hours at 37˚C under humidified 5% CO_2 air. The formazan crystals were then dissolved in DMSO and the absorbance was measured in a microplate reader (measuring filter 540 nm, reference filter 690 nm). The percentage of cellular viability for each entry was calculated relative to the control wells (untreated). The results are expressed as mean % cell viability ± standard deviation (n = 3).

3.6 Data analysis and statistics. The results in the previous sections are expressed as mean ± standard deviation of at least three different experiments (number of experiments n indicated for each case). Statistical comparisons between control and test groups were carried by one-way analysis of variance (ANOVA-1) followed by Dunnett comparison post-test (α = 0.05, 95% confidence intervals). Further details of specific analysis are expressed in the figures. Differences were considered to be significant for p values lower than 0.05. Plots and statistical analysis were performed using GraphPad Software, Inc. La Jolla, CA 92937, USA).

Conclusions

Lipophilic HCA derivatives that retain the biological properties of the parent compounds and display BBB-permeability have been successfully synthesised. Generally, these HCAs derivatives display a wide safety window and did not show significant cytotoxic effects at the tested concentrations. Moreover, some of them successfully prevented 6-OHDA-induced damage in a cellular model of ND and inhibited AChE in the low micromolar range, which may be significant to tackle memory impairment. Direct inhibition of GSK-3β was not observed under the tested conditions. Overall, the *N*-hexylcarboxamides **12**-**14** exhibited the superior results; in particular, compound **14** combines mild AChE inhibition, iron chelation, BBB permeability and encouraging neuroprotection against 6-OHDA. This compound can be used as a lead to develop neuroprotective agents with potential application in

ND. The depicted strategy can thus be a promising approach for the development of new chemical entities able to prevent or reduce the oxidative damage associated with ND.

Acknowledgements

The authors would like to thank the Foundation for Science and Technology (FCT) of Portugal (Pest-C/QUI/UI0081/2013) and QREN (FCUP-CIQ-UP-NORTE-07-0124-FEDER-000065) for funding and T. Silva (SFRH/BD/79671/2011) grant.

Notes and references

[a] CIQ/Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, 4196-007, Porto, Portugal.

[b] Addiction Biology Group, Institute for Molecular and Cell Biology, University of Porto, Rua do Campo Alegre s/n, 4150-180, Porto, Portugal.

[c] REQUIMTE/Laboratory of Toxicology, Department of Biological Sciences, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, 4050-313, Porto, Portugal.

[d] Instituto de Química Médica, CSIC, C/ Juán de la Cierva, 28006, Madrid, Spain.

[e] Present Adress: Centro de Investigaciónes Biológicas, CSIC, C/ Ramiro de Maeztu, 28040 Madrid, Spain.

†Corresponding author: fborges@fc.up.pt

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1 A. Wetzel, R.S Bon, K. Kumar and H. Waldmann, *Angew. Chem. Int. Ed. Engl.*, 2011, **50**, 10800-10826;

2 R.S Bon and H. Waldmann , *Acc. Chem. Res.*, 2011, **43**, 1103-14.

3 W. Wilk, T.J. Zimmermann, M. Kaiser and H. Waldmann, *Biol. Chem.*, 2010, **391**, 491-497.

4 C. Cordier, D. Morton, S. Murrison, A. Nelson and C. O'Leary-Steele, *Nat. Prod. Rep.*, 2008, **25**, 719-737.

5 H.R. El-Seedi, A.M. El-Said, S.A. Khalifa, U. Göransson, L. Bohlin, A.K. Borg-Karlson and R. Verpoorte, *J. Agr. Food Chem.*, 2012, **60**, 10877-10895.

6 H. Maeda and N. Dudareva, *Annu. Rev. Plant Biol.,* 2013, **63**, 73-105.

7 F.A. Silva, F. Borges, C. Guimarães, J.L. Lima, S. Reis, *J. Agr. Food Chem.*, **48**, 2122-2126.

8 Z. Sroka and W. Cisowsski, *Food Chem. Toxicol.*, 2003, **41**, 753-758.

9 F. Borges, C. Guimarães, J.L. Lima, I. Pinto, S. Reis, *Talanta*, 2005, **66**, 670.673.

10 S. Nair, W. Li and A.N. Kong, *Acta Pharmacol. Sin.* 2007, **28**, 459-472.

11 A. Gopalakrishnan and A.N Kong, *Food Chem. Toxicol*, 2008, **46**, 1257-1270.

12 C.T. Yeh, L.C. Ching and G.C Yen, *J. Nutr. Biochem.*, **20**, 163-171.

13 A. Scalbert, C. Manach, C. Morand, C. Rémésy, L. Jiménez, *Crit. Rev. Food Sci. Nutri.*, 2005, **45**, 287-306.

14 J.M. Landete, *Crit. Rev. Food Sci. Nutr.*, 2012, **52**, 936-948.

15 F. Visioli, C.A Lastra, C. Andres-Lacueva, M. Aviram, C. Calhau, A. Cassano, M. D'Archivio, A. Faria, G. Favé, V. Fogliano, R. Llorach, P. Vitaglione, M. Zoratti, M. Edeas, *Crit. Rev. Food Sci. Nutri.*, 2011, **51**, 524-546.

