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Iron complexes of deferiprone and dietary plant catechols as cytoprotective superoxide radical scavengers

Majid Y. Moridani, Peter J. O'Brien*

Faculty of Pharmacy, University of Toronto, Toronto, Ontario M5S 2S2, Canada

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Abstract

Superoxide radicals have been implicated in the pathogenesis of aging, cataract, ischemia–reperfusion, cancer and inflammatory diseases. In the present work, we found that deferiprone (L1), an iron-chelating drug, and dietary dihydroxycinnamic acids (catechols) were much more effective at protecting isolated rat hepatocytes against hypoxia–reoxygenation injury if complexed with Fe^{3+}. Furthermore, the 2:1 catechol–metal complexes with Cu^{2+}, Fe^{2+}, and Fe^{3+} were also more effective than uncomplexed catechols in scavenging superoxide radicals generated enzymically (xanthine oxidase/hypoxanthine). The 2:1 deferiprone:Fe^{3+} complex was less effective at scavenging enzymically generated superoxide radicals even though it was effective at preventing hepatocyte hypoxia–reoxygenation injury. On the other hand, the 1:1 deferoxamine:Fe^{3+} complex, another iron-chelating drug, did not prevent hepatocyte hypoxia–reoxygenation injury and did not scavenge enzymically generated superoxide radicals. Furthermore, hepatocytes readily reduced the 2:1 deferiprone:Fe^{3+} complex but not the deferoxamine:Fe^{3+} complex. These results suggest that the initial step in superoxide radical scavenging (SRS) activity is the formation of a redox complex between Fe^{3+} and deferiprone or catechols. The [deferiprone:Fe^{3+}] complex was more cytoprotective than would be expected from its SRS activity. This suggests that [deferiprone:Fe^{3+}] complex is reduced by a ferrireductase present on the hepatocyte membrane to form [deferiprone:Fe^{2+}] complex, which then scavenges superoxide radicals. Therefore, the clinically used deferiprone (L1) may have therapeutic advantages over deferoxamine in having a double role therapeutically: (a) it chelates iron to alleviate iron overload pathology, and (b) the readily formed iron complex protects hepatocytes from superoxide radical-mediated hypoxia–reoxygenation injury.

Keywords: Iron; Copper; Superoxide dismutase mimics; Ischemia–reperfusion injury; Catechols; Deferiprone; Deferoxamine; Antioxidants

1. Introduction

Cell membrane-permeable SOD mimics have been suggested as a therapy to alleviate the degenerating action of superoxide radicals in the pathogenesis of ischemia–reperfusion injury, aging, cataract, cancer, and other metabolic, degenerative, and inflammatory diseases [1]. Various low-molecular-weight SOD-like complexes of manganese, copper, and iron have also been reported to exhibit SOD mimic activities [2–9]. At a physiological pH, catechols readily form thermodynamically stable *bis* complexes with ferric iron as bidentate ligands. Catechols are also biosynthesised and utilised as iron sequestering agents by microorganisms [10,11]. These catechols have large stability constants for ferric iron and low reduction potentials [12,13]. Deferiprone (L1) is an iron-chelating agent used in the clinic for treating iron overload conditions such as thalassemia major [14–16].

Previously, it was shown in our laboratory that maintaining hepatocytes under a low oxygen concentration induced hepatocyte cell death [17,18]. This cytotoxic effect was attributed to ATP depletion and reductive stress, which resulted in intracellular iron release and a marked increase in oxygen activation. The cytotoxicity and oxygen activation were prevented by catecholic antioxidants (e.g. quercetin, caffeic acid) [17,18]. Later, [catechol:ferric] complexes were found to be much more effective than the uncomplexed catechols (e.g. quercetin, catechin, 4-*tert*-butylcatechol, caffeic acid, protocatechuic acid) at protecting hepatocytes as well as at SRS activity [19].

* Corresponding author. Tel.: +1-416-978-2716; fax: +1-416-978-8511.

E-mail address: peter.obrien@utoronto.ca (P.J. O'Brien).

