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Design, synthesis, and evaluation of novel bifunctional iron-chelators as potential agents for neuroprotection in Alzheimer's, Parkinson's, and other neurodegenerative diseases

Hailin Zheng,[a] Lev M. Weiner,[a] Orit Bar-Am,[b] Silvina Epsztejn,[c] Z. Ioav Cabantchik,[c] Abraham Warshawsky,[a] Moussa B. H. Youdim[b] and Mati Fridkin[a,*]

[a]*Department of Organic Chemistry and Neurobiology, The Weizmann Institute of Science, Rehovot 76100, Israel*
[b]*Eve Topf and USA National Parkinson Foundation Centers of Excellence for Neurodegenerative Diseases and Department of Pharmacology, Technion—Faculty of Medicine, Haifa, Israel*
[c]*Department of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel*

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Abstract—Several novel antioxidant-iron chelators bearing 8-hydroxyoxyquinoline moiety were synthesized, and various properties related to their iron chelation, and neuroprotective action were investigated. All the chelators exhibited strong iron(III) chelating and high antioxidant properties. Chelator **9** (HLA20), having good permeability into K562 cells and moderate selective MAO-B inhibitory activity (IC_{50} 110 μM), displayed the hightest protective effects against differentiated P19 cell death induced by 6-hydroxydopamine. EPR studies suggested that Chelator **9** also act as radical scavenger to directly scavenge hydroxyl radical.

1. Introduction

Parkinson's disease (PD) and Alzheimer's disease (AD) are the most common neurodegenerative disorders. They affect at least 5% of the population above the age of 65 years.[1] At present, drugs used for PD therapy consist mainly of L-dopa and/or dopamine (DA) agonists, monoamine oxidase B inhibitors such as rasagiline and selgiline, catechol-methyl transferase inhibitor, entacapone.[2] However, these drugs can only improve clinical symptoms (symptomatic) but cannot mitigate progression of the disease process underlying PD. For AD the only therapeutically active drugs are cholinesterase inhibitors[3] and glutamate antagonist, memantine.[4] Moreover, severe side effects will appear in the long-term treatment.[2] Therefore, other approaches are critically needed for these progressive neurodegenerative diseases.

Although the etiology of neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, Huntington disease, amyotrophic lateral sclerosis, Friedreich ataxia) is not yet well established, accumulating evidences have shown that iron-dependent oxidative stress, increased levels of iron and monoamine oxidase (MAO)-B activity, and depletion of antioxidants in the brain may be major pathogenic factors in PD and other neurodegenerative diseases.[1,2] In fact, a number of iron chelators, antioxidant or MAO-B inhibitors have been shown to possess neuroprotective activity in animal models. For example, deferoxamine (DFO), a prototype

Abbreviations: L1, 1,2-dimethyl-3-hydroxypyridin-4-one; DFO, desferal; Boc, *tert*-butoxycarbonyl; DMSO, dimethyl sulfoxide; Fmoc, 9-fluorenylmethoxycarbonyl; Boc_2O, di-*tert*-butyl dicarbonate; TFA, trifluoroacetic acid; Trt, trityl; DMF, dimethylformamide; TV3326, *N*-propargyl-3*R*-aminoindan-5yl-ethyl methylcarbamate; TV3279, *N*-propargyl-3*S*-aminoindan-5yl-ethyl methylcarbamate; MAO, monoamine oxidase; MPTP, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridinium; 6-OHDA, 6-hydroxydopamine; ICV, intraventricularly; SIH, salycylaldehyde isonicitinoyl hydrazone; ROS, reactive oxygen species; EGCG, (−)-epigallocatechin-3-gallate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; IC_{50}, the concentration required for 50% inhibition; THF, tetrahydrofuran; *t*Bu, *tert*-butyl.
Keywords: Antioxidant-iron chelator; Lipid peroxidation; Neuroprotection; 8-Hydroxyquinoline.
* Corresponding author. Tel.: +972 08 934 2505; fax: +972 08 934 4142; e-mail: mati.fridkin@weizmann.ac.il

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iron chelator drug that does not cross the blood brain barrier, when injected intraventricularly (ICV), protects against the dopaminergic neurodegeneration induced by 6-hydroxydopamine (6-OHDA).[5] Desferal also has neuroprotective activity in preventing iron and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridinium (MPTP)-induced neurotoxicity in mice.[6] More recently, the antibiotic iron chelator, 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol) has been shown to be able to prevent MPTP-induced neurotoxicity in vivo.[7] Oral administration of clioquinol was reported to inhibit β-amyloid accumulation in an Alzheimer's disease (AD) transgenic mouse model via its actions as a metal chelator.[8] Antioxidants such as vitamin E and ebselen when injected into rats and mice prevent the neurotoxic action of 6-OHDA and MPTP.[9,10] The green tea catechin, (−)-epigallocatechin-3-gallate (EGCG), which is known for its iron chelating and antioxidant properties, is one of the most potent neuroprotective agents against MPTP neurotoxicity in mice.[11] In animal models, L-deprenyl, as well as other MAO-B inhibitors, such as rasagiline, has been shown to protect against the toxic damage induced by (MPTP) and 6-OHDA.[2] However, none of these antioxidants and iron chelators have been successfully introduced for clinical use.[2,7,9]

Recently a new neuroprotective strategy has been proposed, that is, neuroprotection in neurodegenerative diseases may require a drug combining iron chelating with antioxidant capacity, iron chelator, and MAO-B inhibitory properties.[2]

The goal of this study was to develop novel antioxidant-iron chelators with potent selective MAO-B inhibitory activity, which can be used as drug candidates for the treatment and/or prevention of the neurodegenerative diseases. For this purpose, several novel antioxidant chelators with 8-hydroxyoxyquinoline moiety were designed, synthesized, and characterized. Here we selected 8-hydroxyoxyquinoline as a chelating moiety based on the following reasons: first, 8-hydroxyquinoline is a strong iron chelator with antioxidant property.[12,13] Secondly, it can protect plants against oxidative damage caused by herbicide paraquat via blocking the Fenton reaction.[14] Moreover, 8-hydroxyquinoline has been shown to be able to cross the blood brain barrier (BBB).[15] And more recently, as already mentioned, the activity of the antibiotic iron chelator, 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol) in preventing MPTP neurotoxicity in mice has been reported.[7] We also introduced a propargyl moiety and/or piperazine moiety (as a linker) to these new chelators. The propargyl moiety is believed to be responsible for the potent MAO B inhibitory activity since N-propargyl-containing compounds such as rasagiline, pargyline, clorgyline, and deprenyl show high MAO inhibitory activity; removal of the propargyl moiety abolishes the inhibitory activity.[16,28,29] This moiety is also responsible for neuroprotective activity unrelated to MAO inhibition, since the *S*-isomer of rasagiline, TVP-1022, is an extremely weak inhibitor of MAO, but has similar neuroprotective activity in vitro and in vivo to rasagiline.[17] Furthermore recent studies have shown that propargylamine itself possesses the neuroprotective activities of rasagiline and TVP-1022 and other propaergylamines.[18] A piperazine moiety was selected as a spacer since it contains two amino moieties available for convenient chemical modifications. Moreover, due to its hydrophobicity, it may enhance penetration through the BBB.

In this paper, we reported the synthesis and characterization of these novel antioxidant chelators.

