Protection against Oxidative Damage by Iron Chelators: Effect of Lipophilic Analogues and Prodrugs of *N,N′*-Bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic Acid (OR10141)

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N,N′-Bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid (**1**) was recently described as a new type of iron chelator for protection against oxidative damage. It has a low affinity for iron, but the corresponding iron complex undergoes a site-specific oxidation by hydrogen peroxide through intramolecular aromatic hydroxylation into a highly stable iron phenolato complex, which does not catalyze hydroxyl radical formation. The purpose of this local activation process is to minimize toxicity compared to strong iron chelators, which may interfere with normal iron metabolism. **1** efficiently protects biological molecules against oxidative damage in vitro but not intact cells because of poor membrane permeability. We show here that, among a series of prodrug esters and lipophilic analogues, membrane-permeant *N,N′*-bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid diacetoxymethyl ester **(7)** protects human skin fibroblasts against hydrogen peroxide toxicity with an IC_{50} of 3 μM. These results thus demonstrate that, providing sufficient intracellular chelator concentration is reached, **1** efficiently protects cells against the deleterious effects of hydrogen peroxide. This strategy of oxidative activation should help the design of new chelators with better safety margins, which may be useful against oxidative damage under conditions where a prolonged administration is needed.

Introduction

Iron imbalance is known to play an essential role in oxidative damage associated with a variety of conditions ranging from inflammatory disorders,[1] drug toxicities,[2] traumatic or ischemic injuries,[3] neurodegenerative disorders,[4] and ultraviolet light effects.[5] Various iron proteins such as ferritin release iron in the presence of superoxide or other reducing radicals.[6] Once released, free iron can undergo a redox cycling in the presence of physiological reductants and hydrogen peroxide, thereby generating hydroxyl radicals by the Fenton reaction.[7] The use of iron chelators has therefore very broad clinical potential.[8,9] However, strong iron chelators are likely to show side effects by removing safe iron from various sites such as iron-containing enzymes, especially when used for prolonged periods of time. Iron chelators may also possess high affinities for other metals such as copper or zinc with the capacity to induce toxic effects by interfering with the homeostasis of these metals.[10] Strong iron chelators such as desferrioxamine (Desferal), *N,N′*-bis(2-hydroxybenzyl)ethylenediamine-*N,N′*-diacetic acid (HBED), or 1,2-dimethyl-3-hydroxypyridin-4-one (Deferiprone) are potentially very interesting compounds against oxidative injury. Nevertheless, their very high affinities for ferric iron preclude their use for prolonged periods of time in situations unrelated to iron overload.[11]

To minimize the side effects of iron chelators, we have developed a new strategy based on an oxidative activation mechanism. These chelators have an affinity for iron low enough to avoid side effects related to iron mobilization from iron proteins[12] but are able to bind free or loosely bound iron. Moreover, as illustrated in Figure 1 for compound **1** (OR10141), the corresponding complex is quantitatively hydroxylated by hydrogen peroxide into a highly stable phenolato iron complex. A dramatic 10^{12} increase in affinity constant for ferric iron is indeed observed.[12] Intramolecular hydroxylation mechanisms as well as structure−efficiency relationships were recently investigated in this series.[13,14] It was found that intramolecular aromatic hydroxylation is dependent upon the chelating moiety and on the substituents of the aromatic rings. The best profile was found with **1**, i.e., electron-enriched benzyl groups and ethylenediamine-*N,N′*-diacetic acid as chelating moiety.[15] **1** has been shown to efficiently protect biological molecules against oxidative damage in in vitro systems. Compound **1** at 50−100 μM inhibits iron ascorbate-induced lipid peroxidation of rat liver microsomes, decreases the generation of carbonyl residues on various

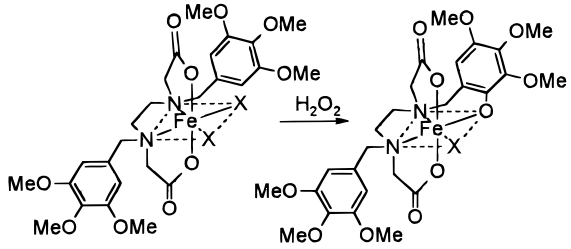

Figure 1. Oxidative activation of **1** iron complex (X = coordinating solvent).