Abbreviations: SOD, superoxide dismutase; SRS, superoxide radical scavenging; NBT, nitro blue tetrazolium; XO, xanthine oxidase; ROS, reactive oxygen species; UV-VIS, ultra-violet visible; Tris, Tris(hydroxymethyl)aminomethane; DETAPAC, diethylenetriaminepenta-acetic acid; NTA, nitrilotriacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; and BPS, bathophenanthrolinedisulfonic acid disodium salt.

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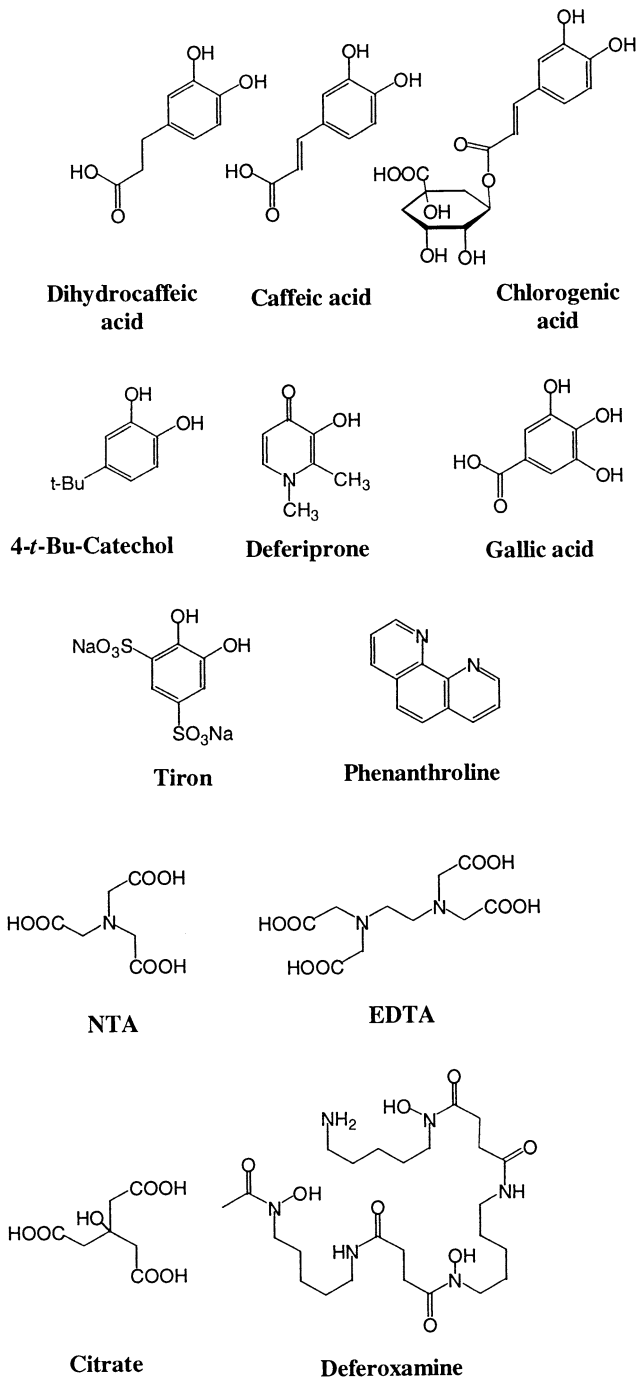


Fig. 1. Chemical structures of ligands.

In the present study, we compared deferiprone, deferoxamine, and dietary plant catechols (Fig. 1) with their Fe^{2+}, Fe^{3+}, and Cu^{2+} complexes for their SRS activities. We also investigated the Fe^{3+} complexes for cytoprotective activity against hypoxia–reoxygenation injury. It was found that catecholic Fe^{2+} and Fe^{3+} complexes had much greater SRS activities than their corresponding uncomplexed catechols. Furthermore, the Fe^{3+} complexes were markedly effective at preventing hypoxia–reoxygenation hepatocyte injury caused by ROS. The Fe^{3+} complex of deferiprone was also markedly effective at preventing hypoxia–reoxygenation injury and was reduced rapidly by the hepatocytes unlike the Fe^{3+} complex of deferoxamine. Deferiprone, in contrast to deferoxamine, may therefore have a double role therapeutically in chelating iron to alleviate iron overload pathology and in scavenging superoxide radicals.