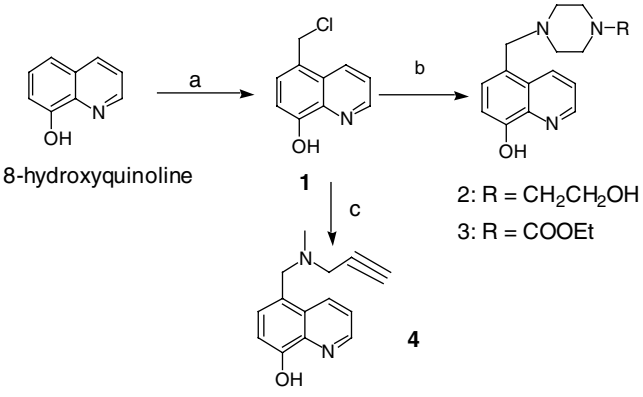
2. Results and discussion

2.1. Chemical synthesis

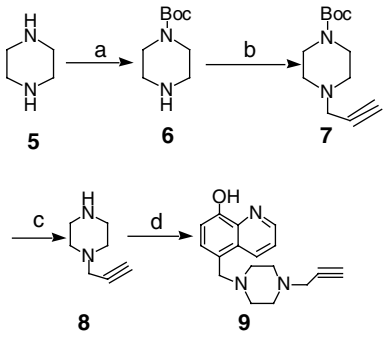
The preparation of chelators **2**, **3**, and **4** is outlined in Scheme 1, starting from commercially available 8-hydroxyquinoline. According to standard methods, 8-hydroxyquinoline was converted to 5-chloromethyl-8-quinoline **1** by treating with hydrochloric acid and formaldehyde. Reaction of **1** with N-substituted piperazines or propargylamine yielded the target compounds **2**, **3**, or **4**.

The synthesis of **9** (HLA20) is shown in Scheme 2. Piperazine **5** was protected to afford its *N*-Boc derivative **6**. Compound **6** reacted with propargyl bromide to obtain *N*-*t*-Boc propargylpiperazine **7**. Deprotection of **7** using trifluroacetic acid yielded propargylpiperazine **8**. Finally, **8** was alkylated with 5-chloromethyl-8-quinoline **1** to give propargyl hydroxyquinoline **9**.

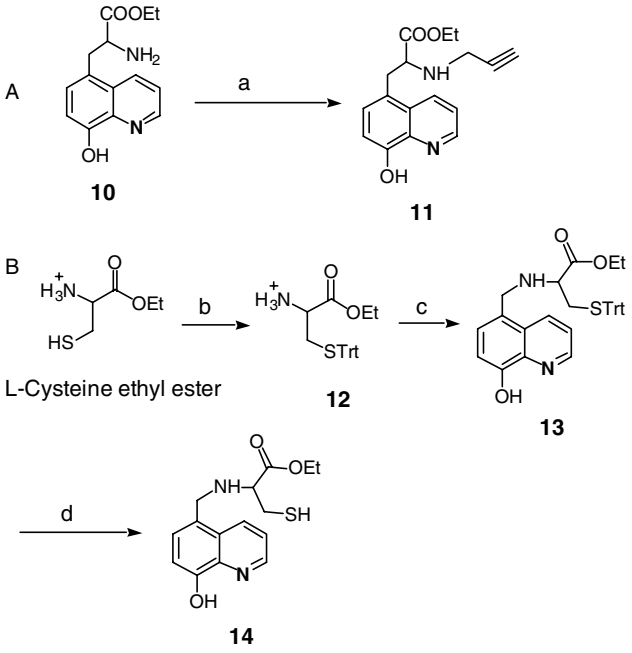
Compounds **11** and **14** were synthesized by the pathways depicted in Scheme 3. Starting from quinolinyl alanine ethyl ester **10**.[19] Compound **11** was obtained by reacting ethyl ester **10** with propargylamine in DMSO with sodium bicarbonate as base. To obtain compound **14**, we started from commercially available L-cysteine ethyl ester, which was first protected as its *S*-Trt derivative **12**. Compound **12** was then treated with 5-chloro-methyl-8-quinoline **1** to give intermediate **13**. Removal of the trityl protecting group in **13** by trifluroacetic acid afforded target compound **14**.



Scheme 1. Reagents and conditions: (a) HCl (32% water), HCHO (37% in water), 0 °C → rt, 8 h; (b) N-substituted piperazine, $(Me_2CH)_2$-NEt, $CHCl_3$, rt, 1 day; (c) propargylamine, $(Me_2CH)_2NEt$, $CHCl_3$, rt, 1 day.



Scheme 2. Reagents and conditions: (a) Boc_2O, MeOH, 0 °C → rt, 1 day; (b) propargyl bromide, $(Me_2CH)_2NEt$, 0 °C → rt, 1 day, (c) F_3CCOOH, 0 °C → rt, overnight; (d) 5-chloromethyl-8-quinoline **2**, $CHCl_3$, $(Me_2CH)_2NEt$, rt, 1 day.



Scheme 3. Reagents and conditions: (a) propargyl bromide, $NaHCO_3$, DMSO, rt; (b) TrCl, DMF, rt; (c) 5-chloromethyl-8-quinoline **2**, $CHCl_3$, $(Me_2CH)_2NEt$, rt; (d) CF_3COOH, rt.

2.2. Iron-chelating properties

2.2.1. Complex formation with Fe(III), and Cu(II). Spectrophotometric study reveals the complex formation of the new chelators with both Fe(III), and Cu(II), but with higher selectivity for Fe(III) over Cu(II). One representative is shown in Figures 1 and 2. The appearance of a pronounced absorption maxima around 460 and 590 nm upon the addition of $Fe_2(SO_4)_3$ (Fig. 1) showed the formation of Fe(III)–**9** complex. As shown in Figure 2b a new band around 375 nm appeared upon the addition of $CuSO_4$ demonstrating the formation of Cu-complex. Addition of $Fe_2(SO_4)_3$ resulted in disappearance of the 375 nm band and appearance of new bands 460 and 590 nm (Fig. 2c), which are the characteristic maximal absorption of Fe(III)–**9** complex. This indicates that **9**

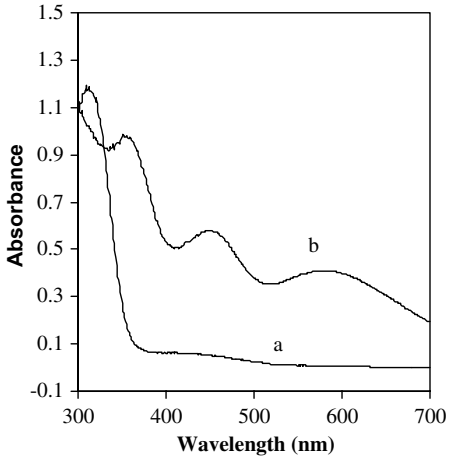


Figure 1. Spectrophotometric detection of Fe(III) complexation with HLA20: (a) spectrum of 0.8 mM compound **9** (HLA20) in Tris buffer pH 7.4; (b) spectrum of 0.8 mM compound **9** (HLA20) in Tris buffer pH 7.4 after the addition of 0.15 mM $Fe_2(SO_4)_3$.

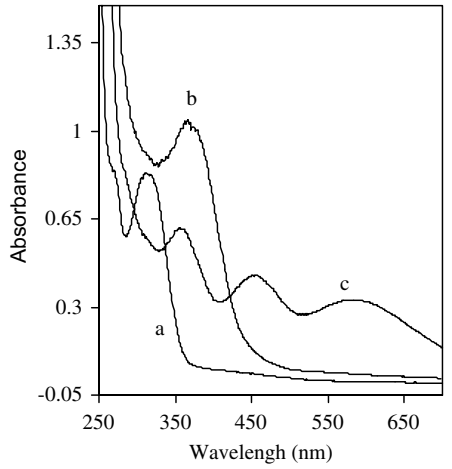


Figure 2. Spectrophotometric detection of the formation of Fe(III)–**9** (HLA20) complex from Cu(II)–**9** (HLA20) complex: (a) spectrum of 0.6 mM compound **9** (HLA20) in Tris buffer pH 7.4; (b) spectrum of (a) after the addition of 0.2 mM $Cu(SO_4)_2$. (c) spectrum of (b) after the addition of 0.1 mM $Fe_2(SO_4)_3$.

