ChemPubSoc Europe

DOI: 10.1002/cmdc.201300295

A Glutathione Derivative with Chelating and in vitro Neuroprotective Activities: Synthesis, Physicochemical Properties, and Biological Evaluation

Ivana Cacciatore,*[a] Catia Cornacchia,[a] Erika Fornasari,[a] Leonardo Baldassarre,[a] Francesco Pinnen,[a] Piera Sozio,[a] Antonio Di Stefano,[a] Lisa Marinelli,[a] Annalisa Dean,[b] Stefania Fulle,[c] Ester Sara Di Filippo,[c] Rita Maria Laura La Rovere,[c] Antonia Patruno,[d] Alessio Ferrone,[d] and Valerio Di Marco[b]

Metal-ion dysregulation and oxidative stress have been linked to the progressive neurological decline associated with neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. Herein we report the synthesis and chelating, antioxidant, and in vitro neuroprotective activities of a novel derivative of glutathione, GS(HQ)H, endowed with an 8-hydroxyquinoline group as a metal-chelating moiety. In vitro results showed that GS(HQ)H may be stable enough to be absorbed unmodified and arrive intact to the blood–brain barrier, that it may be able to remove Cu^{II} and Zn^{II} from the Aβ peptide without causing any copper or zinc depletion in vivo, and that it protects SHSY-5Y human neuroblastoma cells against H_2O_2- and 6-OHDA-induced damage. Together, these findings suggest that GS(HQ)H could be a potential neuroprotective agent for the treatment of neurodegenerative diseases in which a lack of metal homeostasis has been reported as a key factor.

Introduction

Oxidative stress, protein aggregation, and redox-active metal ions can be considered promising pharmacological targets for the treatment of neurodegenerative disorders, such as Parkinson's (PD) or Alzheimer's (AD) diseases.[1] In particular, metal homeostasis is altered during neurodegenerative diseases, and, as a consequence, metals are accumulated in various brain sections with concentrations increased three- to fivefold compared with age-matched controls.[2] Iron levels are higher than those expected during normal aging in both the substantia nigra pars compacta (SNpc) and in the cortex of patients affected by PD and AD, respectively. In fact, ferric iron deposits have been found in the normal protein aggregates typical of neurodegenerative brains: α-synuclein (Lewy bodies) in PD and β-amyloid peptide (Aβ) plaques and neurofibrillary tangles in AD.[3–5] Furthermore, elevated concentrations of Cu and Zn have been detected in amyloid plaques by spectroscopic studies.[6] Both copper and zinc are able to bind Aβ, thus promoting its aggregation and contributing to the production of reactive oxygen species (ROS) and oxidative stress. The same metals have been found in the cerebrospinal fluid (CSF) of patients affected by both AD and PD.[7]

Iron and copper can be considered a potential link between oxidative stress and deposition of protein aggregates in both PD and AD, as they are associated with ROS generation through Fenton reaction:

$$Fe^{II} \text{ or } Cu^{I} + H_2O_2 \rightarrow Fe^{III} \text{ or } Cu^{II} + OH^- + {}^{\bullet}OH$$

In this reaction, iron or copper react with H_2O_2 generated by dopamine metabolism by monoamine oxidase and catechol *O*-methyl transferase. The Fenton reaction leads to formation of the highly reactive hydroxyl radical (•OH), which induces oxidative stress in neurons by lipid membrane peroxidation, DNA damage, and protein oxidation or misfolding.[8,9]

Many reports support the hypothesis that modulation of biometals in the brain represents a promising therapeutic strategy for the treatment of neurodegenerative disorders, especially for PD and AD. Chelators can sequester free iron or copper and thereby prevent their ability to induce oxidative stress as a consequence of reactive hydroxyl radical generation.[10] Several compounds with chelating and antioxidant properties, including desferoxamine, clioquinol, VK-28, ebselen, and (−)-epigallactocatechin-3-gallate, showed neuroprotective activity in animal models of PD and AD.[11,12]

[a] Dr. I. Cacciatore, Dr. C. Cornacchia, Dr. E. Fornasari, Dr. L. Baldassarre, Prof. F. Pinnen, Dr. P. Sozio, Prof. A. Di Stefano, Dr. L. Marinelli
Department of Pharmacy, University "G. D'Annunzio"
Via dei Vestini 31, 66100 Chieti (Italy)
E-mail: cacciatore@unich.it

[b] Dr. A. Dean, Dr. V. Di Marco
Department of Chemical Sciences, University of Padova
Via Marzolo 1, 35131 Padova (Italy)

[c] Prof. S. Fulle, Dr. E. S. Di Filippo, Dr. R. M. L. La Rovere
Department of Neuroscience and Imaging, University "G. D'Annunzio"
Via dei Vestini 31, 66100 Chieti (Italy)

[d] Dr. A. Patruno, Dr. A. Ferrone
Department of Medicine and Aging Science, University "G. D'Annunzio"
Via dei Vestini 31, 66100 Chieti (Italy)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201300295.

Recent medicinal chemistry approaches for the treatment of PD and AD have focused on the development of multi-target-directed ligands (MTDLs), as a single molecule combining antioxidant capacity, chelating, or MAO-B inhibitory properties might be more effective than those directed to a single target.[13] Starting from these considerations, we recently synthesized multifunctional drugs that combine potent antioxidant, chelating, and neuroprotective properties in a single molecule for the treatment of PD and AD.[14–19]

Here, the aim of our study was to develop a novel antioxidant chelator with neuroprotective activity which could be considered as drug candidate for the treatment and/or prevention of neurodegenerative diseases. For this purpose, we chose 8-hydroxyquinoline (8-HQ) as chelating portion as: 1) it is able to cross the blood–brain barrier (BBB),[20] 2) it is a strong iron chelator with antioxidant property,[21–23] and 3) it is able to protect against the precipitation of β-amyloid plaques in the presence of Cu^{2+}, Fe^{3+}, and Zn^{2+} (compared to clioquinol), due to its ability to chelate these metals.[24] For an antioxidant scaffold, we selected the tripeptide GSH, as its metabolism is altered in neurodegenerated brain regions and its levels are decreased both in the SNpc of PD patients and in the cingulated cortex and substantia innominata of AD patients. It is generally accepted that restoring GSH levels may help to manage PD and AD.[25] There are many ways to increase GSH levels (e.g., *N*-acetylcysteine and other derivatives) but, in this context, we preferred the dual advantage of GSH: the antioxidant portion, in addition to directly or indirectly acting as a free radical scavenger, can be used as a carrier. In fact, GSH can be used as a BBB shuttle for the delivery of anti-PD or anti-AD drugs, as the presence of GSH transporters in the BBB is well documented.[26]

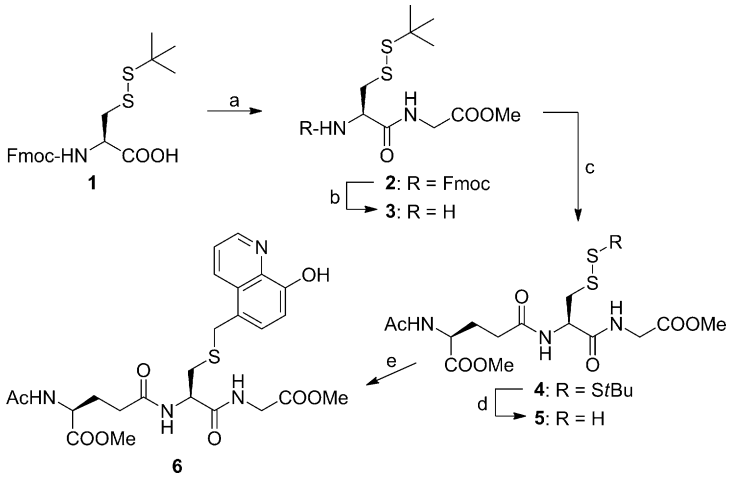
Considering the importance of developing novel antioxidant chelators with neuroprotective activity and their relevance in the treatment of neurodegenerative diseases, herein we report the synthesis of a novel *S*-alkyl GSH thioether (**6**) to enhance the antioxidant, neuroprotective, and chelating properties of GSH. In particular, we synthesized this alkyl sulfur, Ac-Glu[Cys(8-hydroxyquinolin-5-ylmethyl)-Gly-OMe]-OMe [GS(HQ)H, (**6**)], containing a tripeptide GSH linked by its sulfhydryl group to the hydroxymethyl moiety of 8-HQ. The lipophilicity of GSH was increased by conjugation to 8-HQ, acetylation of the amino group, and esterification of the two carboxylic groups of the tripeptide. We also pursued a study of the coordination properties of GS(HQ)H toward Fe^{III}, Zn^{II}, and Cu^{II}, which are involved in neurodegenerative diseases.

Moreover, to better understand the role of GS(HQ)H in protection against H_2O_2- and 6-OHDA-induced toxicity, we used an in vitro cell culture system consisting of differentiated SH-SY5Y cells, a CNS-origin cell line expressing both cholinergic and dopaminergic phenotypes. To investigate the role of GS(HQ)H in the cellular modulation of inflammatory response and immune effectiveness, we evaluated the effect of our compound in U937 cells, used as an in vitro monocytic model for the production of ROS.

Results and Discussion

Synthesis

The synthetic strategy employed to obtain compound **6** involved preparation of the suitably protected GSH peptide (**5**) and then condensation to 5-hydroxymethyl-8-hydroxyquinoline[27] by employing solution phase procedures (Scheme 1). The synthesis of Fmoc-Cys(S*t*Bu)-Gly-OMe (**2**) was achieved in 44% yield starting from readily available Fmoc-Cys(S*t*Bu)-OH (**1**) and H-Gly-OMe·HCl. Then, the treatment of **2** with 95% DBU in dichloromethane at room temperature gave dipeptide **3** in 40% yield. The fully protected tripeptide **4** was synthesized through a coupling reaction between **3** and Ac-Glu-OMe (prepared using a standard acetylation reaction starting from H-Glu-OMe[28]) using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC·HCl). Deprotection of the cysteine-SH group resulted from treatment of the corresponding protected precursor **4** for 1.5 h at room temperature with a small excess (1.2 equiv) of tri-*n*-butylphosphine in a water/*n*-propanol solution, made slightly alkaline (pH 8.5) by aqueous ammonia.[29] Finally, desired compound **6** was obtained in 96% yield, using mild conditions, via condensation of 5-hydroxymethyl-8-hydroxyquinoline[27] and the suitable protected tripeptide **5**.



Scheme 1. *Reagents and conditions*: a) H-Gly-OMe·HCl, HOBt, NMM, DCC, THF, 3 h, 0 °C, then 16 h, 5 °C; b) 95% DBU, CH_2Cl_2, 20 min, RT; c) Ac-Glu-OMe,[28] EDC·HCl, DIPEA, HOBt, DMF, 24 h, RT; d) *n*PrOH/H_2O, NH_4OH, P(*n*Bu)$_3$, 1.5 h, RT; e) 5-hydroxymethyl-8-hydroxyquinoline,[27] TFA, CH_2Cl_2, DMF, 10 min, −10 °C.