16 V. Jain, M.C. Langham and F.W. Wehrli, *J. Cereb. Flow Metab.*, 2010, **30**, 1596-1607.

17 T.A. Roualt, *Nat. Rev. Neurosci.*, 2013, **14**, 551-564.

18 M.T. Lin and M.F. Beal, *Nature*, 2006, **443**, 787-795.

19 J.C. Fernández-Checa, A. Fernández, A. Morales, M. Marí, C. García-Ruiz and A. Colell. *CNS Neurol. Disord. Drug Targets*, 2010, **9**, 493-454.

20 M. Sano, C. Ernesto, R.G. Thomas, M.R. Klauber, K. Schafer, M. Grundman, P. Woodbury, J. Growdon, C.W. Cotman, E. Pfeiffer, L.S. Schneider and L.J. Thal, *N. Engl. J. Med.* 1997, **336**, 1216-1222.

21 M.C. Morris, D.A. Evans, J.L Bienias, C.G. Tangney, D.A. Bennett, N. Aggarwal, R.S. Wilson and P.A. Scherr, *JAMA*, 2002, **287**, 3230-3237.

22 D.R. Galasko, E. Peskind, C.M. Clark, J.F. Quinn, J.M. Ringman, G.A. Jicha, C. Cotman, B. Cottrell, T.J. Montine, R.G. Thomas and Aisen P, *Arch. Neurol.*, 2012, **69**, 836-841.

23 M.J. Engelhart, M.I. Geerlings, A. Ruitenberg, J.C. Swieten, A. Hofman, J.C. Witteman and M.M. Breteler, *JAMA*, 2002, **287**, 3223-3229.

24 N. Farina, M.G. Isaac, A.R. Clark, J. Rusted and N. Tabet, *Cochrane Database Syst. Rev.*, 2012, **11**, CD002854.

25 L.A. Boothby and P.L. Doering PL, *Ann. Pharmacother.*, 2005, **39**, 2073-2080.

26 M. Laguerre, C. Wrutniak-Cabello, B. Chabi, L.J. López Giraldo, J. Lecomte, P. Villeneuve and G. Cabello, *J. Pharm. Pharmacol.*, 2011, **63**, 531-540.

27 C.D. Kamat, S. Gadal, M. Mhatre, K.S. Williamson, Q.N. Pye and K. Hensley K, *J. Alzheimers Dis.*, 2008, **15**, 473-493.

28 S.M. Jakovetić, B.Z. Jugović, M.M. Gvozdenović, D.I. Bezbradica, M.G. Antov, D.Z. Mijin ans Z.D. Knežević-Jugović, *Appl. Biochem. Biotechnol.*, 2013, **170**, 1560-1573.

29 M.P. Murphy and R.A. Smith, *Annu. Rev. Pharmacol. Toxicol.*, 2007, **47**, 629-656.

30 G. Pereira-Caro, B. Sarriá, A. Madrona, J.L. Espartero, L. Goya, L. Bravo and R. Mateos, *J. Agric. Food Chem.*, 2011, **59**, 5964-5976.

31 J.M. Calderón-Montaño, A. Madrona, E. Burgos-Morón, M.L. Orta, S. Mateos, J.L. Espartero, M. López-Lázaro, *J. Agric. Food Chem.*, 2013, **61**, 5046-5053.

32 J. Garrido, A. Gaspar, E.M. Garrido, R. Miri, M. Tavakkoli, S. Pourali, L. Saso, F. Borges and O. Firuzi, *Biochimie*, 2012, **94**, 961-967.

33 F.M. Roleira, C. Siquet, E. Orrù, E.M. Garrido, J. Garrido, N. Milhazes, G. Podda, F. Paiva-Martins, S. Reis, R.A. Carvalho, E.J. Silva and F. Borges, *Bioorg. Med. Chem.*, 2010, **18**, 5816-5825.