(HLA20) had a higher binding selectivity for iron over copper. The fact that the new chelators have binding capability both for iron and copper but with higher selectivity for iron may be important factors for the anti-oxidative-type drugs, since it is the excessive iron stores and iron-mediated generation of free radicals in the brain that are thought to be associated with neurodegenerative diseases.[2,20] Therefore, the novel chelators with these properties would be expected to chelate iron instead of copper and hence would have potential use as drug candidates in neurodegenerative diseases.

2.2.2. Composition of the iron-complex. The composition of the Fe(III)-complex of the novel chelators was established by spectroscopic measurement and mass spectrometry. One example is reported here. Figure 3 indicates that under the experimental conditions a complex with 1:3 [iron:chelator **9** (HLA20)] molar ratio was formed.

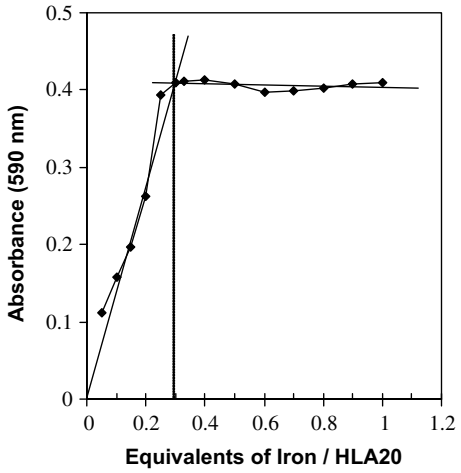


Figure 3. Change in absorption at λ_{max} = 590 nm with [Fe(III)]/[HLA20] molar ratio in Tris buffer (pH 7.4).

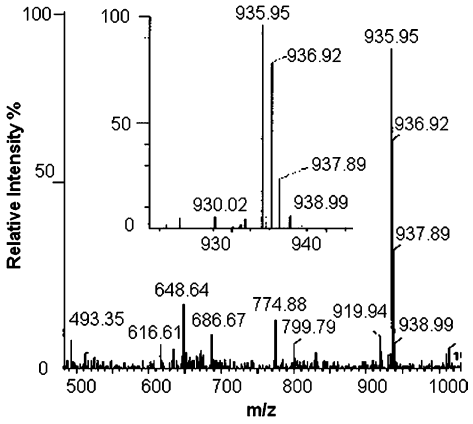


Figure 4. Positive ion electrospray ionization mass spectrum of a solution of iron(III) sulfate and compound **9** (HLA20).

The mass spectra of the complex (Fig. 4) shows that the HLA20–Fe(III) complex gave a value of *m/z* 935.95 corresponding to the ion $[Fe(HLA20)_3 + K]^+$, that is, stoichiometry of 1:3. The same stoichiometry (1:3 iron:chelator) was also found for the other new chelators (data not shown).

2.3. Relative binding affinities

The relative Fe(II) binding affinities of the chelators were determined by a fluorescent probe calcein (CA).[21] In this method, the fluorescene of CA can be quenched by metal ions such as Fe(II), and then dequenched by adding iron chelators. The Fe(II) binding affinity is inversely proportional to the half maximal dequenching concentration $[M]_{1/2}$. Figure 5 shows the fluorescence recovery as a function of the concentration of three new chelators. Similar profiles were also obtained for the other chelators (data not shown). From these profiles, $[M]_{1/2}$ for each chelator was estimated, and the order of chelator capacity for complexing iron in solution was obtained: ($[M]_{1/2}$ in μM): DFO (1.5) (not shown) > compound **14** (4.0) > **3** (4.4) > **2** (4.8) > **9** (5.4) > **11** (6.0) > **4** (6.5) > L1 (8.4). DFO is a prototype iron chelator used clinically for treating iron overload with log stability constant ($\log\beta$) = 31;[22] L1 or 1,2-dimethyl-3-hydroxy-4-pyridinone is an orally active iron chelator with overall log stability constant ($\log\beta_3$) = 36.[22] The above data suggested that all the tested compounds were good iron chelators with rather similar Fe(II) binding ability. Their Fe(II) binding capacity were less potent than that of DFO, but slightly higher than that of L1, capable, as well, in iron binding in 3:1 M ratio,[22] respectively. This affinity for iron should be high enough to inhibit the iron redox cycle in the biological environment. The high affinity of DFO for iron preclude its use for prolonged periods of time in situations unrelated to iron overload due to serious cytotoxicity.[20] It has been reported that this serious cytotoxicity of DFO is very likely attributed to its interaction with iron containing enzymes and protein, resulting in mobilizing iron from these enzymes and proteins.[23] The new chelators with moderate affinities for iron may very well prevent interference with iron metabolism and, therefore, could be used in the therapy of neurodegenerative diseases in which treatment for prolonged periods of time is required. In fact, it has been reported[7,8] that treatment with clioquinol, a 8-hydroxylquinoline derivative with moderate iron binding affinity, does not elicit any apparent adverse general health or behavioral effects, unlike chelators currently used as therapy for iron overload conditions, which have severe side effects.[23]

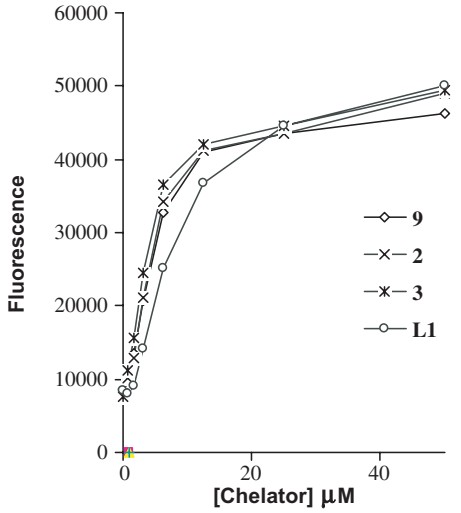


Figure 5. Dequenching of CA–Fe complexes by chelators. Iron precomplexed calcein (CA–Fe) solutions of 1 μM in Hepes 20 mM buffered saline (pH 7.4 = HBS) were incubated with various concentrations of chelators at rt for 1 h. The graph shows the original fluorescence data given in arbitrary units (a.u.) against chelator concentration.

2.4. Antioxidant properties

As shown in Table 1, all the novel chelators inhibited lipid peroxidation with similar IC_{50} values (12–16 μM) comparable to that of desferal, a prototype iron chelator, which does not cross the blood brain barrier. This inhibition may be caused by iron-chelating activity of

Table 1. Inhibitory properties on lipid peroxidation (LPO) and in vitro monoamine oxidase (MAO) of the new chelators

Compd	Inhibition of LPO[a] IC_{50} (μM)	In vitro inhibition[b] IC_{50} (μM)	
		MAO-A	MAO-B
2	13 ± 2	>200	>200
3	14 ± 1.5	>200	>200
4	16 ± 3	<0.1	<0.1
9	12 ± 2	>200	110 ± 10
11	15 ± 3	>200	>200
14	12 ± 2.5	>200	>200
DFO	8 ± 1.5	>1000	>1000

[a] Inhibition of lipid peroxidation by the new chelators in the presence of 1.5 μM FeSO4/50 μM ascorbic acid. The results are the mean ± SEM, $n = 3$, $p < 0.05$.