Conjugation of the free cysteine of GSH to the hydroxyquinoline moiety increases the lipophilicity of the molecule, acting as a GSH carrier to facilitate the diffusion of the compound into cells. In addition, esterification of the two carboxylic groups and acetylation of the amino group of the GSH backbone potentially decreases the overall polarity of the mole-

cule,[30] thus increasing the lipophilicity and membrane permeability of **6**.

In vitro pharmacokinetic studies

Solubility is important in the dissolution process of solid dosage forms; if a compound has a solubility lower than 100 μg mL^{-1}, it could have a poor dissolution rate and thus might be barely absorbed from the GI lumen.[31] The high solubility of GS(HQ)H (much higher than 100 μg mL^{-1}) indicates that our compound may rapidly dissolve in the aqueous GI tract. Because of its high water solubility, the log *D* value—determined by shake-flask method—is negative (Table 1).

Table 1. Physiochemical properties of GS(HQ)H (**6**).

Log *D*[a]			Water solubility[a]
pH 1.4	pH 7.4	pH 9.5	[mg mL^{-1}]
−1.36 ± 0.06	−1.35 ± 0.04	−1.8 ± 0.07	> 12 ± 0.42

[a] Values are means ± SD of three experiments.

Chemical stability and hydrolysis kinetics were determined by evaluating the hydrolysis rate in 0.02 M isotonic buffers (pH 1.3, 5.0, and 7.4) at 37 °C. Physiologically relevant pH values were chosen to study the hydrolysis of compound **6**. Our compound was more stable at pH 1.3 and 5.0 than at pH 7.4 ($t_{1/2} > 7$ h) (Table 2). Apparent first-order kinetics and

Table 2. Kinetic data for chemical and enzymatic hydrolysis of GS(HQ)H (**6**) at 37 °C.

pH 1.3[a]	pH 5.0[a]	pH 7.4[a]		Human plasma	
		$t_{1/2}$ [h]	k_{obs} [h^{-1}]	$t_{1/2}$ [h]	k_{obs} [h^{-1}]
stable	stable	15.37 (±0.32)	4.51×10^{-2} ($\pm9\times10^{-4}$)	8.5 (±0.39)	8.15×10^{-2} ($\pm4\times10^{-4}$)

[a] Data represent the mean of three experiments, with standard deviation given in parentheses.

rate constants were evaluated using initial rates of hydrolysis. The first-order degradation rate constant was determined by plotting the logarithm of co-drug concentration versus time. The hydrolysis of GS(HQ)H was also studied in 80% human plasma at 37 °C and showed that our compound may be stable enough to be absorbed unmodified and arrive intact at the BBB. The collected data show that **6** is chemically stable but can be activated to parent drug in the presence of plasma enzymes.[32,33] On the other hand, the activity of our compound depends not only on stability but also on significant solubility and membrane permeability; for this reason, a PAMPA assay was performed.[34]

The permeability of **6** was determined at pH 7.4 in the PAMPA-BBB assay, while in the PAMPA assay, it was measured at pH 5.0, 6.5, and 7.4 to mimic the physiological conditions of the GI tract.[35,36] As GS(HQ)H possesses high water solubility, we did not use any co-solvent to solubilize the new compound in the donor acceptor. The results (Table 3) showed that, after 2 h of incubation with the GI membrane, **6** displayed a high

Table 3. Permeability coefficient of GS(HQ)H (**6**) determined in the GI assay (2 h incubation) and BBB assay (18 h incubation).[a]

GI assay			BBB assay
pH 7.4	pH 6.5	pH 5.0	pH 7.4
160 nm s^{-1}	129 nm s^{-1}	75 nm s^{-1}	28 nm s^{-1}[b]

[a] The pH given is that of both donor and acceptor compartments. [b] CNS+ (high BBB permeation predicted), $P_e > 60$ nm s^{-1}; CNS+/− (BBB permeation uncertain), P_e: 20–60 nm s^{-1}; CNS− (low BBB permeation predicted), $P_e < 20$ nm s^{-1}.

permeability coefficient (P_e) and was able to cross the intestinal tract; nevertheless, after 18 h of incubation with the BBB membrane, this coefficient considerably decreased. For this reason, compound **6** has to be classified as CNS+/− (P_e from 60 to 20 nm s^{-1}).[36,37]

In vitro complexation studies

Iron, copper, and zinc accumulation in the brain are implicated in neurodegeneration associated with PD and AD. Therefore, by binding free metal ions (especially Fe, Cu, and Zn), metal chelators could prevent metal-mediated toxicity and interfere with Fenton and Haber–Weiss reactions affecting the neurodegeneration process.

To examine the metal binding properties of the new antioxidant chelator **6**, it was first necessary to determine the acid–base properties of the ligand. The acid–base properties of 8-HQ and of GS(HQ)H are listed in Table 4. The first acidity constant refers to the deprotonation of the quinolinic nitrogen, and the second constant refers to the deprotonation of the phenolic oxygen. The pK_{a2} could not be calculated for GS(HQ)H because of its hydrolysis at basic pH. The pK_{a1} is 0.35 log units higher for 8-HQ than for GS(HQ)H, which represents a rather

Table 4. Acid–base constants and metal–ligand stability constants of metal–ligand complexes GS(HQ)H (**6**) and 8-hydroxyquinoline (8-HQ).[a]

Reaction	Log *K*	
	8-HQ	GS(HQ)H (**6**)
$HL + H^+ \rightleftharpoons H_2L^+$	4.91 ± 0.02	4.56 ± 0.02
$L^- + H^+ \rightleftharpoons HL$	9.55 ± 0.03	–
$Cu^{2+} + HL \rightleftharpoons CuL^+ + H^+$	2.15 ± 0.09	1.0 ± 0.1
$Zn^{2+} + HL \rightleftharpoons ZnL^+ + H^+$	−1.15 ± 0.01	−0.52 ± 0.06
$Fe^{3+} + HL \rightleftharpoons FeL^{2+} + H^+$	2.29 ± 0.05	6.37 ± 0.08
$Fe^{3+} + HL \rightleftharpoons FeLH_{-1}^+ + H^+$	−1.69 ± 0.06	2.24 ± 0.09
$Fe^{3+} + 2HL \rightleftharpoons FeL_2^+ + 2H^+$	4.23 ± 0.1	–

[a] Reaction conditions: $T = 25$ °C, [NaCl]: 0.15 M; L^- represents the fully deprotonated form of both **6** and 8-HQ; data represent the mean ± SD of $n = 4$ titrations performed in duplicate.

significant difference, taking into account that the deprotonating groups are the same in both molecules. The higher pK_{a1} of GS(HQ)H can be partially explained by an inductive effect exerted by the GSH substituent and partly by assuming that the tripeptide group may form intramolecular hydrogen bond(s) with the phenolic hydroxy group, thus favoring deprotonation of the quinolinic nitrogen with respect to 8-HQ.

The metal–ligand stability constants of the complexes formed by 8-HQ and GS(HQ)H with Zn^{II}, Cu^{II}, and Fe^{III}, are also reported in Table 4. In the case of Zn^{II} and Cu^{II}, the same simple speciation is obtained for both 8-HQ and GS(HQ)H: the sole complex ML^+ is observed in solution. Similar speciations and stability constants of ZnL^+ and CuL^+ are also reported in the literature for 8-HQ. For example, the log*K* values for ZnL^+ (defined as in Table 4) vary from −1.4 to −0.2,[38] whereas those for CuL^+ are between 2.3 and 3.3.[39]

For all mixtures containing 8-HQ and Cu^{II} or Zn^{II}, the formation of a precipitate near pH 4 was observed during the titrations. These 8-HQ precipitates are the neutral complexes ZnL_2 and CuL_2, which are known to be poorly soluble in aqueous solution; for example, their precipitation products (pK_a values) are between 29.6 and 30.4 for Cu^{II}, and 23.5 for Zn^{II}.[40,41] Precipitation in the titrations of Cu^{II}-GS(HQ)H and Zn^{II}-GS(HQ)H mixtures was also observed at the same pH range (~4) as for 8-HQ; it is therefore likely that these precipitates are again the neutral ZnL_2 and CuL_2 complexes formed by GS(HQ)H. Precipitation of these complexes might not occur in vivo at neutral pH. Assuming that the solubility constants of ZnL_2 and CuL_2 are the same as for 8-HQ complexes, and with a 10^{-5} M total ligand concentration, it follows that the zinc and copper complexes precipitate only if the metal ions exceed 10^{-5}–10^{-6} M and if there is no other endogenous ligand favoring metal solubilization.

The Fe^{III}–ligand speciation is more complicated. Together with FeL^{2+}, also $FeLH_{-1}^{+}$ was detected for both 8-HQ and GS(HQ)H. $FeLH_{-1}^{+}$ is a hydroxo species produced by the deprotonation of FeL^{2+} at one of the coordinated water molecules. At pH greater than 4, precipitation of $Fe(OH)_3$ was observed in all solutions, according to its very low solubility product. Few speciation models are available in the literature for Fe^{3+}–8-HQ and do not agree with each other.[39] An FeL^{2+} species is always reported, but its stability constants range by 1.5 orders of magnitude (log*K* values) between 2.7 and 4.1. FeL_2^{+} was occasionally detected, and the values for its constant are extremely dispersed, varying from 4.5 to 7.2. The hydroxo species $FeLH_{-1}^{+}$ was never detected. The inclusion of this species in the present speciation models was forced by the fitting results. This inclusion likely caused a decrease in the other Fe^{III}–8-HQ constants which, in fact, fall into the lower range of those previously determined. For GS(HQ)H, no FeL_2^{+} complex was detected. This may be due to steric hindrance of the GSH substituent of the ligand bound to Fe^{3+}, which does not allow addition of a second ligand molecule to the metal center. The role of GSH also appears to be very important in enhancing complex stability strength, as the Fe^{3+}–GS(HQ)H complexes are more stable than the Fe^{3+}–8-HQ complexes. Enhancement of the hard character of the quinolinic chelator may be due to the abovementioned intramolecular hydrogen bond(s) formed by GS(HQ)H with the phenolic OH.