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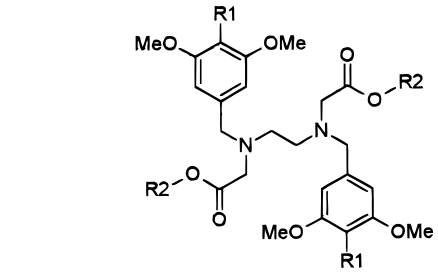

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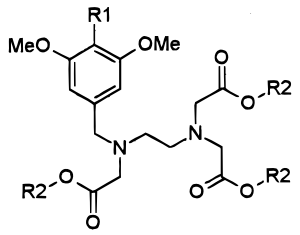

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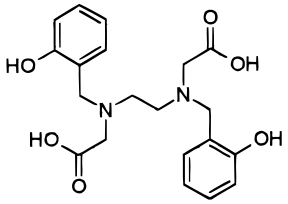

HBED

Figure 2. Iron chelators investigated.

proteins, and protects glucose 6-phosphate dehydrogenase against oxidative inactivation in vitro.[16] This suggests that the affinity of **1** for iron is high enough to partially withdraw nonspecifically bound iron from target molecules in these conditions. However it does not protect intact cells against hydroperoxide toxicity except at very high concentrations (i.e. >1 mM), because of poor membrane permeability. Here we report results of experiments describing cytoprotective effects of various prodrugs or analogues of **1** with enhanced lipophilicity to achieve higher intracellular levels of chelator.

Chemistry

To improve cell protection efficiency against oxidative injury, the effect of structural modifications of **1** were investigated as follows: (i) synthesis of prodrug esters[17] and (ii) addition of lipophilic substituents on the compound without affecting the iron chelating moiety and the intramolecular hydroxylation capacity. The syntheses of *N*,*N*′-bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid (**1**) and *N*-(3,5-dimethoxybenzyl)-ethylenediamine-*N*,*N*′,*N*′-triacetic acid (**11**) were already reported.[14] Compound **2** was obtained as previously described from alkylation of *N*,*N*′-bis(3,4,5-trimethoxybenzyl)ethylenediamine with methyl bromoacetate.[16] Novel compounds **3**−**8** were synthesized by alkylation of disodium *N*,*N*′-bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N*,*N*′-diacetate by corresponding alkyl bromide in DMF. Compounds **9** and **10** were prepared according to the general procedure described previously[14] by reacting bromoacetic acid with the corresponding substituted diamines obtained by reductive alkylation of 4-butyloxy-3,5-dimethoxybenzaldehyde and 4-heptyloxy-3,5-dimethoxybenzaldehyde, respectively. 4-Alkyloxy-3,5-dimethoxybenzaldehyde was prepared from syringaldehyde according to reported procedures.[18] Compounds **12** and **13** were prepared from the same aldehydes, which were reductively coupled to *N*-acetylethylenediamine following the reaction pathway used for **11**.[14]

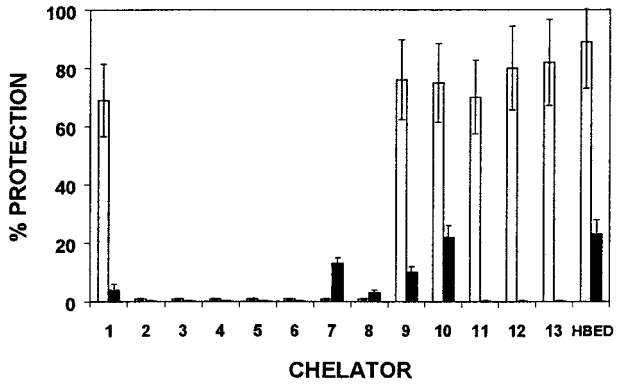

Figure 3. Percent (%) protection afforded by compounds **1**−**13** at 10 μM against DNA SSB induced by the Fenton reaction (white bars) and at 50 μM against cytotoxicity induced by 72 h of contact with 50 μM hydrogen peroxide on human skin fibroblasts (black bars).