[b] In vitro inhibitory action against rat brain MAO-A and MAO-B. The chelators were added to buffer containing 0.05 μM deprenyl (MAO-A) or 0.05 μM clorgylin (MAO-B) and were incubated with the tissue homogenate for 60 min at 37 °C before addition of ^{14}C-5-hydroxy-tryptamine (MAOA) or ^{14}C-phenylethylamine (MAO-B). MAO-A and -B activity in presence of drug was expressed as a percentage of that in control samples. Mean values shown ±SEM, $n = 3$, $p < 0.01$.

the test compounds. It has been reported that the antioxidative properties of iron chelators, such as desferal, rutin, and quercetin, are most likely due to their iron chelation since iron chelators may form inert complexes with iron and interfere with the Fention reaction leading to a decrease of hydroxyl free radical production.[5,6,24] The new chelators might act by similar mechanism to inhibit the free radical formation. However, it is also possible that the novel chelators may, as other antioxidants, directly scavenge free radicals and show inhibitory activity. In a word, whatever the mechanism of new compounds works by, their high potency in inhibiting on lipid peroxidation make them relatively good candidates for the treatment of neurodegenerative disease since lipid peroxidation has been implicated in the pathogenesis of these disease.[25,26]

2.5. Monoamine oxidase (MAO) inhibitory activity in vitro

Selective MAO-B inhibitors have great potential for treating PD.[26,27] Thus, the effects of the novel chelators on MAO-B activity were investigated. The results (Table 1) show that there were distinct differences in MAO inhibitory activities among the tested compounds. While compound **4** caused a significant inhibition of both MAO-A and -B activities with the IC_{50} values less than 0.1 μM. Compounds **2**, **3**, **11**, and **14** have almost no effect on MAO-A and MAO-B activity ($IC_{50} > 200$ μM). Chelator **9** exhibited more potent inhibition on MAO-B (IC_{50} 110 ± 10 μM) than on MAO-A ($IC_{50} > 200$ μM). The high MAO inhibitory activity of **4** may be attributed to the N-propargyl group in the molecule since N-propargyl-containing compounds such as rasagiline, pargyline, clorgyline, and deprenyl are potent MAO inhibitors. Removal of the propargyl moiety from these molecules abolishes the inhibitory activity.[28,29] It is well established that the propargyl moiety binds covalently mole/mole with the N-5 of flavin-adenosine-dinucleotide (FAD) cofactor at the active site of MAO enzyme to inhibit it.[28,29] The poor activity of compounds **9** and **11** as compared with **4** may be due, at least partially, to the presence of a bulky somewhat group (piperazinyl in **9**, ethyloxyacyl in **11**) near the propargyl moiety. The steric effect from a bulky group may affect the chelators binding to the enzyme. The fact that the new chelators, except **4**, do not inhibit MAO-A may be advantageous for their use in the treatment of neurodegenerative diseases since the inhibition of MAO-A activity is related to the serious side effect, known as 'cheese reaction'.[30]

2.6. Transport properties

2.6.1. Cell permeability. The method used for assessing the relative cell permeability of the test chelators is based on chelator-evoked increase in the fluorescence of iron-quenched calcein.[31] Generally the rate of fluorescence dequenching induced by addition of a chelator depends on its cell permeability and binding affinity for iron. However, in a biological system such as in K562 cells (erythroleukemia cell lines, which have been extensively studied in term of their iron-regulatory properties),[31,32] chelator permeation into cells is the rate-limiting step in the chelation of iron. Fast permeating chelators such as salycylaldehyde isonicitinoyl hydrazone (SIH) evokes swift increases in cell calcein fluorescence but poorly permeable chelators such as DFO acts only after prolonged incubations with cells.[31] Thus, the rate constant of fluorescence recovery is considered to be proportional to the permeation of chelators across the cell membrane. In the present experiments, the permeation properties of the test chelators were assessed in K562 cells. Figure 9 shows the time-dependent fluorescence recovery of CA-loaded cells evoked by adding (at 310 s) various chelators at equivalent concentrations (5 μM). As indicated in Figure 9, the fluorescence dequenching rates varied greatly with different chelators. While chelators DFO had no effect on dequenching in the employed conditions (data not shown), SIH, **4**, and **9** acted quickly with $t_{1/2}$ 145, 170, and 181 s, respectively. The rate constant of chelator permeation was determined from the reciprocal of the half time ($t_{1/2}$) of the maximum fluorescence recovery after chelator addition. Taking SIH as 100, the order was SIH (100 ± 7) > **4** (85 ± 3) > **9** (80 ± 5) > **3** (75 ± 8) > **11** (36 ± 4) > **2** (27 ± 6) > **14** (20 ± 8) ≫ DFO (not shown). As seen in Figure 10, **4** and **9** had a good permeability in K562 cells, about 80% permeation into K562 as compared with control (SIH 100%). In contrast, the other test chelators showed a much lower permeability [<40% permeation as compared to control (100%)]. As expected, DFO, a poorly permeant chelator, did not pass K562 cell membrane in our experimental conditions.

2.6.2. Lipophilicity. Lipophilicity is thought to be a major determinant in the permeability of a drug. In order to correlate the permeability of the new chelators to their lipophilicity, we used the shake-flask method to assess their lipophilicity in water (pH 7).[33] The log D (logarithm of the n-octanol/water distribution coefficients) is the most commonly used measure of the lipophilicity of a drug candidate.[33] The log D values of the

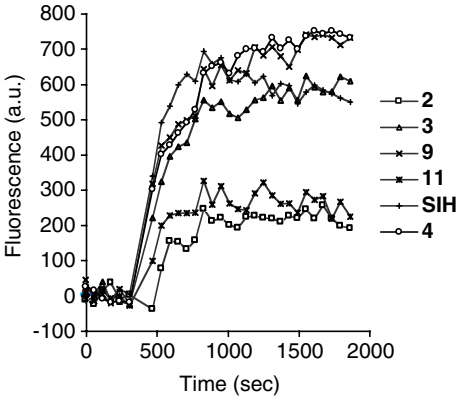


Figure 6. Dequenching of CA/iron complexes in K562 cells by extracellularly added the test chelators. CA-loaded K562 cells were treated with various chelators (5 μM concentration) at the time indicated (arrow). Scale, arbitrary fluorescence units (a.u.) against time (s).

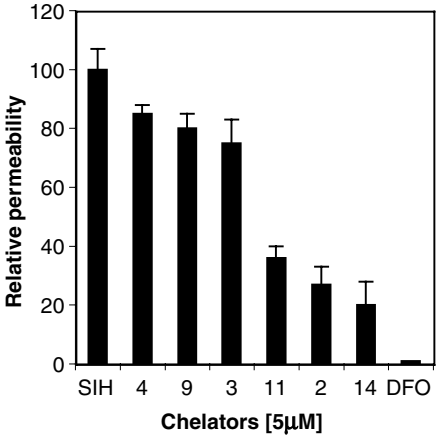


Figure 7. Relative permeability of iron chelators in K562 cells. Values were mean ± SEM ($n = 6$), given relative to those obtained with SIH, for which 100 represents an apparent rate constant of $0.0069\,s^{-1}$ (equivalent to a $t_{1/2}$ of ingress of 145 s for a 5 μM chelator solution).

new chelators were as follows (log *D* value): compound **4** (1.92) > **9** (1.79) > **3** (1.57) > **11** (0.71) > **2** (0.54) > **14** (0.21). These data indicated that there is a good correlation between the lipophilicity values and the permeability of the new chelators, that is, the permeability is proportional to their lipophilicity values. Chelators **4**, **9**, and **3** with high log *D* (good lipophilicity) were found to have a good permeability in K562 cells; **11**, **2**, and **14** with low log *D* exhibited poor permeability in K562 cells. The above results suggest that chelators **4**, **9**, and **3** may cross the blood brain barrier due to their lipid solubility, as opposed to **11**, **2**, and **14** with higher hydrophilicity (Figs. 6 and 7).