Faller and Hureau[42] proposed calculation of the dissociation constant K_D for the given metal–chelator complexes ($K_D = [M]_{free} \cdot \Sigma[H_xL]/\Sigma[M_wH_yL_z]$, computed at pH 7.4 for $C_M = 25$ μM, $C_L = 50$ μM), in order to state if, from a chemical point of view, the proposed ligand is a suitable chelator for neurodegenerative diseases. To this aim, the metal–chelator K_D is compared with the corresponding value of the metal–Aβ-peptide complex. According to these authors, calculation of the K_D is particularly useful for Cu^{II} and for Zn^{II}, as these ions are proven to interact in vivo with the Aβ-peptide. K_D values for 1:1 of Cu^{II}–Aβ-peptide and Zn^{II}–Aβ-peptide complexes reported in the literature vary with the type of buffer used in the measurements and the methods employed for their determination and are 0.01–100 nM and 1–20 μM, respectively.[42]

A good metal chelator for neurodegenerative diseases should have lower K_D values to be able to extract the metal ion from the Aβ-peptide, but they cannot be much lower, in order to avoid massive copper and zinc depletion from the brain. According to Faller and Hureau,[42] recommended K_D values should be ~1–10 pM and 0.1–10 μM for Cu^{II} and Zn^{II} chelators, respectively. The experimental K_D values calculated for the investigated Cu^{II}–ligand systems are 280 pM for both ligands, whereas those calculated for Zn^{II}–ligand systems are 0.57 μM. These values suggest that 8-HQ and GS(HQ)H are likely unable, or only partially able, to remove Cu^{II} from the Aβ-peptide. On the other hand, the quinolinic ligands should be able to remove Zn^{II} from the Aβ-peptide without causing any zinc depletion in the brain, as the experimental K_D values for this ion are within the recommended range.

In vitro antioxidant and neuroprotective studies

Prompted by the results obtained for GS(HQ)H as a metal binding chelator, we used human SH-SY5Y neuroblastoma cells differentiated with RA (retinoic acid) and RA/phorbol 12-myristate 13-acetate (PMA)—to obtain the cholinergic and dopaminergic neuronal phenotypes, respectively—to study its antioxidant and neuroprotective effects following two chemical insults, such as from H_2O_2 and 6-OHDA.[43]

Initially, to define a suitable concentration range, the effects on cell proliferation of GSH, HQ, and GS(HQ)H were evaluated using the colorimetric MTT assay. The dose–response experiments suggested that GSH, HQ, and GS(HQ)H (at concentrations of 1, 10, and 100 μM) added to cells had no effect on their proliferative capacity during a 24 h incubation period. As there was no toxic effect on cell viability (data not shown), the compound concentration used in MTT in vitro experiments was 1 μM, the highest dose that demonstrated no cytotoxic effects.

The antioxidant profile of GS(HQ)H was evaluated using human SH-SY5Y neuroblastoma cells differentiated with RA (10 μM) and lesioned with increasing concentrations (25, 150, 300 μM) of H_2O_2 (Figure 1). Results showed that both GSH and GS(HQ)H had a considerable antioxidant effect in RA-differentiated cells, but GS(HQ)H exhibited a major neuroprotective ca-

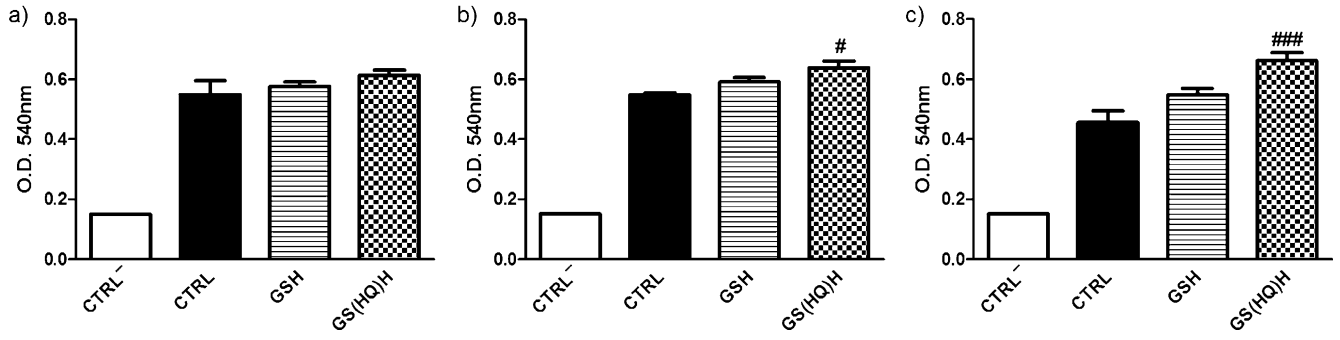


Figure 1. MTT reduction assay in RA-differentiated and H_2O_2-lesioned SHSY-5Y human neuroblastoma cells in the presence of GSH (1 μM) and GS(HQ)H (1 μM). Cells were differentiated with RA (10 μM) for 7 days, and were then incubated with test compounds for 24 h before and during a 24 h incubation period with increasing concentrations [a) 25, b) 150, or c) 300 μM] of H_2O_2. After this period, cell viability was quantified by measuring MTT reduction. CTRL⁻: control without toxic agent; CTRL (cells only in the presence of H_2O_2). Mean values were derived from three different experiments (each with $n=16$; $^{###}p<0.0001$, $^{##}0.0001<p<0.001$, $^{#}0.001<p<0.05$, n.s. $p>0.05$). #: SH-SY5Y human neuroblastoma cells treated with a combination of neuroprotective compound and toxin.

pability at all concentrations of H_2O_2, especially at higher concentrations. In Figure 1, we omitted the effect of H_2O_2 in the presence of HQ because, as previously observed, the simultaneous presence of the two compounds have a synergic toxic effect.[27] HQ, as a metal chelator of iron, copper, and zinc (transition metals that easily react with ROS), avoids the Haber–Weiss reaction between the metal and the superoxide anion; under these oxidative stress conditions, superoxide anion is overproduced, thereby damaging cells. On the other hand, H_2O_2 directly produces a high quantity of ROS, further damaging cells. These two combined actions determine a deleterious synergic effect on cells; for this reason, in the following experiment (Figure 2), we assayed only GSH and GS(HQ)H compounds.

To accurately measure ROS and the cellular capability to counteract the H_2O_2 insult, we used cell-permeable fluorescent and chemiluminescent probes. 2′,7′-Dichlorodihydrofluorescein diacetate (HDCF-DA) is one of the most widely used techniques for directly measuring the redox state of a cell.[44] In the experiment reported in Figure 2, we observed that the oxidant insult, represented by H_2O_2 against differentiated cells toward a cholinergic phenotype, was strongly counteracted by the tested compounds, which were stable over the observation time. In particular, our results showed that at t_0–t_5, the tested concentrations of H_2O_2, GSH, and mainly GS(HQ)H, exerted an antioxidant effect reducing ROS levels (Figure 2). GS(HQ)H, restoring the fluorescence values close to those of cells without H_2O_2, showed a significantly higher antioxidant capacity with respect to GSH alone.

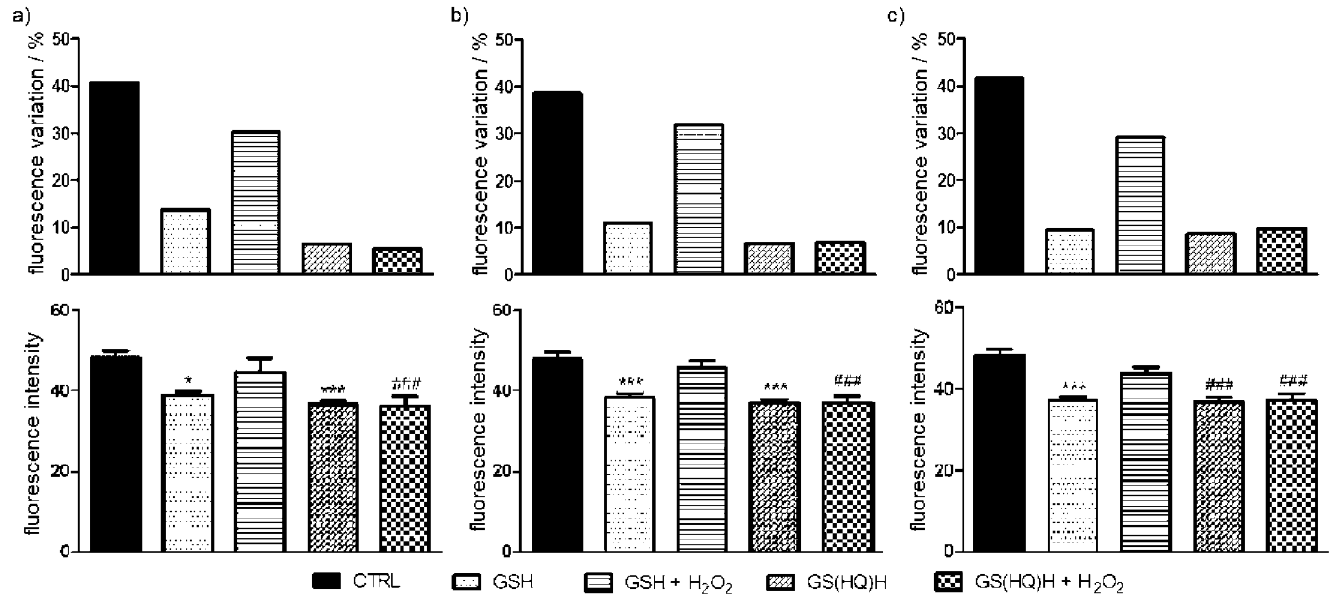


Figure 2. Quantitative analyses of ROS expression. ROS measurement by DCFH-DA fluorescence in RA-differentiated SH-SY5Y human neuroblastoma cells exposed to GSH (1 μM) or GS(HQ)H (1 μM) 24 h before and during a 5 min incubation period with 100 nM H_2O_2. Shown is the percent fluorescence variation relative to control at three points during the time course: a) t_0, b) $t_{2.3}$, and c) t_5. CTRL: control with H_2O_2; mean values ± SEM are derived from two different experiments (each with $n=10$).

The concentration of superoxide anion radical ($O_2^{\bullet-}$) in SH-SY5Y neuroblastoma cells was determined by spectrophotometric method using a semi-quantitative nitroblue tetrazolium (NBT) assay.[45] This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane-permeable, water-soluble, yellow-colored NBT (Y-NBT) by $O_2^{\bullet-}$. There is linear correlation between Y-NBT formation and the concentration of $O_2^{\bullet-}$.

The NBT test showed that 8-HQ, as a metal chelator of iron, copper, and zinc—transition metals that react easily with ROS—does not react with the superoxide anion, leaving it free and available to react with NBT, thus generating more formazan (Figure 3). This hypothesis would explain the higher level of formazan in the presence of only 8-HQ. Furthermore, in Figure 3, data showed the typical antioxidant capacity of GSH and no significant antioxidant effect of GS(HQ)H with respect to GSH alone. The lowered antioxidant capability of GS(HQ)H could be due to the alkylated sulfhydryl group, which is not able to directly act as a scavenger of $O_2^{\bullet-}$.