2. Materials and methods

2.1. Chemicals

NBT, XO, hypoxanthine, caffeic acid, dihydrocaffeic acid, chlorogenic acid, gallic acid, 4-*tert*-butylcatechol, tiron, Tris, sodium phosphate monobasic, sodium phosphate dibasic, DETAPAC, EDTA, NTA, histidine, deferoxamine, citric acid, BPS, MOPS, HEPES, ferrous sulfate, ferric nitrate, cupric acetate, DMSO, and 1-octanol were obtained from Sigma/Aldrich Chemical Co., Canada. The stock solutions of the chemicals were prepared in DMSO, Millipore filtered water, or buffer. A 5-min pre-mixed stock solution of the [ligand:metal] complexes was used for addition to the reaction mixture or hepatocytes whenever [ligand: metal] complexes were tested.

2.2. UV-VIS spectroscopy of deferiprone and catecholic metal complexes

A solution of the compound (25 μM) was prepared in a cuvette containing phosphate buffer (10 mM, pH 5.5), and the absorption spectrum was recorded between 200 and 700 nm using a Shimadzu UV-VIS spectrophotometer (UV-240) [20]. Spectra were also recorded 5 min after the addition of 50 μM ferrous sulfate or ferric nitrate, and/or cupric acetate. Finally, spectra were recorded 5 min after the addition of 125 μM and 1.25 mM EDTA to the above solution.

2.3. SRS activity measurement

An aliquot of 10 μL hypoxanthine (3.5 mM) was added to a mixture of catecholic compound (or deferiprone or deferoxamine) (50 μM) or [ligand:metal] complex (10 μM:5 μM), XO (25 mU/mL), and NBT (50 μM) in 1 mL Tris/HCl buffer (0.1 M, pH 7.4). The absorption of the reaction mixture was monitored spectrophotometrically at 560 nm for 10 min using a Pharmacia Ultraspec model 1000. The results were expressed as percent inhibition.

2.4. XO inhibition assay

A modified method described by Cotelle *et al.* [21] was used to measure uric acid production. Briefly, hypoxanthine (35 μM) was added to a mixture of XO (25 mU/mL) and the ligands (10–50 μM) or Fe^{3+} (5 μM) or [ligand:Fe^{3+}] (10 μM:5 μM) in Tris/HCl buffer (pH 7.4, 0.1 M). The change

in absorbance was monitored at 293 nm at room temperature for 5 min using a Shimadzu UV-VIS spectrophotometer (UV-240).

2.5. Hepatocyte cytotoxicity induced by hypoxia–reoxygenation

Adult male Sprague–Dawley rats, 250–300 g, were obtained from Charles River Canada Laboratories, fed *ad lib.*, and allowed to acclimatise for 1 week on clay chip bedding. Hepatocytes were isolated from the rats by collagenase perfusion of the liver according to Moldéus and co-workers [22]. Isolated hepatocytes (10^6 cells/mL) (10 mL) were suspended in Krebs-Henseleit buffer (pH 7.4) containing HEPES (12.5 mM) in continually rotating round-bottomed 50-mL flasks, under an atmosphere of 95% O_2 and 5% CO_2 in a water bath of 37° for 30 min. Then ligands (20 μM) and their [ligand:Fe^{3+}] (20 μM:10 μM) complexes were introduced into the flasks, and the hepatocytes were incubated under an atmosphere of 95% N_2 and 5% CO_2 for 90 min. Before re-oxygenation in a 1% O_2, 94% N_2, and 5% CO_2 atmosphere, the first assessment (90 min) of cell viability was carried out by determining trypan blue (0.1%, w/v) exclusion from the hepatocytes [22].

2.6. Reduction of the deferiprone:ferric complex by hepatocytes

Ferrous or ferric (20 μM) complexes with BPS or deferiprone or deferoxamine (40 μM) were premixed before addition to the hepatocytes (1×10^6 cells/mL), which were then incubated at 37° for 15 min. The cells were pelleted by centrifugation (500 *g* for 1 min at 45°) before monitoring the absorbance of the supernatant at 535 nm using a Shimadzu UV-VIS spectrophotometer (UV-240). Phosphate buffer (0.1 M, pH 7.4) was used for the preparation of metal complexes as controls. A minimum of three independent samples was used.