2.7. In vitro neuroprotective activity

The cell death induced by 6-hydroxydopamine (6-OHDA) either in PC12 cells or P19 cells is a good model for studying oxidative stress associated with PD and AD.[34–36] As a nerve-cell-model substitute, we used P19 cells (mouse embryonal carcinoma cell line) that can be induced to differentiate into post-mitotic neuron-like cells in the presence of high dose of retinoic acid.[37,38] It has been reported that 6-OHDA triggered mostly necrosis and less than 5% apoptosis in PC12 cells, whereas 6-OHDA-induced death in P19 cells was apoptotic.[35,36]

Figure 8 shows protective effects of a selection of chelators on differentiated P19 cells against neurotoxin 6-hydroxydopamine. These results indicate that chelator **9** (HLA20) exhibits very good neuroprotection (61%), and **3** also had moderate protective activity (31%). However, no neuroprotection were obtained with both **2** and apomorphine. Even for DFO, very poor neuroprotective action (12%) was observed. We chose these compounds for the above experiments based on the following reasons: (a) both **2** and **3** do not show appreciable inhibition on MAO-A or -B in vitro in the range of 0.1–100 μM, **2** having poor but **3** good membrane permeability. (b) Chelator **9** has good membrane permeability and selectively inhibits MAO B in vitro with IC_{50} of 110 μM. (c) DFO is a prototype iron chelator and potent radical scavenger, and apomorphine is a highly potent radical scavenger with iron-chelating potency.[39] The good neuroprotection of chelator **9** in differentiated P19 cells may be due to the combined effects of iron chelation, antioxidant, MAO B inhibition, and good permeability. This finding is consistent with the hypothesis that neuroprotection in neurodegenerative diseases requires a permeable drug combining iron chelating with antioxidant capacity, iron chelator, and MAO-B inhibitory properties.[2] Another possible explanation might be related to glutamate excitotoxicity. Differentiated P19 cells exhibit *N*-methyl-D-aspartate (NMDA) receptor-mediated intracellular calcium responses to glutamate.[40] Studies have shown that pretreatment of differentiated P19 cells with NMDA antagonists such as dizocilpine, protected against glutamate-induced cell death.[41] Compound **9**

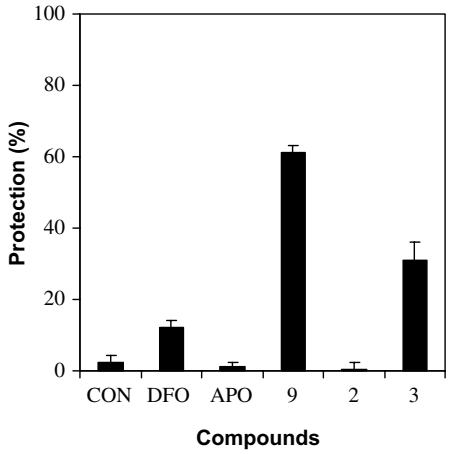


Figure 8. Effects of apomorphine and iron chelators **2**, **3**, and **9** and desferal on 6-OHDA-toxicity in differentiated P19 cells. Differentiated P19 cells were treated for 6 h with 50 μM 6-hydroxydopamine (6OHDA) in full medium containing either 5 μM of indicated chelators or 1 μM apomorphine. The cell viability was measured with Alamar blue assay 4 h later. % Protection = the % activity relative to the system treated with no 6OHDA. Data are means ± SEM, $n = 3$. $p < 0.05$, compared with control.

(HLA20), but neither **2** nor DFO may act as NMDA antagonist to block NMDA receptors and thus protect against cell death. However, this hypothesis need to be further confirmed.

2.8. Radical-scavenging property

Chelator **9**, which shows very good neuroprotection in differentiated P19 cells, was also evaluated for its radical-scavenging property by EPR. The EPR spectrum [a quartet (1:2:2:1) with hyperfine splitting of 14.9 G] in Figure 9A is characteristic for the DMPO-·OH spin adduct.[42] As can seen in Figure 9B–D, the addition of the increasing concentration of **9** to the control (Fig. 9A) reduces the intensity of the DMPO-·OH signal. To quantify the relative amount of ·OH present in a given sample, the third peak of each spectrum was used for calculation. The result (Fig. 10) demonstrated that compound **9** (HLA20) reduced the DMPO-·OH signal in a concentration-dependent manner. At room temperature and the reagent concentration used in this study, the IC_{50} for compound **9** (HLA20) is about 0.15 mM (Fig. 10). It is known that the formation of ·OH by photolysis of H_2O_2 is iron-independent but catalyzed by light.[43] Compound **9** (HLA20) remarkably decrease the intensity of the DMPO-·OH signal produced in photolysis system suggesting that **9** (HLA20) acts as a radical scavenger to directly scavenge ·OH. This result is consistent with published data since, as reported before,[24,44] flavanoids, and other phenolic compounds act as radical scavenger due to the presence of phenolic moiety. It is the ability of the phenolic moiety to neutralize lipid radicals by donating hydrogen atoms to them and form a poorly reactive phenoxy radical that offers these compounds radical-scavenging activity. Therefore, it is perhaps not surprising that **9** (HLA20), due to the presence of the phenolic group, possesses radical-scavenging property.

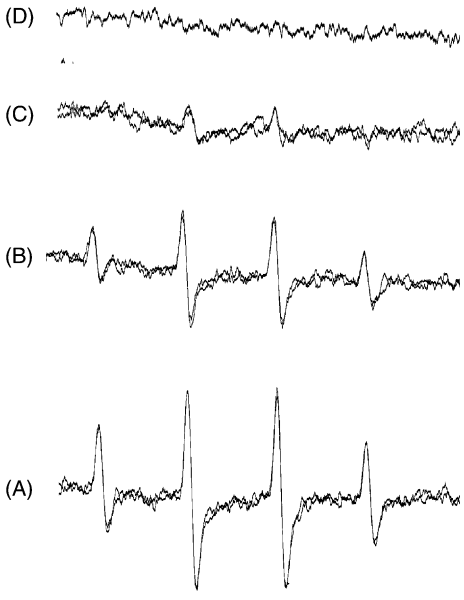


Figure 9. Typical EPR spectra of DMPO-·OH. Derived from hydroxyl radicals generated by photolysis of a 0.6% H_2O_2 pH 7.4 PBS buffer containing 0.1 M DMPO, with different irradiation time: A, B, and C: 40 and 46 min; D 40 min; E 46 min. (A) Control. (B) 0.1 mM **9** (HLA20). (C) 0.5 mM **9** (HLA20). (D) 1.0 mM **9** (HLA20). (E) 1.0 mM **9** (HLA20).