Table 1. Iron Chelators Investigated

compd	R1	R2
1	OCH_3	H
2	OCH_3	CH_3
3	OCH_3	C_2H_5
4	OCH_3	$CH(CH_3)_2$
5	OCH_3	C_4H_9
6	OCH_3	C_7H_{15}
7	OCH_3	CH_2OCOCH_3
8	OCH_3	$CH_2CON(C_2H_5)_2$
9	OC_4H_9	H
10	OC_7H_{15}	H
11	H	H
12	OC_4H_9	H
13	OC_7H_{15}	H

Biological Evaluation

The antioxidant effects of the chelators were assessed by measuring both the protection of isolated DNA against single-strand breaks induced by Fenton chemistry and the cytoprotection of cultured cells against hydrogen peroxide toxicity. HBED was taken as a positive control.

Supercoiled plasmid nicking has been extensively used as a very sensitive measure of DNA damage.[16,19] This assay allows the quantification of both open circular form, generated by a single nick in supercoiled plasmid leading to single-strand breaks (SSB), and linear form, generated by multiple hits at the same locus leading to double-strand breaks (DSB). In the present study, only SSB were evaluated since, in our conditions, linear DNA formation was not significantly detected. This absence of DSB is probably due to the fact that redox cycling of iron cannot take place since no reductant was added to the reaction medium. As expected, none of the esters **2**−**8** tested in vitro against DNA SSB afforded protection, consistent with the need for free carboxylate for iron coordination. On the other hand, HBED and compounds **1** and **9**−**13** inhibit DNA SSB formation, in agreement with the chelation capacity of these compounds[14] (Figure 3). These results confirm the ability of these chelators to form unreactive iron complexes, which do not generate DNA damaging species, contrary to other chelators such as EDTA.[16]

In a first run of the cytoprotection experiment, the same compounds were investigated at 50 μM against the toxicity of 50 μM H_2O_2. It was checked that neither

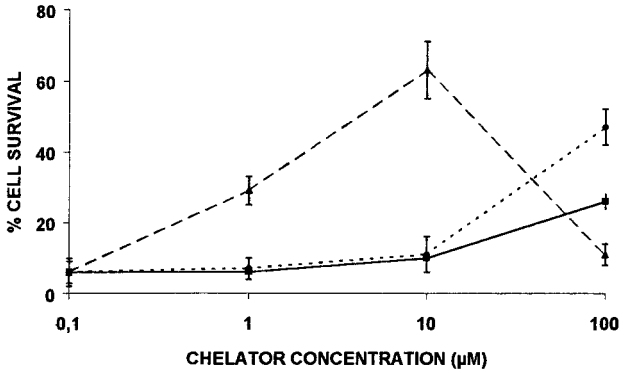

Figure 4. Dose-dependent protection of human skin fibroblasts against cytotoxicity of 100 μM hydrogen peroxide for compounds **7** (—), HBED (· · ·), and **10** (- - -).

compound tested was cytotoxic by itself at this concentration except **7** which was found to reduce cell growth after 72 h of contact, thereby probably minimizing its own protective effect. Figure 3 shows that only compounds **7**, **9,** and **10** afforded a significant, although weak, protection against the deleterious effect of H_2O_2. None of the simple alkyl esters **2**—**6** or diethylaminocarbonylmethyl ester **8** protected cells. Since these compounds do not contain a fluorescent moiety, it was not possible to monitor their intracellular concentrations and therefore try to correlate the observed effects with their intracellular fates. However, a low rate of cellular hydrolysis of these esters is a likely explanation for their absence of cytoprotective effects. Triacids **11**—**13** did not protect cells either, probably because these molecules, which are negatively charged at physiological pH, have insufficient bioavailability, despite the lipophilic alkyloxy chains of **12** and **13**.