34 R. Sultana, *Biochim. Biophys. Acta*, 2012, **1822**, 748-752.

35 H. Pajouhesh and G.R. Lenz, *NeuroRx*, 2005, **2**, 541-553.

36 C.F. Lipinski, F. Lombardo, B. W. Dominy, and P. J. Feeney, *Adv. Drug Deliv. Rev.*, 2001, **46**, 3-26.

37 V. Nienaber, *Curr. Top. Med. Chem.*, 2009, **9**, 1688-1704.

38 R.B. Silverman¸ in *The Organic Chemistry of Drug Design and Discovery*; Academic Press; 2nd edition; 2004; pp-26-27.

39 R. Schliebs and T. Arendt, *Behav. Brain Res.*, 2011, **221**, 555-563.

40 R.M. Lane, M. Kivipelto and N.H. Greig, *Clin. Neuropharmacol.*, 2004, **27**, 141-149.

41 M.C. Dinamarca, M. Arrázola, E. Toledo, W.F. Cerpa, J. Hancke and N.C. Inestrosa, *Chem. Biol. Interact.*, 2008, **175**, 142-149.

42 J. Anwar, R.M. Spanevello, G. Thomé, N. Stefanello, R. Schmatz, J. Gutierres, J. Vieira, J. Baldissarelli, F.B. Carvalho, M.M. da Rosa, M.A. Rubin, A. Fiorenza, V.M. Morsch and M.R. Schetinger, *Pharmacol. Biochem. Behav.*, 2012, **103**, 386-94.
43 G. Oboh, O.M. Agunloye, A.J. Akinyemi, A.O. Ademiluyi and S.A. Adefegha, *Neurochem. Res.*, 2013, **38**, 413-419.
44 K.A. Khan, N. Kumar, P.G. Nayak, M. Nampoothiri, R.R. Shenoy, N. Krishnadas, C.M. Rao and J. Mudgal, *J. Pharm. Pharmacol.*, 2013, **65**, 1745-52.
45 S.B. Salem, A. Jabrane, F. Harzallah-Skhiri and H.B. Jannet, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 4248-4252.
46 R. Pi, X. Mao, X. Chao, Z. Cheng, M. Liu, X. Duan, M. Ye, X. Chen, Z. Mei, P. Liu, W. Li and Y. Han, *PLoS One*, 2012, **7**, e31921.
47 H. Zhang, S. Mak, W. Cui, W. Li, R. Han, S. Hu, M. Ye, R. Pi and Y. Han, *Neurochem. Int.*, 2011, **59**, 981-989.
48 X. Chao, X. He, Y. Yang, X. Zhou, M. Jin, S. Liu, Z. Cheng, P. Liu, Y. Wang, J. Yu, Y. Tan, Y. Huang, J. Qin, S. Rapposelli and R. Pi, Bioorg. Med. Chem. Lett., 2012, **22**, 6498-6502.
49 G.L. Ellman, K.D. Courtney, V Andres and R.M. Feather-Stone, *Biochem. Pharmacol.*, 1961, **7**, 88-95.
50 G.V. Rayasam, V.K. Tulasi, R. Sodhi, J.A. Davis and A. Ray, *Br. J. Pharmacol.*, 2009, **156**, 885-898.
51 F. Hernández, E.B.G. Barreda, A. Fuster-Matanzo, J.J. Lucas and J. Avila, *Exp. Neurol.*, 2010, **223**, 322-325.
52 C. Hooper, R. Killick and S. Lovestone, *J. Neurochem.*, 2008, **104**, 1433-1439.
53 Y. Huang, M. Jin, R. Pi, J. Zhang, M. Chen, Y. Ouyang, A. Liu, X. Chao, P. Liu, J. Liu, C. Ramassamy and J. Qin, *Neurosci. Lett.*, 2013, **535**, 146-151.
54 E.S. Lee, J.O. Lee, S.K. Lee, J.H. Kim, J.H. Jung, B. Keum, S.H. Park and H.S. Kim, *Life Sci.*, 2009, **84**, 755-759.
55 M. Liu, L. Fu, C. Liu, X. Xiong, X. Gao, M. Xiao, H. Cai, H. Hu, X. Wang and C. Mei, *Invest. New Drugs*, 2010, **28**, 783-790.
56 M. Kern, G. Pahlke, Y. Ngiewih and D. Marko, *J. Agric. Food Chem.*, 2006, **54**, 7041-7046.
57 J.J. Kim, Y. Tan, L. Xiao, Y.L. Sun and X. Qu, *Biomed. Res. Int.*, 2013, **2013**, 920128.
58 S. Miyai, A. Yamaguchi, T. Iwasaki, F. Shamsa and K. Ohtsuki, *Biol. Pharm. Bull.*, 2010, **33**, 1932-1937.
59 T. Polgár, A. Baki, G.I. Szendrei and G.M. Keseru, *J. Med. Chem.*, 2005, **48**, 7946-7959.
60 K. Jomova and M. Valko, *Curr. Pharm. Des.*, 2011, **17**, 3460-3473.