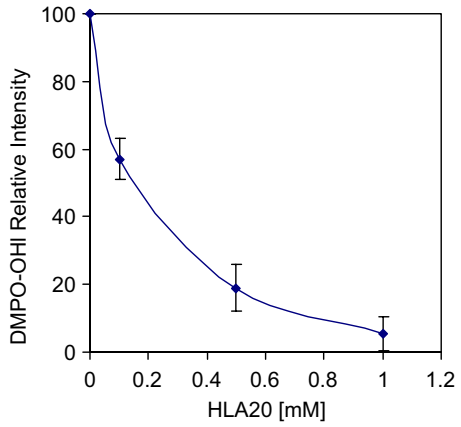


Figure 10. Concentration–response curves for inhibition at room temperature and pH = 7.4 (PBS buffer containing 0.1 M DMPO) of EPR DMPO-·OH peaks by HLA20.

The clinical application of neuroprotective drugs possessing hydroxyquinoline moiety depends, of course, on minimization of potential toxicity. In this regard, it is worth noting that an old antibiotic iron chelator 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol or CQ) was withdrawn from the market due to its possible neurotoxicity, including nerve cell degeneration in the hippocampus, ataxia in rats, and subacute myelo-neuropathy (SMON) in patients.[45,46] As with most drugs, toxicity may occur at very high doses, but for useful agents, not within their effective therapeutic window. More recent studies have found no evidence for CQ toxicity with bioeffective dosing in mice.[47] It was proposed that indiscriminate synaphigh-dose CQ use aggravated B12 deficiency in postwar Japan and led to SMON in a subset of vulnerable patients. More significantly, in a phase II clinical trial, therapeutic CQ has been coadministered with B12 to Alzheimer patients with no evidence of drug-dependent adverse events.[7,47]

3. Conclusion

Several novel antioxidant-iron chelators with 8-hydroxyquinoline moiety have been synthesized, and their various properties related to iron chelation, antioxidant, and neuroprotective activities have been investigated. Studies have shown that all the new compounds are strong chelators for iron and copper with higher selectivity for iron, and chelate iron(III) in a 3:1 M ratio, respectively. They have slightly higher affinity for iron(III) than that of L1, an orally active iron chelator with the overall log stability constant ($\log\beta_3$) = 36. The new compounds inhibited Fe/ascorbate induced mitochondrial membrane lipid peroxidation with IC_{50} in the range of 12–16 μM, which are comparable to that of desferal, a prototype iron chelator which does not

cross the blood brain barrier. Further studies selected Chelator **9** (HLA20) as a possible lead for further development, based on the following criteria: (a) strong iron chelator with high free radical-scavenging capability, (b) moderate MAO-B inhibitory activities and good permeability through K562 cell membranes, and (c) effective neuroprotection in differentiated P19 cell model. Further experiments both in vitro and in vivo are underway to evaluate **9** as a potential drug candidate for treating Parkinson's disease.

4. Experimental

4.1. Chemical synthesis

All chemicals were obtained from commercial suppliers (Aldrich, Merck, or Fluka). Proton NMR spectra were measured on a Bruker DPX-250 spectrometer. Flash column chromatography separations were performed on silica gel Merck 60 (230–400 mesh ASTM). UV–vis spectra were measured on a Hewlett-Packard 8450A diode array spectrophotometer. TLC was performed on E. Merck Kieselgel 60 F254 plates. Mass spectra (DI, EI-MS) were measured on a VG-platform-II electrospray single quadropole mass spectrometry (Micro Mass, UK). Reverse-phase HPLC was performed with a Spectra-Physics SP8800 liquid chromatography system (Spectra-Physics, San Jose, CA) equipped with an applied Biosystem 757 variable-wavelength absorbance detector.

4.1.1. 5-Chloromethyl-8-quinolinol hydrochloride (1). A mixture of 14.6 g (0.1 mol) of 8-quinolinol, 16 mL of 32% HCl in water, and 16 mL (0.1 mL) of 37% formaldehyde in water at 0 °C was treated with hydrogen chloride gas for 6 h. The solution was allowed to stand at room temperature for 2 h without stirring. The yellow solid obtained was collected on a filter, washed with 90% alcohol, and dried under vacuum to give 5-chloromethyl-8-quinolinol hydrochloride **1** (19.0 g, 98%); ^{1}H NMR (250 MHz, $CDCl_3$): 5.32 (s, 2H), 7.53 (m, 1H), 7.85 (m, H), 8.12 (m, 1H), 9.12 (m, 1H), 9.28 (m, 1H).

4.1.2. 5-(4-(2-Hydroxyethyl)piperazin-1-ylmethyl)-8-hydroxyquinoline (2). To a mixture of 5-chloromethyl-8-quinolinol hydrochloride **1** (2.707 g, 11.8 mmol) and diisopropylethylamine (2.1 mL, 20.4 mmol, 2 equiv) in 50 mL $CHCl_3$ at 0 °C was added 4-(2-hydroxyethyl)-piperazine (1.7 mL, 10.2mmol, 1 equiv). The mixture was stirred for 24 h at room temperature. $CHCl_3$ (100 mL) was added and the solution obtained was washed with 5% $NaHCO_3$ (3 × 50 mL), brine (2 × 50 mL), and then dried over Na_2SO_4. The solution was filtered and evaporated to dryness. The residue was crystallized from a mixture of benzene–hexane (1:1) to yield **2** as white solid. (2.2 g, 75% yield). Mp = 127–128 °C; ^{1}H NMR (250 MHz, $CDCl_3$), 2.51 (m, 8H), 2.54 (dd, J = 5.58, 3.56 Hz, 2H), 3.59 (dd, J = 5.28, 5.49 Hz, 2H), 3.80 (s, 2H), 7.06 (d, J = 7.71 Hz, 1H), 7.32 (m, 1H), 7.49 (dd, J = 8.42, 4.10 Hz, 1H), 8.66 (dd, J = 8.46, 1.55 Hz, 1H), 8.79 (dd, J = 4.28. 1.34 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) 52.86, 52.94, 57.65, 59.20, 60.50, 108.72, 121.35, 124.48, 127.84, 128.88, 134.06, 138.63, 147.49, 151.76. Mass spectrometry: calcd for $C_{16}H_{21}N_3O_2$ *m/z* $[M+H]^+$ = 288.36, found $[M+H]^+$ = 288.21.

4.1.3. Ethyl 4-(8-hydroxyquinolin-5-ylmethyl)-1-piperazinecarboxylate (3). Compound **3** was synthesized following the procedure above for **2**. (42% yield, mp = 95–96 °C). ^{1}H NMR (250 MHz, $CDCl_3$), 1.25 (dd, J = 7.1, 7.1 Hz, 1H), 2.42 (s, 4H), 3.43 (s, 4H), 3.81 (s, 2H), 4.14 (dd, J = 14.21, 7.12 Hz, 2H), 7.08 (d, J = 7.72 Hz, 1H), 7.31 (m, 1H), 7.47 (dd, J = 8.52, 4.20 Hz, 1H), 8.66 (dd, J = 8.56, 1.58 Hz, 1H), 8.79 (dd, J = 4.18, 1.54 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) 14.49, 43.47, 52.49, 60.40, 61.09, 108.73, 121.22, 123.77, 127.67, 128.92, 133.83, 138.48, 147.42, 151.91, 151.27. Mass spectrometry: calcd for $C_{17}H_{21}N_3O_3$ *m/z* $[M+Na]^+$ = 238.37, found $[M+Na]^+$ = 238.22.