2.7. Statistical analysis

Statistically significant differences between control and test compounds were determined using Student's *t*-test. The acceptable values were $P \leq 0.05$.

3. Results

Deferiprone showed a maximal UV spectra absorbance at 278 nm. There was a red shift of 5–20 nm upon the addition of Cu^{2+}, Fe^{2+}, and Fe^{3+} to deferiprone at pH 5.5; however, the original spectra were recovered upon the addition of EDTA, with a very small shift (1–3 nm) toward the hypsochromic region. Deferoxamine showed a maximal absorbance at 220 nm. When deferoxamine was complexed with Cu^{2+}, there was a red shift of 30 nm. However, upon the addition of EDTA, there was a further shift to 275 nm, characteristic of [EDTA:Cu^{2+}] complex formation. The complexation of deferoxamine with iron developed a peak at ~425 nm, which was not affected by EDTA.

Interactions of Cu^{2+}, Fe^{2+}, and Fe^{3+} at pH 5.5 with 4-*tert*-butylcatechol (with a UV absorbance at 278 nm), gallic acid (257 nm), and dihydrocaffeic acid (280 nm) at a 2:1 catecholic compound–metal ratio produced very small shifts (1–5 nm) in the absorbance spectra, but upon the addition of EDTA the original spectra were recovered without any significant peak shifts. Caffeic acid (280 and 305 nm) interaction with the cupric ion resulted in a new absorbance maxima at 375 nm which was not seen with the ferrous and ferric ions. The addition of EDTA to the [caffeic acid:Cu^{2+}] complex resulted in the restoration of the caffeic acid spectra (280 and 305 nm). The addition of Cu^{2+} to chlorogenic acid produced no shift in the 321 nm absorbance peak, but a new absorbance peak at 295 nm was formed upon the addition of EDTA. Interaction of Fe^{2+} with chlorogenic acid demonstrated a new absorbance peak at 295 nm in addition to the original 321 nm absorbance peak. A small shift (5 nm) to the hypsochromic region of the absorbance peaks was observed upon competition of EDTA with chlorogenic acid for Fe^{2+} and Fe^{3+}.

The SRS activities of the catecholic compounds deferoxamine and deferiprone (L1), and their Cu^{2+}, Fe^{2+}, or Fe^{3+} complexes were determined using an enzymically superoxide radical generating system of hypoxanthine/XO. As shown in Table 1, the SRS activities for the uncomplexed ligands (50 μM), found in decreasing order of effectiveness, were: 4-*tert*-butylcatechol, chlorogenic acid, caffeic acid > gallic acid > dihydrocaffeic acid [≫] tiron, deferiprone. Deferoxamine, citrate, NTA, and EDTA (50 μM) did not demonstrate any SRS activity. Except for chlorogenic acid and caffeic acid, none of these compounds inhibited XO activity. XO activity was assayed by following uric acid production at 293 nm under the same conditions used for the SRS activity assay but without NBT. Ferric ion (5 μM) also did not affect XO activity (results not shown).

The SRS activities for the 2:1 [ligand:Fe^{3+}] complexes in order of decreasing effectiveness were: gallic acid > 4-*tert*-butylcatechol > chlorogenic acid > caffeic acid > EDTA > dihydrocaffeic acid > NTA, tiron > deferiprone, but not deferoxamine or citrate (Table 1). The SRS activities for the 2:1 [ligand:Fe^{2+}] complexes in order of decreasing effectiveness were: gallic acid > chlorogenic acid > caffeic acid > 4-*tert*-butylcatechol > EDTA > dihydrocaffeic acid ≫ NTA, tiron > deferiprone, but not deferoxamine or citrate. Generally, there was an increase of several-fold in SRS activity of the tested ligand compound when complexed with either Fe^{2+} or Fe^{3+}, although this increase was not observed for the [deferoxamine:Fe^{2+}] and [deferoxamine:Fe^{3+}] complexes. For instance, the SRS activity of the 2:1 [deferiprone:Fe^{3+}] complex was increased 11-fold in comparison to deferiprone alone, whereas uncomplexed de-

Table 1
Deferiprone, deferoxamine, and catecholic compounds and their Fe^{2+}, Fe^{3+}, and Cu^{2+} complexes as superoxide radical scavengers