The neuroprotective profile of GS(HQ)H was evaluated in RA/PMA-differentiated SH-SY5Y neuroblastoma cells using 6-OHDA as a neurotoxic agent (Figure 4). 6-OHDA is a potent inhibitor of the mitochondrial respiratory chain complexes I and IV; it induces apoptosis by enhancing the generation of mitochondrial ROS.[46] The cells were treated with GSH, HQ, and GS(HQ)H for 1 h, and then exposed to increasing concentrations of 6-OHDA (25, 50, 75, 150 μM). After a further 24 h of incubation, the cultures were assessed for viability by MTT assay (Figure 4). Results showed that GS(HQ)H had a significant neuroprotective capability, counteracting the toxic effect at all tested concentrations of 6-OHDA.

All of our data confirmed that GS(HQ)H was able to combat in vitro oxidative stress, showing good antioxidant and neuroprotective capabilities both in the cholinergic and dopaminergic phenotypes exposed to different toxic insults. In particular, GS(HQ)H showed a remarkable direct protective ability against oxidative stress, counteracting the H_2O_2 insult in cholinergic differentiated cells. Moreover, GS(HQ)H was able to successfully block the 6-OHDA-induced free radical formation in dopaminergic differentiated cells.

A study of the protective functions of GSH on inhibition of the inflammatory response and correction of the important oxidant/antioxidant imbalance in patients with neurodegenerative diseases led us to further investigate the role of our compounds in the cellular modulation of inflammatory response, antioxidant capability, and immune effectiveness. Thus, we evaluated the effect of GSH,

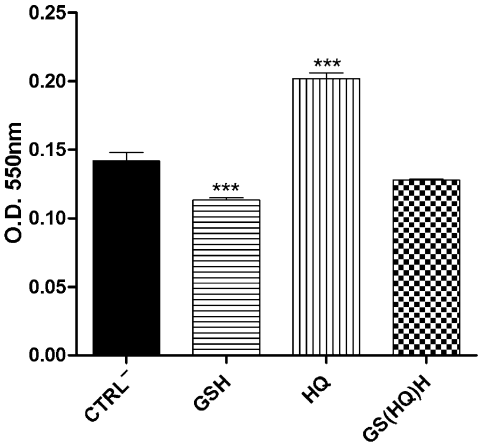


Figure 3. Antioxidant activity of GSH, HQ, and GS(HQ)H against oxidative stress measured by the NBT test using undifferentiated SH-SY5Y neuroblastoma cells. Shown are results obtained after 24 h of incubation with GSH (1 μM), HQ (1 μM), or GS(HQ)H (1 μM). CTRL⁻: control without toxic agent. Mean values ± SEM were derived from three different experiments (each with $n = 10$; ***$p < 0.0001$, **$0.0001 < p < 0.001$, *$0.001 < p < 0.05$, n.s. $p > 0.05$). ∗: SH-SY5Y human neuroblastoma cells treated only with the neuroprotective compound.

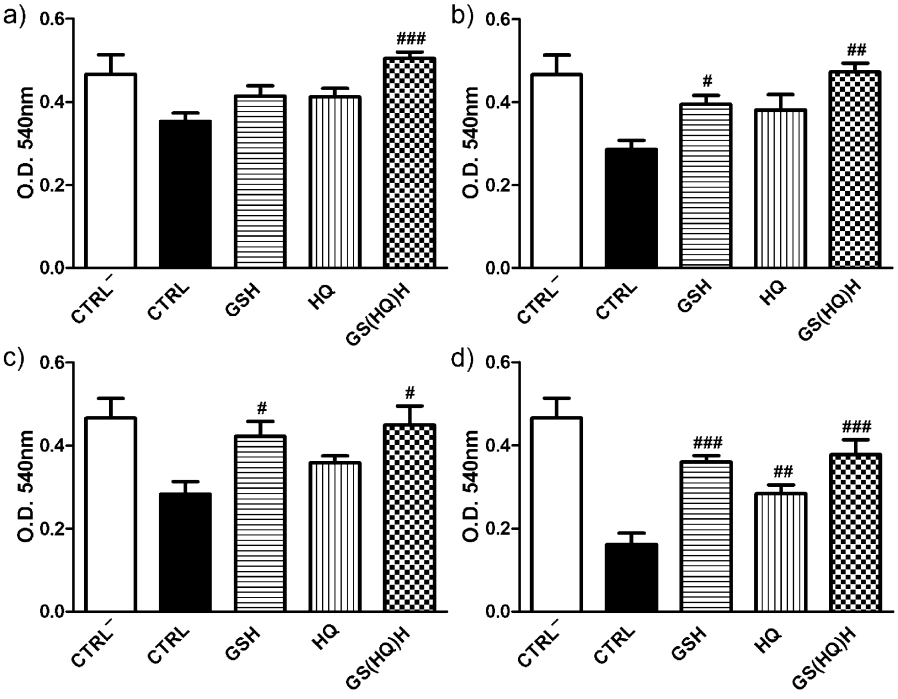


Figure 4. Neuroprotective effect of GSH (1 μM), HQ (1 μM), and GS(HQ)H (1 μM) in RA/PMA-differentiated and 6-OHDA-lesioned SH-SY5Y human neuroblastoma cells. Cells were incubated with test compounds 1 h before and during a 24 h incubation period with increasing concentrations of 6-OHDA: a) 25, b) 50, c) 75, and d) 150 μM. After this period, cell viability was quantified by measuring MTT reduction. CTRL⁻: control without toxic agent; CTRL: control with 6-OHDA. Mean values ± SEM were derived from three different experiments (each with $n = 16$; ###$p < 0.0001$, ##$0.0001 < p < 0.001$, #$0.001 < p < 0.05$, n.s. $p > 0.05$). #: SH-SY5Y human neuroblastoma cells treated with a combination of neuroprotective compound and toxin.

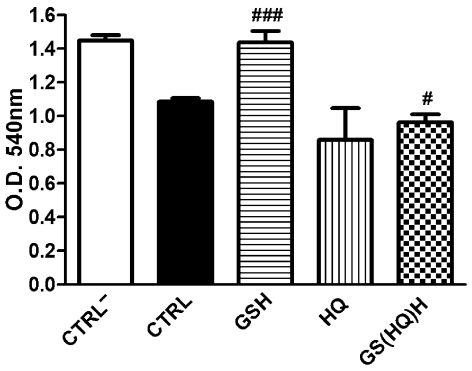


Figure 5. Cell proliferation effects of GSH (100 μM), HQ (100 μM), and GS(HQ)H (100 μM) in the U937 cell line, measured by MTT assay. Cells were pre-incubated for 40 min with the compounds and then treated with H_2O_2 (500 μM) for a further 2 h. CTRL⁻: control without toxic agent; CTRL: control with H_2O_2. Mean values ± SEM were derived from three different experiments (each with $n = 12$; $^{###}p < 0.0001$, $^{##}0.0001 < p < 0.001$, $^{#}0.001 < p < 0.05$, n.s. $p > 0.05$). #: SH-SY5Y human neuroblastoma cells treated with a combination of the neuroprotective compound and the toxin.

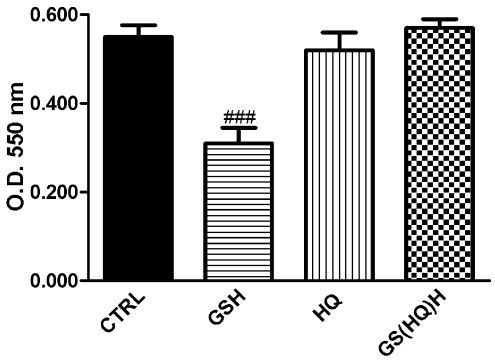


Figure 6. Antioxidant capacity of GSH (100 μM), HQ (100 μM), and GS(HQ)H (100 μM) against oxidative stress assayed by the NBT test using U937 cells. Cells were pre-incubated for 40 min with the compounds and then treated with H_2O_2 (500 μM) for a further 2 h. CTRL: control with H_2O_2. Mean values ± SEM were derived from three different experiments (each with $n = 12$; $^{###}p < 0.0001$, $^{##}0.0001 < p < 0.001$, $^{#}0.001 < p < 0.05$, n.s. $p > 0.05$). #: SH-SY5Y human neuroblastoma cells treated with a combination of the neuroprotective compound and the toxin.

HQ, and GS(HQ)H in U937 cells, used as a monocytic model capable of generating ROS upon challenge (Figure 5).[47,48]

Firstly, we analyzed U937 cellular vitality in the presence of our compounds (1, 10, and 100 μM) for 24 h. There was no effect on cellular viability (data not shown); thus, the compound concentration used in these in vitro experiments was 100 μM, the highest dose with no cytotoxic effects. Then, we investigated the effects of GSH, HQ, and GS(HQ)H on U937 cells under strong oxidative stress conditions (U937 cells stimulated for 2 h with H_2O_2 at a concentration of 500 μM). In this toxicity model, cells incubated with 500 μM H_2O_2 had significantly decreased cell viability (Figure 5). The viability increased in U937 cells pre-treated with GSH relative to control cells. Conversely, for both compounds HQ and GS(HQ)H, antiproliferative activity was observed, not showing significant differences relative to the control. Also in this experiment, we observed the same synergic toxic effect of the combined administration of HQ and H_2O_2 as previously reported.[27]

The antioxidant capacity of GSH, HQ, and GS(HQ)H against strong oxidative stress conditions was also assayed by the NBT test in U937 cells (Figure 6). As expected, the ability of cells to reduce NBT was markedly increased in the presence of GSH compared with the control, while both HQ and GS(HQ)H were not able to affect ROS reduction in H_2O_2-stimulated U937 cells.

Different results were obtained following the two toxic insults, 6-OHDA and H_2O_2, and two different cellular lines, human SHSY-5Y neuroblastoma and U937 cells. Our data confirmed that GS(HQ)H was able to counteract an overproduction of ROS, showing good antioxidant capability in both the cholinergic and dopaminergic cells exposed to H_2O_2 and 6-OHDA, respectively. This behavior can be explained on the basis that the synergic effect of GSH and HQ is more evident when the two molecules are combined with respect to the individual compounds. GS(HQ)H likely exerts its action through a direct mechanism of metal chelation, due to the HQ moiety, and an antioxidant mechanism against H_2O_2- and 6-OHDA-induced free radical formation.