For the three remaining compounds found partially active at 50 μM, the effect of varying the concentration of the chelator was investigated. Only compounds **7** and **10** afforded a significant dose-dependent protection in the low micromolar range (Figure 4), although the magnitude was weak for **10**. On the other hand, cells loaded by means of acetoxymethyl ester **7** were efficiently protected with an IC_{50} of 3 μM, i.e., better protected than by reference chelator HBED. This result is consistent with the well-known ability of acetoxymethyl esters to be cleaved by nonspecific membrane esterases.[20] However, dose-dependent protection by **7** shows a biphasic curve suggesting that cytotoxicity of the ester becomes predominant at concentrations above 30 μM.

These results demonstrate that, providing sufficient bioavailability is reached, cells are efficiently protected by **1** against the deleterious effects of hydrogen peroxide, thus demonstrating the relevance of the oxidative activation mechanism depicted in Figure 1. Such a minimal intracellular concentration has to be achieved to compete for endogenous ligands, such as citrate, which can bind iron.[9] After weakly bound-iron displacement by **1**, protection is most likely the result of strong iron chelation by oxidized ligand, as previously suggested.[15] Overall, although the effectiveness of structural modifications of the present series toward cytoprotection is only limited, our results demonstrate that this goal is achievable. Other structural modifications around the core structure of compound **1** to improve bioavailability and protection efficiency against oxidative injury will be the subject of further investigations.

Experimental Section

Chemistry. All starting materials were purchased from Fluka or Sigma Aldrich Co. unless otherwise stated. *N,N′*-Bis-(2-hydroxybenzyl)ethylenediamine-*N,N′*-diacetic acid (HBED) was purchased from Strem. Column chromatography utilized Merck silica gel (230—400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on Brucker 500, 400 or 200 MHz spectrometers. All elemental analyses are within 0.4% of the calculated values. Mass spectra were obtained by desorption chemical ionization (DCI) with a Finnigan Mat SSQ 710 mass spectrometer or with a Fisons Platform mass spectrometer equipped with an atmospheric pressure ion source in the electro spray ionization (ESI) mode. Compounds **1**, **2**, and **11** were prepared as described previously.[14,16]

***N,N′*-Bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic Acid Diethyl Ester (3).** A mixture of 10.0 mmol disodium *N,N′*-bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetate[14] and 60.0 mmol 1-bromoethane was heated in 50 mL DMF at 55 °C for 48 h. The mixture was evaporated off and the residue was solubilized in dichloromethane, washed with water, dried and evaporated off. The crude ester was recrystallized from ethanol to give a white powder (71% yield): mp 74 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.17 (t, 6H), 2.71 (s, 4H), 3.38 (s, 4H), 3.63 (s, 6H), 3.65 (s, 4H), 3.70 (s, 12H), 4.06 (q, 4H), 6.60 (s, 4H); MS (ESI$^+$) m/z = 593 [M + H]$^+$, 615 [M + Na]$^+$. Anal. ($C_{30}H_{44}N_2O_{10}$) C, H, N, O.

***N,N′*-Bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid diisopropyl ester (4)** was obtained according to the same procedure from 2-bromopropane as a white powder (60% yield): mp 112 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.16 (d, 12H), 2.70 (s, 4H), 3.35 (s, 4H), 3.63 (s, 6H), 3.65 (s, 4H), 3.70 (s, 12H),4.89 (m, 2H), 6.59 (s, 4H); MS (ESI$^+$) m/z = 621 [M + H]$^+$, 643 [M + Na]$^+$. Anal. ($C_{32}H_{48}N_2O_{10}$) C, H, N, O.

***N,N′*-Bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid dibutyl ester (5)** was obtained according to the same procedure from 1-bromobutane as a white powder (40% yield): mp 61 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.90 (t, 6H), 1.34 (m, 4H), 1.57 (m, 4H), 2.81 (s, 4H), 3.38 (s, 4H), 3.71 (s, 4H), 3.78 (s, 12H), 3.80 (s, 6H), 4.06 (t, 4H), 6.56 (s, 4H); MS (ESI$^+$) m/z = 649 [M + H]$^+$, 671 [M + Na]$^+$. Anal. ($C_{34}H_{52}N_2O_{10}$) C, H, N, O.