4.1.4. 5-(*N*-Methyl-*N*-propargylaminomethyl)-8-hydroxyquinoline (4). Chelator **4** was prepared as described above for **2**. (80%). Mp = 232–233 °C (hydrochloric salt); ^{1}H NMR (250 MHz, $CDCl_3$), 2.30 (dd, J = 2.15, 2.14 Hz, 1H), 2.33 (s, 3H), 3.27 (d, J = 2.20 Hz, 2H), 3.86 (s, 2H), 7.06 (d, J = 7.72 Hz, 1H), 7.31 (m, 1H), 7.37 (d, J = 7.73 Hz, 1H), 7.46 (dd, J = 8.52, 4.2 Hz, 1H), 8.60 (dd, J = 8.52, 1.47 Hz, 1H), 8.76 (dd, J = 4.01, 1.50 Hz, 1H). ^{13}C NMR (100 MHz, hydrochloric salt in D_2O) 42.46, 47.54, 56.35, 74.18, 83.77, 118.22, 1198.17, 15.83, 131.63, 132.06, 138.96, 145.46, 145.97, 152.20. Mass spectrometry: calcd for $C_{17}H_{21}N_3O_3$ *m/z* $[M+Na]^+$ = 249.27, found $[M+Na]^+$ = 249.23.

4.1.5. *tert*-Butyl 1-piperazinecarboxylate (6). A solution of di-*tert*-butyl dicarbonate (2.93 g, 12.77 mmol) in MeOH (25 mL) was slowly added to a stirring solution of piperazine (2.00 g, 23.22 mmol) in MeOH (50 mL) at 0 °C. The mixture was then stirred for 2 days at room temperature, and the solvent removed in vacuum. The crude solid was redissolved in diethyl ether (100 mL) with warming, and the white precipitate left was filtered off. The product was extracted from the mother liquor with 1 M citric acid solution (3 × 50 mL), and the aqueous layer was washed with Et_2OAc (3 × 50 mL), basified with Na_2CO_3 (pH 11), and extracted with Et_2OAc (3 × 50 mL). The organic layer was dried over Na_2SO_4 and evaporated in vacuum to give *tert*-butyl 1-piperazinecarboxylate **6** as a waxy white solid (crude, 1.57 g, 66%), mp = 53–54 °C. ^{1}H NMR (250 MHz, $CDCl_3$) 1.42 (s, 9H), 1.89 (s, 1NH), 2.78 (m, 4H), 3.36 (m, 4H).

4.1.6. *tert*-Butyl 4-propargylpiperazine-1-carboxylate (7). Propargyl bromide (356.9 mg, 3 mmol) was added slowly to a mixture of *tert*-butyl 1-piperazinecarboxylate **6**. (558.8 mg, 3 mmol) and diisopropylethylamine (407.1 mg, 3.15 mmol) in $CHCl_3$ (25 mL) at 0 °C. The mixture was stirred for 24 h at room temperature. $CHCl_3$ (50 mL) was then added and the solution obtained was washed with 5% $NaHCO_3$ (3 × 50 mL), brine (2 × 50 mL), and then dried over Na_2SO_4. The solution was filtered and evaporated to dryness. The residue was crystallized from a mixture of benzene–hexane (1:1) and gave *tert*-butyl 4-propargylpiperazine-1-carboxylate **7** (337 mg, 86%). ^{1}H NMR (250 MHz, $CDCl_3$):

1.42 (s, 9H), 2.22 (s, 1H), 2.46 (s, 4H), 3.26 (s, 2H), 3.41 (s, 4H).

4.1.7. *N*-Propargylpiperazine (8). *tert*-Butyl 4-propargylpiperazine-1-carboxylate **7** (570 mg, 2.545 mmol) was dissolved in a mixture of trifluoroacetic acid (10 mL) and water (2.5 mL). The solution was stirred at room temperature overnight, and then was evaporated to dryness in vacuum. The residue was dissolved in water (10 mL) and then based with Na_2CO_3 (pH 11), and extracted with Et_2OAc (3×50 mL). The organic layer was washed with brine (2×50 mL) and dried over Na_2SO_4 overnight. Evaporation in vacuum gave *N*-propargylpiperazine **8** as white solid. (193 mg, 62% yield). ^1H NMR (250 MHz, $CDCl_3$) 1.64 (s, 1NH), 2.26 (m, 1H), 2.55 (dd, $J = 4.73$, 4.50 Hz, 4H), 2.93 (dd, $J = 4.96$, 4.84 Hz, 4H), 3.29 (d, $J = 2.44$ Hz, 2H).

4.1.8. 5-(4-Propargylpiperazin-1-ylmethyl)-8-hydroxyquinoline (9). To a mixture of 5-chloromethyl-8-quinolinol hydrochloride **2** (323 mg, 1.407 mmol) and diisopropylethylamine (0.26 mL, 1.477 mmol, 1.05 equiv) in 6 mL $CHCl_3$ at 0 °C was added *N*-propargylpiperazine **8** (173 mg, 1.407 mmol, 1 equiv). The mixture was stirred for 24 h at room temperature. $CHCl_3$ (10 mL) was then added and the solution was washed with 5% $NaHCO_3$ (3×50 mL), brine (2×50 mL), and then dried over Na_2SO_4. The crude product was purified by FC to give 5-(4-propargylpiperazin-1-ylmethyl)-8-hydroxyquinoline **12** as white solid. (337 mg, 86% yield). Mp = 183–184 °C (hydrochloric salt). Mass spectrometry: calcd for $C_{17}H_{21}N_3O_3$ *m/z* $[M+H]^+ = 282.35$, found $[M+H]^+ = 282.29$. ^1H NMR (250 MHz, $CDCl_3$), 2.23 (m, 1H), 2.54 (s, 8H), 3.28 (d, $J = 2.44$ Hz, 2H), 3.80 (s, 2H), 7.08 (m, 1H), 7.36 (d, $J = 10.59$, 1H), 7.46 (dd, $J = 8.55$, 4.18 Hz, 1H), 8.67 (dd, $J = 8.56$, 1.55 Hz, 1H), 8.78 (dd, $J = 4.18$. 1.51 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) 46.77, 51.98, 52.87, 60.55, 73.05, 78.92, 108.60, 121.40, 124.58, 127.87, 128.87, 134.10, 138.68, 147.50, 151.78.

4.1.9. DL-*N*-Propargyl-3-(8-hydroxyquinolin-5-yl)alanine ethyl ester (11). A mixture of $NaHCO_3$ (17 mg, 0.2 mmol) and compound **10** (26 mg, 0.1 mmol, synthesized as described before[4]) was dissolved in 5 mL DMSO, and the solution was stirred at room temperature for 2 h. To the solution propargyl bromide (11.9 mg, 0.1 mmol) was slowly added, and the solution was stirred at room temperature for 24 h. The solvent was removed by vacuum, and the crude product was purified by preparative HPLC [VYDAC RP-18 column (250×22 mm, Hesperia, CA). Solvent A = water, 0.1% v/v TFA; solvent B = MeCN–water = 3:1, 0.1% v/v TFA; $t_R = 25.4$ min (linear gradient 0–80% B over 55 min)] to give the title compound **11**: 21 mg, 70% yield. Mp = 34–35 °C. ^1H NMR (250 MHz, D_2O) 0.92 (dd, $J = 28.5$, 8.2 Hz, 3H), 2.91 (s, 1H), 3.68 (m, 2H), 4.01 (m, 2H), 4.36 (dd, $J = 7.7$, 7.5 Hz, 1H), 5.04 (d, $J = 2.3$ Hz, 1H), 7.59 (d, $J = 8.0$ Hz, 1H), 7.70 (d, $J = 8.2$ Hz 1H), 8.06 (dd, $J = 8.7$, 5.4 Hz, 1H), 8.97 (d, $J = 5.4$ Hz, 1H), 9.13 (d, $J = 8.7$ Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) 14.04, 31.08, 37.12, 55.53, 60.98, 70.34, 84.72, 109.31, 121.58, 123.76, 127.17, 128.89, 132.57, 138.61, 147.44, 151.40, 174.96. Mass spectrometry: calcd for $C_{17}H_{18}N_2O_3$ *m/z* $[M+H]^+ = 299.34$, found $[M+H]^+ = 299.30$.