On the other hand, our compound did not show the same antioxidant profile in the U937 cellular line. The U937 cells may have been subjected to an H_2O_2 concentration that was too high (500 μM), suggesting a decreased capacity of GS(HQ)H to counteract the reactive oxygen species formed. Under strong oxidative stress conditions, the direct antioxidant activity was confirmed by GSH alone, likely due to its free thiol group. Our conjugate contains a covalent bond between the sulfhydryl group of GSH and 8-HQ, which is not a cleavable linkage and is difficult to be hydrolyzed by enzymes. In this case, the chelating and antioxidant properties of GS(HQ)H are not sufficient to contrast high H_2O_2 concentrations; direct antioxidant action through the sulfhydryl group of cysteine seems to be necessary, as confirmed by GSH alone. In particular, our compound—containing a phenolic portion such as 8-hydroxyquinoline—possesses free radical-scavenging and antioxidant activities, as it may participate in radical-scavenging reactions as an electron donor of the hydroxy group to form stable radicals. In spite of this, the capacity of the free thiol group of GSH in ROS scavenging activity is stronger than that of the hydroxy group of 8-hydroxyquinoline; thus, it is able to counteract strong oxidative stress conditions applied to U937 cells.

Finally, to assess whether GS(HQ)H is able to permeate into the cells, SH-SY5Y human neuroblastoma cells were incubated with a solution of GS(HQ)H (1 μM) for 24 h at 37 °C. At different time points, the medium was deproteinized and analyzed by HPLC. Our results showed that, by prolonging the incubation time, the concentration of drug in the medium decreased: this decrease depended not only on chemical degradation at pH 7.4, but also on the permeation of drug through the cell membrane, which was faster than we expected (Figure 7). On the contrary, by increasing incubation time, the intracellular drug concentration was increased. These data demonstrate the ability of GS(HQ)H to penetrate the cells.

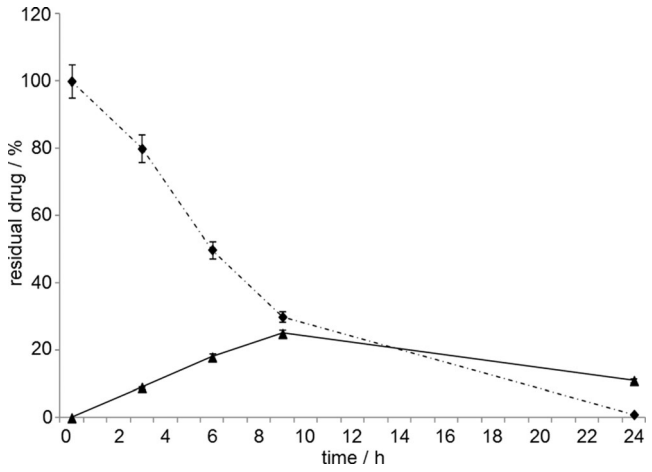


Figure 7. Percentage of drug GS(HQ)H in the cell culture medium (–•–•◆–•–•) and in cells (——▲——) at various incubation times. Data are expressed as mean values ± SEM; each experiment was performed in triplicate.

Conclusions

In this work, we report the synthesis and in vitro pharmacokinetic, chelating, and neuroprotective properties of a novel GSH derivative containing antioxidant, chelating, and neuroprotective moieties with the aim of creating a multi-target therapy to increase neuronal protection and prevent the progression of neurodegeneration. In vitro cellular studies showed that our compound markedly prevents cellular death against H_2O_2-induced oxidative stress and 6-OHDA-induced neurotoxicity. GS(HQ)H might be therapeutically useful as it is stable at pH 7.4 with a $t_{1/2}$ value equal to 8.5 h; this time is sufficient to reach the BBB without undergoing peripheral metabolism prior to CNS uptake. On the other hand, the PAMPA test showed a low value of BBB permeability for GS(HQ)H, but the presence of GSH transporters in the BBB could certainly improve the uptake. Moreover, the good chelating properties of GS(HQ)H suggest that it may be able to partially and quantitatively remove Cu^{II} and Zn^{II}, respectively, from the Aβ-peptide without causing any copper or zinc depletion in vivo.

In conclusion, the antioxidant and neuroprotective profile of GS(HQ)H suggests that it could be a novel candidate for in vivo studies to further investigate its effects in those pathologies characterized by oxidative stress and lack of metal homeostasis, such as neurodegenerative diseases.

Experimental Section

Chemistry

General procedures: H-Gly-OMe·HCl, H-Glu-OMe, and Fmoc-Cys(S*t*Bu)-OH were purchased from Bachem. Human SH-SY5Y neuroblastoma cells (EGACC) were obtained from Sigma–Aldrich (UK). All other chemicals were of the highest purity commercially available. Chromatographic purifications were performed on silica gel using column chromatography (Merck 60, 70–230 mesh ASTM silica gel), and compounds were detected with UV light ($\lambda = 254$ nm). Optical rotations were taken at 20 °C with a PerkinElmer 241 polarimeter. IR spectra were recorded with a Varian 1000 FT-IR spectrometer. NMR spectra were recorded with a Varian VXR-300 spectrometer. Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane (Me_4Si). Mass spectra were obtained by electrospray ionization (ESI) in positive mode using a LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300 °C and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N_2) as both the sheath gas and the auxiliary gas. The LC–MS–MS instrument was an Agilent (Santa Clara, CA, USA) U-HPLC 1200 equipped with a Q-TOF 6520 MS–MS detector. TOF (reflectron) was characterized by medium-high resolution (FWHM 18000) and good mass accuracy ($\delta = 2$ and 4 ppm, respectively, in MS and MS–MS mode). MS operating conditions: ESI mode, positive; capillary voltage, 3500 V; nebulizer gas, 30 psig; drying gas, 8 L min^{-1}, 350 °C; fragmentor, 120–240 V. The solvent used was H_2O/CH_3CN (50:50).

The identity of all intermediates was confirmed by NMR and MS spectra; homogeneity was confirmed by TLC on silica gel Merck 60 F_{254}. The purity and chemical structure of GS(HQ)H were confirmed by ^{1}H NMR,^{13}C NMR, IR, MS, and HRMS. Before performing biological studies, the purity of GS(HQ)H was determined by analytical HPLC using a Waters 600 HPLC equipped with a X-Bridge BEH130 C_{18}, 5 μm, 4.6×250 mm column with a Waters 2996 PDA detector, and H_2O/CH_3CN (0.1% TFA) as a solvent system in the form of a linear gradient from 10–90% CH_3CN in 70 min and a flow rate of 3 mL min^{-1}. GS(HQ)H was obtained with a purity greater than 99%, determined by analytical HPLC at 254 and 280 nm (see Supporting Information).

Fmoc-Cys(S*t*Bu)-Gly-OMe (2): H-Gly-OMe·HCl (5.85 g, 46.57 mmol) and NMM (5.13 mL, 46.57 mmol) in dry THF (50 mL) at 0 °C were added to a stirred solution of Fmoc-Cys(S*t*Bu)-OH (**1**; 20.1 g, 46.57 mmol) and HOBt (6.29 g, 46.57 mmol) in dry THF (100 mL), followed by portionwise addition of a solution of DCC (9.61 g, 46.57 mmol) in dry THF (12.5 mL). After 3 h at 0 °C and 16 h at 5 °C, the reaction mixture was filtered, and the resulting solution was evaporated under vacuum. The residue was re-dissolved in $CHCl_3$ and washed with $KHSO_4$ (1 N), $NaHCO_3$ (ss), and brine; the organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum. Chromatography was performed on silica gel with CH_2Cl_2/EtOAc (8:1) as eluant to give 10.41 g of the corresponding dipeptide **2** (Yield: 44%): $R_f = 0.59$, CH_2Cl_2/EtOAc (5:1); ^{1}H NMR ($CDCl_3$): $\delta = 1.36$ (9H, s, S*t*Bu), 3.12–3.16 (2H, m, Cys β-CH_2), 3.71 (3H, s, OMe), 3.90–4.01 (2H, m, Gly α-CH_2), 4.20–4.24 (1H, m, Cys α-CH), 4.35–4.43 (2H, m, CH_2 Fmoc), 4.56–4.59 (1H, t, CH Fmoc), 5.82 (1H, d, Cys NH), 6.97 (1H, s, Gly NH), 7.22–7.76 ppm (8H, m, Ar); ^{13}C NMR ($CDCl_3$): $\delta = 30.08$ (S*t*Bu), 38.01 (Cys β-CH_2), 40.42 (Gly α-CH_2), 45.53 (S*t*Bu), 47.41 (CH Fmoc), 52.12 (OCH_3), 53.81 (Cys α-CH), 57.77 (CH_2 Fmoc), 155.81 (NH Cys) 120.53–140.33 (Ar), 169.04, and 172.31 ppm (2×CO).

H-Cys(S*t*Bu)-Gly-OMe (3): Fmoc-Cys(S*t*Bu)-Gly-OMe (10.33 g, 20.49 mmol) was dissolved in dry CH_2Cl_2 (151 mL), then 95% DBU (3.21 mL) was added to the stirring solution. The reaction mixture was stirred at room temperature for 20 min and then dried under vacuum. The crude mixture was purified by chromatography on silica gel with CH_2Cl_2/EtOAc (1:9) as eluant to give the deprotected dipeptide **3** (2.29 g, yield: 40%): $R_f = 0.34$, EtOAc; ^{1}H NMR ($CDCl_3$): $\delta = 1.34$ (9H, s, S*t*Bu), 1.82 (2H, s, Cys NH_2), 2.65–2.75 (1H, m, Cys β-CH_A), 3.20–3.33 (1H, m, Cys β-CH_B), 3.65–3.70 (1H, m, Cys α-CH), 3.72 (3H, s, OMe), 3.99–4.05 (2H, m, Gly α-CH_2), 7.90 ppm (1H, s, Gly NH); ^{13}C NMR ($CDCl_3$): $\delta = 30.12$ (S*t*Bu), 41.95 (Cys β-CH_2), 45.36

(Gly α-CH_2), 48.59 (S*t*Bu), 54.38 (Cys α-CH), 52.58 (OCH_3), 169.19 and 173.56 ppm (2×CO).

Ac-Glu[Cys(S*t*Bu)-Gly-OMe]-OMe (4): A solution of Ac-Glu-OMe[28] (1.74 g, 8.58 mmol), EDC·HCl (1.65 g, 8.58 mmol), DIPEA (4.48 mL, 25.74 mmol), HOBt (1.16 g, 8.58 mmol), and H-Cys(S*t*Bu)-Gly-OMe (2.18 g, 7.80 mmol) in dry DMF (21 mL) was stirred at room temperature for 24 h. The solvent was removed, and the residue was re-dissolved in $CHCl_3$ and treated with 5% citric acid, $NaHCO_3$ (ss), and brine; the organic layer was dried over anhydrous Na_2SO_4 and evaporated under vacuum. Product **4** (2.07 g) was obtained as a white foam after chromatography on silica gel with EtOAc/MeOH (98:2) as an eluant system (yield: 57%): R_f = 0.24, EtOAc/MeOH (98:2); ^{1}H NMR ($CDCl_3$): δ = 1.33 (9H, s, S*t*Bu), 2.01 (3H, s, Glu Ac), 2.15–2.38 (4H, m, Glu β- and γ-CH_2), 3.02–3.15 (2H, m, Cys β-CH_2), 3.71 (6H, s, 2×OMe), 3.88–4.10 (2H, m, Gly α-CH_2), 4.57–4.62 (1H, m, Glu α-CH), 4.75–4.83 (1H, m, Cys α-CH), 6.83 (1H, d, Glu NH), 7.11 (1H, d, Cys NH), 7.45 ppm (1H, t, Gly NH); ^{13}C NMR ($CDCl_3$): δ = 23.25 (Glu Ac), 28.16 (Glu β-CH_2), 30.03 (S*t*Bu), 32.43 (Glu γ-CH_2), 41.68 (Gly α-CH_2), 42.29 (Cys β-CH_2), 48.54 (S*t*Bu), 51.83 (Glu α-CH), 52.84 (Cys α-CH), 52.94 (OCH_3), 53.03 (OCH_3), 170.42, 170.87, 171.01, 172.75, and 173.01 ppm (5×CO).