***N,N′*-Bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid diheptyl ester (6)** was obtained according to the same procedure from 1-bromoheptane as a white powder (58% yield): mp 57 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.86 (t, 6H), 1.28 (m, 16H), 1.58 (m, 4H), 2.82 (s, 4H), 3.39 (s, 4H), 3.71 (s, 4H), 3.78 (s, 12H), 3.80 (s, 6H), 4.05 (t, 4H), 6.56 (s, 4H); MS (ESI$^+$) m/z = 733 [M + H]$^+$, 755 [M + Na]$^+$. Anal. ($C_{40}H_{64}N_2O_{10}$) C, H, N, O.

***N,N′*-Bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid diacetoxymethyl ester** (7) was obtained from acetoxymethyl bromide following a similar procedure. The crude ester was purified by chromatography on silica gel (eluant dichloromethane) and crystallized from pentane as a white powder (35% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.08 (s, 6H), 2.83 (s, 4H), 3.46 (s, 4H), 3.71 (s, 4H), 3.79 (s, 12H), 3.81 (s, 6H), 5.73 (s, 4H), 6.56 (s, 4H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 20.63, 51.39, 53.78, 56.03, 58.58, 60.81, 78.98, 105.49, 134.38, 137.06, 153.23, 169.50, 170.14; MS (ESI$^+$) m/z = 681 [M + H]$^+$. Anal. ($C_{32}H_{44}N_2O_{14}$) C, H, N, O.

***N,N′*-Bis(3,4,5-trimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid *N,N*-diethylaminocarbonylmethyl ester (8)** was obtained from 2-chloro-*N,N*-diethylacetamide following the same procedure. The crude ester was solubilized in ethanol and precipitated with concentrated hydrochloric acid to give **8** as a white dihydrochloride (25%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.09 (s, 6H), 1.22 (s, 6H), 3.12 (q, 4H), 3.33 (q, 4H), 3.84 (s, 6H), 3.88 (s, 2H), 3.90 (s, 12H), 3.95 (m, 4H), 4.25 (m, 4H), 4.61 (s, 4H), 4.91 (s, 4H), 7.10 (s, 4H). Anal. ($C_{38}H_{58}N_4O_{12}$·2HCl·H_2O) C, H, N, O, Cl.

***N,N′*-Bis(4-butyloxy-3,5-dimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid (9)** was prepared from 4-butyloxy-3,5-dimethoxybenzaldehyde[18] following a three steps procedure described previously for **1**.[14] The crude product was recrystallized from ethanol/water (1:1) to yield the pure compound as a white powder (39% overall yield): mp 210 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.90 (t, 6H), 1.43 (m, 4H), 1.59 (m, 4H), 3.52 (s, 4H), 3.77 (s, 12H), 3.84 (m, 8H), 4.21 (s, 4H), 6.86 (s, 4H); DCI/MS (NH_3) m/z = 619 [M − H]$^-$. Anal. ($C_{32}H_{48}N_2O_{10}$·1.7HCl·0.2 HBr·$^1/_2H_2O$) C, H, N, O, Cl.

***N,N′*-Bis(4-heptyloxy-3,5-dimethoxybenzyl)ethylenediamine-*N,N′*-diacetic acid (10)** was prepared from 4-heptyloxy-3,5-dimethoxybenzaldehyde[18] according to the same procedure as a white powder (33% overall yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.89 (t, 6H), 1.30−1.44 (m, 16H), 1.64 (m, 4H), 3.30 (s, 4H), 3.69 (s, 4H), 3.78 (s, 12H), 3.88 (t, 4H), 4.06 (s, 4H), 6.77 (s, 4H); DCI/MS (NH_3) m/z = 703 [M − H]$^-$. Anal. ($C_{38}H_{60}N_2O_{10}$·1.5HCl·0.3HBr) C, H, N, O, Cl.