61 M. Wessling-Resnick, *Annu. Rev. Nutr.*, 2010, **30**, 105-122.
62 M. Valko, C.J. Rhodes, J. Moncol, M. Izakovic and M. Mazur, *Curr. Biol. Interact.*, 2006, **160**, 1-40.
63 K. Jomova and M. Valko, *Toxicology*, 2011, **283**, 65-87.
64 O. Weinreb, S. Mandel, M.B. Youdim and T. Amit t, *Free Rad. Biol. Med.*, 2013, **62**, 52-64.
65 V. Dias, E. Junn and M.M. Mouradian, *J. Parkinsons Dis.*, 2013, **3**, 461-491.
66 C.Y. Huang, R. Zhou, D.C. Yang and P.B. Boon Chock, *Biophys. Chem.*, 2003, **100**, 143-149.
67 G. Morabito, D. Trombetta, K.S. Brajendra, K.P. Ashok, S.P. Virinder, C. Naccari, F. Mancari, A. Saija, M. Cristani, O. Firuzi and L. Saso, *Biochimie*, 2010, **92**, 1101-1107.
68 P. Mladenka, K. Macáková, L. Zatloukalová, Z. Reháková, B.K. Singh, A.K. Prasad, V.S. Parmar, L. Jahodár, R. Hrdina and L. Saso, *Biochimie*, 2010, **92**, 1108-1114.
69 M.D. Engelmann, R. Hutcheson and I.F. Cheng, *J. Agric. Food Chem.*, 2005, **53**, 2953-2960.
70 M.T. Fernandez, M.L. Mira, M.H. Florêncio and K.R. Jennings, *J. Inorg. Biochem.*, 2002, **92**, 105-111.
71 P. Mladěnka, K. Macáková, T. Filipský, L. Zatloukalová, L. Jahodář, P. Bovicelli, I.P. Silvestri, R. Hrdina and L. Saso, *J. Inorg. Biochem.*, 2011, **105**, 693-701.
72 N.R. Perron and J.L. Brumaghim, *Cell Biochem. Biophys.*, 2009, **53**, 75-100.
73 L. Di, E.H. Kerns, K. Fan, O.J. McConnell and G.T. Carter, *Eur. J. Med. Chem.*, 2003, **38**, 223-232.
74 L. Di, E.H. Kerns, I.F. Bezar, S.L. Petusky and Y. Huang, *J. Pharm. Sci.*, 2009, **98**, 1980-1991.
75 A. Avdeef, S. Bendels, L. Di, B. Faller, M. Kansy, K. Sugano and Y. Yamauchi, *J. Pharm. Sci.*, 2007, **96**, 2893-2909.
76 R. Soto-Otero, E. Méndez-Alvarez, A. Hermida-Ameijeiras, A.M. Muñoz-Patiño and J.L. Labandeira-Garcia, *J. Neurochem.*, 2000, **74**, 1605-1612.
77 P. Pereira, P.A. Oliveira, P. Ardenghi, L. Rotta, J.A. Henriques J.N. Picada, *Basic Clin. Pharmacol. Toxicol*, 2006, **99**, 374-378.
78 S.E. Im, H. Yoon, T.G. Nam, H.J. Heo, C.Y. Lee and D.O. Kim, *J. Med. Food*, 2010, **13**, 779-784.
79 C.Y. Cheng, S.Y. Su, N.Y. Tang, T.Y. Ho, S.Y. Chiang and C.L. Hsieh, *Brain Res.*, 2008, **1209**, 136-150.
80 J.H. Sung, S.A. Gim and P.O. Koh, *Neurosci. Lett.*, 2014, **566**, 88-92.
81 M.P. Gleeson, *J. Med. Chem.*, 2008, **51**, 817-834.
82 N.E. Leadbeater and M. Marco, *J. Org. Chem.*, 2003, **68**, 888-892.
83 N. Milhazes, T. Cunha-Oliveira, P. Martins, J. Garrido, C. Oliveira, A.C. Rego and F. Borges, *Chem. Res. Toxicol.*, 2006, **19**, 1294-1304.
84 J. Teixeira, T. Silva, S. Benfeito, A. Gaspar, E.M. Garrido, J. Garrido and F. Borges, *Eur. J. Med. Chem.*, 2013, **62**, 289-296.
85 A. Gaspar, T. Silva, M. Yáñez, D. Vina, F. Orallo, F. Ortuso, E. Uriarte, S. Alcaro and F. Borges, *J. Med. Chem*., 2011, **54**, 5165-5173.
86 X. Lei and J.A. Porco, *J. Am. Chem. Soc.*, 2006, **128**, 14790-14791.
87 D. Michelot and M. Meyer, *Nat. Prod. Res.*, 2003, **17**, 41-46.
88 A. Baki, A. Bielik, L. Molnár, G. Szendrei and G.M. Keserü, *Assay Drug. Dev. Technol.*, 2007, **5**, 75-83.
89 D. Loebner, *J. Neurosci. Methods*, 2000, **96**, 147-152.