Ac-Glu[Cys-Gly-OMe]-OMe (5): Ac-Glu[Cys(S*t*Bu)-Gly-OMe]-OMe (**4**) (1.97 g, 4.23 mmol) was dissolved in *n*PrOH/H_2O (2:1, 72 mL), then the solution was raised to pH 8–9 by adding aqueous ammonia to the stirring solution under N_2 flux. After the addition of P(*n*Bu)$_3$ (1.27 mL, 5.08 mmol), the reaction mixture was left at room temperature for 1 h and 30 min, then dried under vacuum. Chromatographic purification on silica gel with $CHCl_3$:MeOH (9:1) as eluant gave the corresponding free thiolic tripeptide **5** (910 mg, yield: 57%): R_f = 0.57, $CHCl_3$/MeOH (9:1); ^{1}H NMR ([D$_6$]DMSO): δ = 1.72–1.97 (2H, m, Glu β-CH_2), 1.83 (3H, s, Glu Ac), 2.19–2.25 (2H, t, Glu γ-CH_2), 2.29 (1H, s, SH), 2.61–2.77 (2H, m, Cys β-CH_2), 3.59 (6H, s, 2×OMe), 3.82 (2H, d, Gly α-CH_2), 4.19–4.23 (1H, m, Glu α-CH), 4.38–4.41 (1H, m, Cys α-CH), 8.10 (1H, t, Glu NH), 8.27 (1H, t, Cys NH), 8.43 ppm (1H, m, Gly NH); ^{13}C NMR ([D$_6$]DMSO): δ = 22.95 (Glu Ac), 26.84 (Cys β-CH_2) 27.48 (Glu β-CH_2), 31.99 (Glu γ-CH_2), 41.32 (Gly α-CH_2), 52.43 (OCH_3), 52.52 (OCH_3), 55.46 (Glu α-CH), 53.01 (Cys α-CH), 170.17, 170.83, 171.15, 172.07, and 173.24 ppm (5×CO).

Ac-Glu[Cys(8-hydroxyquinolin-5-ylmethyl)-Gly-OMe]-OMe (GS(HQ)H, 6): TFA (3.44 mL, 44.94 mmol) was poured dropwise into a stirring solution of Ac-Glu[Cys-Gly-OMe]-OMe (**5**) (808 mg, 2.14 mmol) in dry CH_2Cl_2 (18.4 mL) at −10 °C. The reaction mixture was added to a solution of 5-hydroxymethyl-8-hydroxyquinoline (378 mg, 2.16 mmol) in CH_2Cl_2/DMF (2:1, 10 mL) and stirred for 10 min before removing the solvent. The residue was treated with diethyl ether and then chromatographed on silica gel with CH_2Cl_2/MeOH (9:1) as eluent, giving the final product **6** (1.09 g, 96% yield): R_f = 0.67, CH_2Cl_2/MeOH (9:1); $[\alpha]_D^{20}$ = −69.6 (*c* = 1 in MeOH); ^{1}H NMR ([D$_6$]DMSO): δ = 1.75–1.92 (1H, m, Glu β-CH_A), 1.82 (3H, s, Glu Ac), 1.92–1.97 (1H, m, Glu β-CH_B), 2.17–2.23 (2H, m, Glu γ-CH_2), 2.52–2.56 (1H, m, Cys β-CH_A), 2.77–2.81 (1H, m, Cys β-CH_B), 3.55 and 3.61 (6H, s, 2×OMe), 3.84–3.86 (2H, d, Gly α-CH_2), 4.09–4.15 (2H, m, HQ CH_2), 4.19–4.21 (1H, m, Glu α-CH), 4.59–4.63 (1H, m, Cys α-CH), 6.93–7.57 (3H, m, HQ Ar), 8.17 (1H, brt, Cys NH), 8.25 (1H, brt, Glu NH), 8.59 (1H, brt, Gly NH), 8.52–8.83 (2H, m, HQ Ar), 9.73 ppm (1H, s, HQ OH); ^{13}C NMR ([D$_6$]DMSO): δ = 22.95 (Glu Ac), 27.60 (Glu β-CH_2), 32.06 (Glu γ-CH_2), 32.75 (Cys β-CH_2), 34.38 (S-CH_2), 41.32 (Gly α-CH_2), 52.19 (Glu α-CH), 52.30 (Cys α-CH), 52.41 (OCH_3), 52.51 (OCH_3), 111.35–153.07 (9×HQ CH), 170.30, 170.73, 171.65, 172.12, and 173.20 ppm (5×CO); IR (KBr): $\tilde{\nu}$ = 3576, 3370, 3280, 3055, 2950, 2879, 2740, 1649, 1550, 1380, 1201 cm^{-1}; MS (ESI) *m/z* 557.3 $[M+Na]^+$; HRMS *m/z* $[M+H]^+$ calcd for $C_{24}H_{30}N_4O_8S$: 534.1763, found: 535.1836.

Pharmacokinetic studies

HPLC analyses were carried out on a Waters 600 pump (Waters Corp., Milford, MA, USA), equipped with a Waters 2996 photodiode array detector, a 20 μL Rheodyne injector, and a computer integrating apparatus. A Waters X-Terra RP_8 (4.6×150 mm, 5 μm) column was used, with a water (pH 3)/CH_3CN (10:90) mixture as the mobile phase at a flow rate of 1.5 mL min^{-1} and the UV detector set at a wavelength of 246 nm.[49]

Aqueous solubility: The aqueous solubility of GS(HQ)H was determined in deionized water. An excess of compound was added to 1 mL of water, the suspension was shaken at 25 °C for 15 min to ensure the solubility equilibrium, and the supernatant was filtered (Millipore 0.45 μm). The filtered solution was analyzed by HPLC.

Log D determination by shake-flask method: The partition coefficients were determined by placing ~5 mg of the compound in 1 mL of anhydrous *n*-octanol, shaking vigorously for ~2 min and then filtering. An equal volume of buffer (pH 7.4, 1.4, or 9.5) was added, and the mixture was equilibrated by repeated inversions up to 200 times for 5 min and then allowed to stand for 30 min for the phases to fully separate. Thereafter, the respective phases were analyzed by HPLC.

PAMPA method: The protocol applied to measure the permeability coefficient (P_e) through the artificial membrane and to predict oral absorption and BBB permeation has been previously described.[14] The artificial membrane for GI permeability was a 2% solution (*w/v*) of egg lecithin (phosphatidylcholine, PC, >98%) in dodecane, as PC is the most common lipid component of mammalian membranes.[50] The studies were performed at three different pH values, to simulate the regions on the GI tract, for an incubation time of 4 h.[51] For BBB permeability, a phospholipid mixture from porcine polar brain lipid extract was used; this mixture was composed of PC (12.6%), phosphatidylethanolamine (PE, 33.1%), phosphatidylserine (PS, 18.5%), phosphatidylinositol (PI, 4.1%), phospatidic acid (PA, 0.8%), and 30.9% of other compounds (Avantis polar lipids, Alabaster, AL). The incubation time was 18 h.[52]

Kinetics of chemical hydrolysis: A 0.02 M hydrochloric acid buffer of pH 1.3, as a non-enzymatic simulated gastric fluid (SGF), and a 0.02 M phosphate buffer of pH 7.4 were used in this study. Reactions were initiated by adding 1 mL of a MeOH stock solution of the compound **6** to 10 mL of the appropriate temperature (37 ± 0.5 °C) aqueous buffer solution containing 20% CH_3CN. At appropriate time intervals, samples of 20 μL were withdrawn and analyzed by HPLC. Pseudo-first-order rate constants (k_{obs}) for hydrolysis of the compound were then calculated from the slopes of the linear plots of log (% residual compound) against time. Experiments were run in triplicate and the mean values of the rate constants were calculated.[53]

Kinetics of enzymatic hydrolysis: Plasma from humans was obtained by centrifugation of blood samples containing 0.3% citric acid at 3000×*g* for 15–20 min. Plasma fractions (4 mL) were diluted with 0.02 M phosphate buffer (pH 7.4) to give a final volume of 5 mL (80% plasma). Incubation was performed at 37 ± 0.5 °C using a shaking water bath. The reaction was initiated by adding 200 μL of a stock solution of drug (1 mg mL^{-1} in MeOH) to 5 mL of preheated plasma, as previously described.[54]

Complexation studies

All potentiometric measurements were performed with an automatic Metrohm 765 Dosimat titrator equipped with 1, 5, and 20 mL burettes and with two independent potentiometric channels. Solutions were prepared by weight, using water purified with a Milli-Q/plus apparatus (Millipore), and analyte concentrations were expressed in the molality scale (mol kg^{-1} of water). HCl was obtained by azeotropic distillation of concentrated HCl (J. T. Baker). From this solution, a working solution (~0.1 M) was prepared and standardized with sodium carbonate (Aldrich, 99.95–100.05%). An NaOH solution (~0.1 M) was prepared from a material with a low carbonate content (Aldrich, nominal purity 99.99%, CO_2 max. 0.3%), and it was standardized with HCl. 8-HQ (Riedel de Haen, 99% min.) and GS(HQ)H were used without further purification and were directly added as solids to the vessel before being titrated. The Zn^{II}, Cu^{II}, and Fe^{III} solutions were prepared by dissolution of ZnO (Carlo Erba, 99.5% min.), $CuSO_4$ (Riedel de Haen, 99–100.5%), and iron pellets (Aldrich, 99.999%) in an HCl solution of known composition, respectively. Concentrations of the Zn^{II} and Cu^{II} solutions were determined by titration with standard EDTA (Carlo Erba, 0.1 M), and were both 0.05 M. The concentration of the Fe^{III} solution was obtained as previously described[55] and was 0.04 M. The ionic strength of all solutions was adjusted to 0.15 M NaCl. NaCl (Prolabo RP Normapur, minimal purity 99.5%) was recrystallized from water and calcinated at 600 °C.