***N*-(4-Butyloxy-3,5-dimethoxybenzyl)ethylenediamine-*N,N′,N′*-triacetic acid (12)** was obtained from 4-butyloxy-3,5-dimethoxybenzaldehyde[18] according to the five steps procedure already reported to prepare **11**[14] and recrystallized from ethanol−water (1:1) to yield the pure compound as a white powder (21% overall yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.91 (t, 3H), 1.42 (m, 2H), 1.59 (m, 2H), 3.15 (m, 2H), 3.36 (m, 2H), 3.53 (s, 4H), 3.78 (s, 6H), 3.86 (t, 2H), 4.09 (s, 2H), 4.38 (s, 2H), 6.88 (s, 2H); MS (ESI$^-$) m/z = 455 [M − H]$^-$. Anal. ($C_{21}H_{32}N_2O_9$·2HCl·H_2O) C, H, N, O, Cl.

***N*-(4-Heptyloxy-3,5-dimethoxybenzyl)ethylenediamine-*N,N′,N′*-triacetic acid (13)** was obtained from 4-heptyloxy-3,5-dimethoxybenzaldehyde[18] according to the same procedure as a white powder (23% overall yield): ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.87 (t, 3H), 1.27 (m, 6H), 1.40 (m, 2H), 1.61 (m, 2H), 3.14 (m, 2H), 3.35 (m, 2H), 3.53 (s, 4H), 3.78 (s, 6H), 3.85 (t, 2H), 4.10 (s, 2H), 4.38 (s, 2H), 6.87 (s, 2H); MS (ESI$^-$) m/z = 497 [M − H]$^-$. Anal. ($C_{24}H_{38}N_2O_9$·2HCl) C, H, N, O, Cl.

Biological Evaluation. Analysis of in vitro SSB induced by the Fenton reaction: Supercoiled DNA from pBR322 (Boehringer) was incubated for 15 min at 25 °C in 10 mM Tris-HCl buffer (pH 7.5) with 10 μM chelators, 100 μM H_2O_2 and 10 μM $Fe^{(II)}(NH_4)_2SO_4$ (Mohr's salt). Reactions were performed on 0.25 μg of DNA in a final volume of 20 μL and stopped by addition of 10 μL of stop solution (4 M urea, 50% sucrose, 50 mM EDTA 0.1% bromophenol blue). Samples were analyzed by electrophoresis in 1% agarose gel in 40 mM Tris acetate/10 mM EDTA buffer. The gel was stained with ethidium bromide. Untreated plasmid DNA revealed a major band corresponding to the intact supercoiled form and a minor band corresponding to nicked circular form. Related intensity of each bands was quantified using an image analyzer and the Bioprofil software (Vilbert Lourmat, France). All experiments were performed in duplicate.

Cytotoxicity assays: Human fetal foreskin fibroblasts were grown in Dulbecco's modified essential medium (DMEM) supplemented by 10% fetal calf serum, penicillin (50 units/ml), streptomycin (50 μg/mL) and glutamine (1 mM). Cells were seeded in 24-well plates at a final density of 10^4 cells in 0.5 mL complete medium. 24 h later, the medium was removed and replaced by 1 mL fresh medium containing H_2O_2 and investigated chelators. Plates were then incubated at 37 °C for 72 h. All chelators were directly solubilized in PBS except compounds **2**−**8** for which concentrated stock solutions were prepared in DMSO, which was always kept in the final medium at a concentration below 0.1%. Cell viability was evaluated after 72 h of contact with H_2O_2 and chelator using the XTT assay (cell proliferation kit II, Boehringer Mannheim Biochemica) according to manufacturer's instructions. Data from these experiments were expressed as the percent ratio of living cells for treated cells versus untreated cells. Challenge with 50 μM H_2O_2 alone resulted in 80 ± 10% reduction of cell viability. IC_{50}'s represent the concentrations of the iron chelators producing a 50% reduction in the level of cell death. All experiments were preformed in quadruplicate.

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