4.1.10. *N*-(8-Hydroxyquinolin-5-ylmethyl)-L-cysteine ethyl ester (14). L-Cysteine hydrochloride (250 mg, 1.1 mmol) and trityl chloride (675 mg, 2.4 mmol) were stirred in 1 mL DMF for 2 days at room temperature. A 10% sodium acetate solution (9 mL) was then added, and the precipitate obtained was filtered and washed with distilled water. The solid residue was stirred in acetone at 50 °C for 30 min and filtered after cooling to 0 °C, and washed with acetone and diethyl ether. After drying in vacuo, 500 mg (86%) of **12** was obtained as a white powder. Compound **12** (39 mg, 0.1 mmol) was dissolved in DMSO (1 mL), to the solution solid $NaHCO_3$ (17 mg, 0.2 mmol) was added, and the mixture was stirred for 30 min at room temperature. Then powdered 5-chloromethyl-8-quinolinol hydrochloride (19 mg, 0.1 mmol) was added, and the solution was stirred at room temperature for 24 h. The solvent was removed by vacuum, and the crude product was dissolved in 3 mL solution of TFA–H_2O_2–triethylsilane–thioanisole (85:5:5:5). After 2 h, the solvent was removed in vacuo, and the crude product was purified by preparative HPLC [VYDAC RP-18 column (250×22 mm, Hesperia, CA); solvent A = water, 0.1% v/v TFA; solvent B = MeCN–water = 3:1, 0.1% v/v TFA; $t_R = 15.4$ min (linear gradient 0–80% B over 55 min)] to give the title compound **14**: 21 mg 60% based on **12**. Mp = 117–118 °C. Mass spectrometry: calcd for $C_{15}H_{18}N_2O_3S$ *m/z* $[M+Na]^+ = 229.38$, found $[M+Na]^+ = 229.27$. ^1H NMR (250 MHz, D_2O) 0.95 (dd, $J = 7.1$, 7.1 Hz, 3H), 2.94 (m, 1H), 3.42 (dd, $J = 14.2$, 7.1 Hz, 2H), 4.15 (m, 2H), 4.75 (s, 2H), 7.01 (d, $J = 8.0$ Hz, 1H), 7.38 (d, $J = 7.8$ Hz, 1H), 7.83 (m, 1H), 8.73 (m, 1H), 8.99 (m, 1H).

4.2. EPR spectra

The EPR spectra were recorded on E-12 (Varian) spectrometer in a flat sealed (volume 200 μL) at room temperature (22 °C). The experimental conditions were as follows: microwave power 20 mW; sweep width 100 G; modulation amplitude 1.0 G; receive gain 4.0×10^5.

4.3. Determination of distribution coefficients

Distribution coefficients of the novel chelators were determined using the shake-flask method. 1-Octanol (spectrophotometric grade from Aldrich Chemie, Steinheim, Germany) and distilled deionized water were used as the partitioning solutions. The water was shaken with an excess of 1-octanol for presaturating and was then allowed to stand overnight before use. 1-octanol was also presaturated with an excess of water and allowed to settle overnight. A 1 mL of the test chelators (0.1 mM) in water (pH 7) was stirred vigorously with 9 mL of 1-octanol in10-mL stoppered centrifuge tubes for 1 h. The tubes were centrifuged for 20 min at 1000–2000*g*, and the two layers were separated and analyzed by UV spectrophotometry.

4.4. Lipid peroxidation assay

Lipid peroxidation was measured in rat brain mitochondrial homogenates as previously described.[39] Lipid peroxidation was induced by 50 μM ascorbic acid and 1.5 μM $FeSO_4$ The absorption of thiobarbituric acid derivatives is measured photometrically at 532 nm.

4.5. Monoamine oxidase (MAO) assay

4.5.1. Preparation of brain MAO. Rats were decapitated and the brains were quickly taken into a weighted ice-cold sucrose buffer (10 mM Tris–HCl buffer, pH 7.4, containing 0.32 M sucrose), and their weights were determined. All subsequent procedures were performed at 0–4 °C. The brains were homogenized in 0.32 M sucrose (one part tissue to 20 parts sucrose) in a Teflon glass homogenizer followed by the addition of sucrose buffer to a final concentration of 10% homogenate. The homogenates were centrifuged at 600*g* for 15 min. The supernatant fraction was taken out and centrifuged at 4500*g* for 30 min. The pellet was diluted in 0.32 M sucrose buffer and kept frozen for later assaying of MAO. Protein concentration was determined with Bradford reagent at 595 nm, using bovine serum albumin as a standard.

4.5.2. Determination of MAO activity. The activity of MAO-A and MAO-B were determined by the adapted method of Tipton and Youdim.[48] Briefly, the drug under test was added to a suitable dilution of the enzyme preparation (70 μg protein for MAO-B and150 μg MAO-A assay) in 0.05 M phosphate buffer (pH 7.4). The mixture was incubated together with 0.05 μM deprenyl (for determination of MAO-A) or 0.05 μM clorgylin (for determination of MAO-B). Incubation was carried on 1 h at 37 °C before addition of ^{14}C-5-hydroxytryptamine binoxalate (100 μM) for determination of MAO-A, or ^{14}C-phenylethylamine 100 μM for determination of MAO-B and incubation continued for 30 min or 20 min, respectively. The reaction was stopped with 2 M ice-cold citric acid, and the metabolites were extracted and determined by liquid-scintillation counting in cpm units.

4.6. Fluorescence assay

The relative binding affinity and permeability of the new compounds were measured by fluorescence assay as previously described.[31,32] For determination of relative binding affinity, iron precomplexed calcein (Fe-CAL) solutions of 1 μM in Hepes buffered saline (20 mM, pH 7.4) were incubated with various concentrations of different chelators at room temperature. The fluorescence intensity (475 nm > 520 nm) was followed with time in a Tecan fluorescence plate reader, using 96 well culture plates (Nunc) at 100 μL final volume. For measure of permeability, K562 cells were preloaded with calcein by incubation with 0.25 mM calcein-acetoxymethyl ester for 5 min at 37 °C, washed, and incubated in HBS medium containing anti-calcein antibodies to quench traces of extracellular-associated fluorescence. The suspensions were placed in 96 well plates (100 μL final volume) and analyzed while maintaining the system at 37 °C. At the indicated time, the chelator was added so as to reach a final 5 μM concentration.

4.7. Determination of neuroprotective effects on differentiated P19 cells

P19 cells were obtained from the American Type Culture Collection. They were maintained in alpha minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Before stimulation, P19 cells were allowed to differentiate into neurons during 4 days in complete medium containing 100 nM of retinoic acid as previously described.[34] Differentiated P19 neuronal cells grown in 96 well culture plate were treated for 6 h with 50 μM 6-OHDA in full medium containing either 5 μM of the indicated chelator or 1 μM apomorphine. The cells were subsequently washed and resuspended in fresh medium containing 5% Alamar blue, incubated for 4 h and the fluorescence monitored at 450 nm exc 590 nm emission (Tecan Safire fluorescence plate reader). Data are given as % protection which is equivalent to the % activity relative to the system treated with no 6OHDA.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2004.10.037.

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