Potentiometric titrations were carried out in a 10 mL water-jacketed cell at 25.0 ± 0.1 °C under nitrogen flow, to avoid carbon dioxide contamination, using a Metrohm 6.0262.100 combined glass electrode. At the beginning of each experiment, a 0.15 M NaCl solution (2.3 g water) was prepared directly in the cell, and the glass electrode was calibrated by multiple additions of HCl (final concentration ~0.01 M); the proper amounts of ligand and, when required, of metal ion, were added subsequently, and the solution was titrated with NaOH. Acid–base titrations gave a carbonate content of 0.5 ± 0.5% and the water dissociation constant as $pK_w = 13.55 \pm 0.02$. Titrations of each ligand (in the absence of the metal ion) were performed to determine its acid–base properties and to obtain its concentration in the titration vessel. Ligand concentration ranged from 0.6×10^{-3} to 1.4×10^{-3} M. The GS(HQ)H titrations were limited to pH 5.5 (upper limit) because of the tendency of this ligand to hydrolyze at basic pH values. At the end of each ligand titration, the solution was acidified to pH 2, the proper amount of metal solution was added, and then the solution was titrated with NaOH. The metal/ligand ratio varied from 2:1 to 1:5. The pK_w, ligand protonation constants, and metal–ligand complex stability constants were calculated by the program PITMAP.[56] This program refines equilibrium constants by iterative nonlinear least-squares fit of potentiometric titration data through a set of simultaneous mass balance equations for all components. Formation constant values of metal hydroxo complexes at 25 °C and at an ionic strength near that of 0.15 M NaCl were taken from a published source[40] and were held constant during data optimization.

Biological studies

SH-SY5Y and U937 cell cultures: Human SH-SY5Y neuroblastoma cells (EGACC, Sigma–Aldrich, UK) were grown at 37 °C in 5% CO_2 humidity, in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), and 1% L-glutamine. SH-SY5Y cells were plated in 96-well plates (2700 cells per well). Undifferentiated cells (UN) were grown for 24 h in normal medium and then incubated with the compounds (GSH, HQ, and GS(HQ)H). After 24 h incubation, the cultures were assessed for viability using a colorimetric assay based on the ability of living cells to reduce a tetrazolium-based compound to a blue formazan product (MTT assay).

To obtain a cholinergic phenotype, SH-SY5Y cells were grown in a medium containing retinoic acid (RA) (10 μM) for 3 days, then the medium was removed and replaced with fresh RA medium (10 μM) for a further 3 days of differentiation.[57] Alternatively, to have a dopaminergic phenotype, the cells were treated with RA (10 μM) for 3 days; then the medium was removed and replaced with growth medium containing phorbol 12-myristate 13-acetate (PMA, 80 nM) for a subsequent 3 days. The neuroprotective effects of GSH, HQ, and GS(HQ)H at a concentration of 1 μM were evaluated in differentiated cells exposed to 6-OHDA (25, 50, 75, 150 μM) or H_2O_2 (25, 150, 300 μM).

The undifferentiated and differentiated cells were plated in 96-well plates. For experiments with the neurotoxin H_2O_2, SH-SY5Y cells were pretreated with compounds for 24 h and then exposed to 25, 150, or 300 μM H_2O_2 for further 24 h. Cell viability was detected using the MTT assay. For experiments with the neurotoxin 6-OHDA, SH-SY5Y cells were treated with compounds for 1 h and then exposed to 25, 50, 75, and 150 μM 6-OHDA. After a further 24 h of incubation, the cultures were assessed for viability by the MTT assay. After incubation, the cells were treated with 20 μL of MTT solution (5 mg mL^{-1} in PBS) in each well, followed by incubation at 37 °C for 3 h. The plate was centrifuged at 2000 rpm for 15 min. The supernatant was removed, and DMSO (200 μL) was added to each well and incubated for 30 min at 37 °C. Finally, the plate was read at 540 nm on a Titertek Multiscan microeliza reader (Flow Laboratories, Urvine, UT, USA).

U937 mononuclear cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in a 5% CO_2 atmosphere in RPMI 1640 medium (GIBCO, Invitrogen) containing 10% fetal calf serum, 100 ng mL^{-1} streptomycin, 100 U mL^{-1} penicillin, and 2 mM L-glutamine. At sub-confluence (80%), U937 cells cultured in 24-well plates were pre-exposed to test drugs for 40 min, followed by H_2O_2 (500 μM) for 2 h. Cell viability was measured using the MTT test as described above.[58]

Measurement of intracellular ROS: Intracellular ROS were quantified by the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay using a Microplate Fluorometer SPECTRAmax Gemini XS (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 480 nm and 540 nm, respectively, and were analyzed by SOFTmax Pro software (version 5.0, Molecular Devices). DCFH-DA, a non-fluorescent ester dye, is a useful indicator of ROS because, after penetration into the cells, it is hydrolyzed by intracellular esterases to the relevant DCFH. DCFH can be rapidly oxidized by ROS (i.e., H_2O_2) to the highly fluorescent 2,7-dichlorofluorescein (DCF). SHSY-5Y human neuroblastoma cells were plated (2000 cells per well) into special optics 96-well plates (Corning-Costar), and 24 h later, were washed three times with imaging buffer (125 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 5 mM glucose, 25 mM HEPES, and 2 mM $CaCl_2$). After addition of 10 μM DCFH-DA media solution, the plates were incubated at 37 °C for 30 min. Subsequently, cells were washed twice with imaging buffer cells and treated with 100 nM H_2O_2 for immediate fluorescence measurement. The measured fluorescence intensity is proportional to the ROS levels present in the cell cytosol. Plates, kept at 25 °C, were monitored every 30 s for 0–5 min for kinetic data analysis.[59]

NBT assay: The NBT assay is based on the reduction of NBT in formazan by O_2^-. The formazan was read by a spectrophotometer. Detection of higher levels of formazan correspond to greater reduction of NBT by O_2^-. In the presence of potential antioxidant substances, the superoxide is detoxified (scavenger action) and decreases the amount of NBT reduced, so less formazan is detected. The SH-SY5Y cells (10^6 cells) were incubated for 24 h with 1 μM GSH, HQ, or GS(HQ)H, then were detached, centrifuged for 5 min at 170×g, and were resuspended in 1 mL 0.9% NaCl with NBT dissolved (1 mg mL^{-1}, Sigma–Aldrich). The cells were left for 3 h at 37 °C (incubator), centrifuged for 5 min at 500×g, resuspended in 1 mL DMSO, and incubated for 20 min at 37 °C. For the assay, cells were plated in a 96-well plate (2×10^5 cells per well) and read by spectrophotometer at 550 nm in a scanning multi-well spectrophotometer (Cary50MPR, Varian).

The same NBT test was performed on U937 cells, but initially, the cells were treated with 100 μM GSH, HQ, or GS(HQ)H and then stimulated with 500 μM H_2O_2.

Determination of intracellular GS(HQ)H uptake: SH-SY5Y human neuroblastoma cells were incubated with GS(HQ)H (1 μM) at 37 °C. PBS alone was used as a negative control. The amount of GS(HQ)H was assayed in cell medium and in neuroblastoma cells after different incubation times (3, 6, 9, and 24 h).[60] Aliquots (100 μL) of each medium were deproteinized by mixing with 200 μL of 0.01 M HCl in MeOH, centrifuged at 10000 rpm for 10 min, filtered, and injected into the HPLC system.[61] The amounts of remaining intact codrug were plotted as a function of incubation time. Neuroblastoma cells were suspended in 100 μL of PBS, added to 200 μL of 0.01 M HCl in MeOH, centrifuged at 10000 rpm for 10 min, filtered, and injected into the HPLC system. The amounts of permeated intact codrug were plotted as a function of incubation time.

Statistical analysis: Statistical analysis (unpaired *t*-test) was performed using GraphPad Prism software version 5.0. One-way ANOVA was computed for each level of treatment, followed by Dunnett's *t*-test post hoc.

Acknowledgements

The authors thank Dr. Antonio Pivato, Maristella Feltracco, and Dr. Annamaria Cassini, University of Padova, for obtaining the HRMS spectra of GS(HQ)H and for performing Fe^{III}–ligand potentiometric titrations. This work was supported by the Italian Ministry for Education, University and Research (MIUR) with 60% grants 2012 to I.C, F.P. and A.D.S.

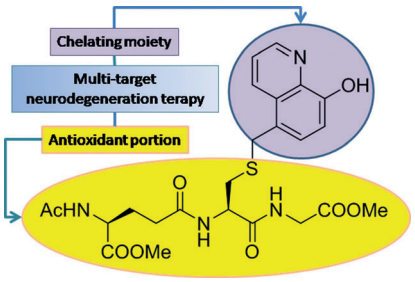
Keywords: antioxidants · chelation therapy · glutathione · 8-hydroxyquinolines · neurodegenerative diseases · peptides

[1] K. Jomova, M. Valko, *Toxicology* **2011**, *283*, 65 – 87.
[2] M. A. Lovell, J. D. Robertson, W. J. Teesdale, J. L. Campbell, W. R. Markesbery, *J. Neurol. Sci.* **1998**, *158*, 47 – 52.
[3] L. Zecca, M. B. H. Youdim, P. Riederer, J. R. Connor, R. R. Crichton, *Nat. Rev. Neurosci.* **2004**, *5*, 863 – 873.
[4] M. A. Smith, P. L. R. Harris, L. M. Sayre, G. Perry, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 9866 – 9868.
[5] R. J. Castellani, S. L. Siedlak, G. Perry, M. A. Smith, *Acta Neuropathol.* **2000**, *100*, 111 – 114.
[6] J. Dong, C. S. Atwood, V. E. Anderson, S. L. Siedlak, M. A. Smith, G. Perry, P. R. Carey, *Biochemistry* **2003**, *42*, 2768 – 2773.
[7] H. Kozlowski, M. Luczkowski, M. Remelli, D. Valensin, *Coord. Chem. Rev.* **2012**, *256*, 2129 – 2141.
[8] L. M. Sayre, M. A. Smith, G. Perry, *Curr. Med. Chem.* **2001**, *8*, 721 – 738.
[9] E. R. Stadtman, *Science* **1992**, *257*, 1220 – 1224.
[10] M. Valko, H. Morris, M. T. D. Cronin, *Curr. Med. Chem.* **2005**, *12*, 1161 – 1208.
[11] R. B. Mounsey, P. Teismann, *Int. J. Cell Biol.* **2012**, 983245.
[12] C. A. Perez, Y. Tong, M. Guo, *Curr. Bioact. Compd.* **2008**, *4*, 150 – 158.
[13] R. León, A. G. Garcia, J. Marco-Contelles, *Med. Res. Rev.* **2013**, *33*, 139 – 189.
[14] I. Cacciatore, L. Baldassarre, E. Fornasari, C. Cornacchia, A. Di Stefano, P. Sozio, L. S. Cerasa, A. Fontana, S. Fulle, E. S. Di Filippo, R. M. L. La Rovere, F. Pinnen, *ChemMedChem* **2012**, *7*, 2021 – 2029.
[15] A. Minelli, C. Conte, I. Cacciatore, C. Cornacchia, F. Pinnen, *Amino Acids* **2012**, *43*, 1359 – 1367.
[16] P. Sozio, L. S. Cerasa, S. Laserra, I. Cacciatore, C. Cornacchia, E. S. Di Filippo, S. Fulle, A. Fontana, A. Di Crescenzo, M. Grilli, M. Marchi, A. Di Stefano, *Eur. J. Pharm. Sci.* **2013**, *49*, 187 – 198.
[17] P. Sozio, A. Iannitelli, L. S. Cerasa, I. Cacciatore, C. Cornacchia, G. Giorgioni, M. Ricciutelli, C. Nasuti, F. Cantalamessa, A. Di Stefano, *Arch. Pharm.* **2008**, *341*, 412 – 417.
[18] A. Minelli, C. Conte, E. Prudenzi, I. Cacciatore, C. Cornacchia, E. Taha, F. Pinnen, *Free Radic. Biol. Med.* **2010**, *49*, 31 – 39.
[19] I. Cacciatore, C. Cornacchia, L. Baldassarre, E. Fornasari, A. Mollica, A. Stefanucci, F. Pinnen, *Mini-Rev. Med. Chem.* **2012**, *12*, 13 – 23.
[20] C. J. Frederickson, E. J. Kasarskis, D. Ringo, R. E. Frederickson, *J. Neurosci. Methods* **1987**, *20*, 91 – 103.
[21] T. D. Turnquist, E. B. Sandell, *Anal. Chim. Acta* **1968**, *42*, 239 – 245.
[22] R. Kayyali, A. S. Pannala, H. Khodr, R. C. Hider, *Biochem. Pharmacol.* **1998**, *55*, 1327 – 1332.
[23] D. B. Shachar, N. Kahana, V. Kampel, A. Warshawsky, M. B. H. Youdim, *Neuropharmacology* **2004**, *46*, 254 – 263.
[24] M. G. Dickens, K. J. Franz, *ChemBioChem* **2010**, *11*, 59 – 62.
[25] I. Cacciatore, C. Cornacchia, F. Pinnen, A. Mollica, A. Di Stefano, *Molecules* **2010**, *15*, 1242 – 1264.
[26] I. Cacciatore, L. Baldassarre, E. Fornasari, A. Mollica, F. Pinnen, *Oxid. Med. Cell. Longevity* **2012**, *2012*, 1 – 12.
[27] I. Cacciatore, E. Fornasari, L. Baldassarre, C. Cornacchia, S. Fulle, E. Di Filippo, T. Pietrangelo, F. Pinnen, *Pharmaceuticals* **2013**, *6*, 54 – 69.
[28] I. Cacciatore, A. Cocco, M. Costa, M. Fontana, G. Lucente, L. Pecci, F. Pinnen, *Amino Acids* **2005**, *28*, 77 – 83.
[29] I. Cacciatore, A. M. Caccuri, A. Di Stefano, G. Luisi, M. Nalli, F. Pinnen, G. Ricci, P. Sozio, *Farmaco* **2003**, *58*, 787 – 793.
[30] F. Pinnen, I. Cacciatore, C. Cornacchia, P. Sozio, A. Iannitelli, M. Costa, L. Pecci, C. Nasuti, F. Cantalamessa, A. Di Stefano, *J. Med. Chem.* **2007**, *50*, 2506 – 2515.
[31] A. M. El-Gendy, A. Adejare, *Int. J. Pharm.* **2004**, *280*, 47 – 55.
[32] D. Gupta, S. V. Gupta, K. D. Lee, G. L. Amidon, *Mol. Pharmaceutics* **2009**, *6*, 1604 – 1611.
[33] A. Di Stefano, P. Sozio, I. Cacciatore, A. Cocco, G. Giorgioni, B. Costa, M. Montali, A. Lucacchini, C. Martini, G. Spoto, F. Di Pietrantonio, E. Di Matteo, F. Pinnen, *J. Med. Chem.* **2005**, *48*, 2646 – 2654.
[34] M. Kansy, F. Senner, K. Gubernator, *J. Med. Chem.* **1998**, *41*, 1007 – 1010.
[35] E. H. Kerns, L. Di, S. Petusky, M. Farris, R. Ley, P. Jupp, *J. Pharm. Sci.* **2004**, *93*, 1440 – 1453.
[36] L. Di, E. H. Kerns, K. Fan, O. J. McConnell, G. T. Carter, *Eur. J. Med. Chem.* **2003**, *38*, 223 – 232.
[37] S. Carrara, V. Reali, P. Misiano, G. Dondio, C. Bigogno, *Int. J. Pharm.* **2007**, *345*, 125 – 133.
[38] L. M. Ramenskaya, O. V. Kraeva, *Russ. J. Phys. Chem.* **2006**, *80*, 90 – 94.
[39] a) L. D. Pettit, H. K. J. Powell, "The IUPAC Stability Constant Database", Academic Software, Royal Society of Chemistry, London (UK), **1997**; b) S. Novick, "Biography of Rotational Spectra of Weakly Bound Complexes", **1999**: https://wesfiles.wesleyan.edu/home/snovick/SN_webpage/vdw.pdf (accessed September 3, 2013).
[40] L. M. Ramenskaya, O. V. Kraeva, T. V. Vladimirova, *Zh. Fiz. Khim.* **2004**, *78*, 1433 – 1437.
[41] Z. A. Muzykantova, V. N. Aseeva, *Izv. Sib. Otd. Akad. Nauk SSSR Ser. Khim. Nauk* **1986**, *1*, 29 – 36.
[42] P. Faller, C. Hureau, *Dalton Trans.* **2009**, 1080 – 1094.
[43] H. R. Xie, L. S. Hu, G. Y. Li, *Chin. Med. J.* **2010**, *123*, 1086 – 1092.
[44] C. P. LeBel, H. Ischiropoulos, S. C. Bondy, *Chem. Res. Toxicol.* **1992**, *5*, 227 – 231.

[45] B. Halliwell, J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, **1989**, pp. 188 – 266.
[46] V. Lehmensiek, E. M. Tan, S. Liebau, T. Lenk, H. Zettlmeisl, J. Schwarz, A. Storch, *Neurochem. Int.* **2006**, *48*, 329 – 340.
[47] M. K. Cathcart, G. M. Chisolm, A. K. McNally, D. W. Morel, *In Vitro Cell. Dev. Biol.* **1988**, *24*, 1001 – 1008.
[48] A. Patruno, S. Franceschelli, M. Pesce, C. Maccallini, M. Fantacuzzi, L. Speranza, A. Ferrone, M. A. De Lutiis, E. Ricciotti, R. Amoroso, M. Felaco, *Biochim. Biophys. Acta Gen. Subj.* **2012**, *1820*, 2095 – 2104.
[49] D. Agbaba, A. Mihajlovic, P. Beljanski, D. Zivanov-Stakic, S. Vladimirov, *Chromatographia* **1997**, *45*, 145 – 148.
[50] A. Fortuna, G. Alves, P. Soares-da-Silva, A. Falcao, *J. Pharm. Sci.* **2012**, *101*, 530 – 540.
[51] K. K. Hwang, N. E. Martin, L. Jiang, C. Zhu, *J. Pharm. Pharm. Sci.* **2003**, *6*, 315 – 320.
[52] M. Malakoutikhah, R. Prades, M. Teixido, E. Giralt, *J. Med. Chem.* **2010**, *53*, 2354 – 2363.
[53] F. Pinnen, P. Sozio, I. Cacciatore, C. Cornacchia, A. Mollica, A. Iannitelli, E. D'Aurizio, A. Cataldi, S. Zara, C. Nasuti, A. Di Stefano, *Arch. Pharm.* **2011**, *344*, 139 – 148.
[54] P. Sozio, E. D'Aurizio, A. Iannitelli, A. Cataldi, S. Zara, F. Cantalamessa, C. Nasuti, A. Di Stefano, *Arch. Pharm.* **2010**, *343*, 133 – 142.
[55] V. B. Di Marco, A. Tapparo, G. G. Bombi, *Ann. Chim.* **2001**, *91*, 595 – 603.
[56] V. B. Di Marco, PhD Thesis, University of Padova (Italy), **1998**.
[57] F. M. Lopes, R. Schröder, M. L. C. da Frota, Jr., A. Zanotto-Filho, C. B. Müller, A. S. Pires, R. T. Meurer, G. D. Colpo, D. P. Gelain, F. Kapczinski, J. C. F. Moreira, M. da Cruz Fernandes, F. Klamt, *Brain Res.* **2010**, *1337*, 85 – 94.
[58] L. Speranza, M. Pesce, A. Patruno, S. Franceschelli, M. A. De Lutiis, A. Grilli, M. Felaco, *Mar. Drugs* **2012**, *10*, 890 – 899.
[59] L. Menghini, L. Leporini, N. Scanu, G. Pintore, R. La Rovere, E. S. Di Filippo, T. Pietrangelo, S. Fulle, *J. Biol. Regul. Homeost. Agents* **2011**, *25*, 27 – 35.
[60] M. Zampagni, D. Wright, R. Cascella, G. D'Adamio, F. Casamenti, E. Evangelisti, F. Cardona, A. Goti, B. Nacmias, S. Sorbi, G. Liguri, C. Cecchi, *Free Radical Biol. Med.* **2012**, *52*, 1362 – 1371.
[61] F. Pinnen, I. Cacciatore, C. Cornacchia, A. Mollica, P. Sozio, L. S. Cerasa, A. Iannitelli, A. Fontana, C. Nasuti, A. Di Stefano, *Amino Acids* **2012**, *42*, 261 – 269.

Received: July 5, 2013
Revised: August 23, 2013
Published online on ■■ ■■, 0000

Multi-target multitasking: We report the synthesis of a novel and chemically stable *S*-hydroxyquinoline glutathione derivative. This compound was shown to prevent H_2O_2- and 6-OHDA-mediated oxidative stress in in vitro models and to partially and quantitatively remove Cu^{II} and Zn^{II}, respectively, from the Aβ peptide.



I. Cacciatore, C. Cornacchia, E. Fornasari, L. Baldassarre, F. Pinnen, P. Sozio, A. Di Stefano, L. Marinelli, A. Dean, S. Fulle, E. S. Di Filippo, R. M. L. La Rovere, A. Patruno, A. Ferrone, V. Di Marco*

■■ – ■■

A Glutathione Derivative with Chelating and in vitro Neuroprotective Activities: Synthesis, Physicochemical Properties, and Biological Evaluation