Chelator	λ_{max} (nm)	Superoxide radical scavenging activity (%)			
		Ligand (50 μM)	[Ligand:Fe^{2+}] (10 μM:5 μM)	[Ligand:Fe^{3+}] (10 μM:5 μM)	[Ligand:Cu^{2+}] (10 μM:5 μM)
Clinical chelators					
Deferoxamine	220	0	0	0	63 ± 3
Deferiprone	278	5 ± 2	10 ± 1	11 ± 2	83 ± 4
Catechols					
4-*tert*-Butylcatechol	278	84 ± 4	61 ± 4	68 ± 3	83 ± 4
Gallic acid	257	80 ± 3	80 ± 5	76 ± 4	72 ± 3
Dihydrocaffeic acid	280	69 ± 3	42 ± 3	43 ± 3	68 ± 3
Chlorogenic acid	321	84 ± 2	74 ± 4	63 ± 4	67 ± 2
Caffeic acid	280, 305	83 ± 4	65 ± 4	55 ± 2	80 ± 3
Citrate		0	0	0	66 ± 3
NTA		0	26 ± 3	19 ± 3	63 ± 2
Tiron		7 ± 2	17 ± 2	15 ± 2	67 ± 3
EDTA		0	52 ± 5	49 ± 6	60 ± 4
None			0	0	65 ± 2
Histidine					31 ± 4

The reaction mixture contained the ligand (50 μM), XO (25 mU/mL), and NBT (50 μM) in Tris/HCl buffer (0.1 M, pH 7.4) to which hypoxanthine (35 μM) was added at the end. The absorption of the reaction mixture was monitored spectrophotometrically at 560 nm at 10 min. A 5-min pre-mixed solution of the [ligand:metal] (100 μM:50 μM) complex was used as a stock solution for the experiment. The activity of the superoxide radicals is expressed as a percentage of the superoxide radicals scavenged with respect to the control. Data are averages of 3 independent determinations.

feroxamine had no SRS activity. There was no SRS activity observed with Fe^{2+} or Fe^{3+}. In principle, the SRS activities of the 2:1 [ligand:Fe^{3+}] complexes were almost the same as those of the 2:1 [ligand:Fe^{2+}] complexes.

The SRS activity of 10 μM:5 μM [ligand:Cu^{2+}] complexes was much higher than for Cu^{2+} alone or the uncomplexed ligands. However, the histidine:Cu^{2+} complex (the Cu^{2+} complex found in the plasma) was less effective than Cu^{2+}. Deferoxamine, chlorogenic acid, dihydrocaffeic acid, citrate, NTA, tiron, and EDTA were the least effective among the [ligand:Cu^{2+}] complexes tested in scavenging superoxide radicals. The SRS activities for Cu^{2+} complexes in order of decreasing activity were: 4-*tert*-butylcatechol, deferiprone > caffeic acid > gallic acid > dihydrocaffeic acid, chlorogenic acid, tiron, citrate > NTA, deferoxamine, and EDTA. Deferiprone was much more effective in scavenging superoxide radicals when complexed with Cu^{2+} than when uncomplexed.

As shown in Table 2, the Fe^{3+} complexes of the catecholic compounds (dihydrocaffeic acid, caffeic acid, chlorogenic acid, gallic acid, and 4-*tert*-butylcatechol), deferoxamine, and deferiprone were much more effective than the uncomplexed ligands at preventing ROS-mediated hypoxia–reoxygenation injury in isolated rat hepatocytes. The order of effectiveness at inhibiting the ROS-mediated cytotoxicity by the [ligand:Fe^{3+}] complexes was: deferiprone, gallic acid > dihydrocaffeic acid > chlorogenic acid, caffeic acid ≫ deferoxamine. The [deferoxamine:Fe^{3+}] complex did not prevent hypoxia–reoxygenation injury mediated by ROS.

Table 2
Prevention of hypoxia–reoxygenatin injury cytotoxicity in isolated rat hepatocytes by the catechol–, deferiprone, and deferoxamine–Fe^{3+} complexes

Treatment	Hepatocyte toxicity (% of trypan blue uptake)		
	90 min	3 hr	3 hr
Hypoxia–reoxygenation	45 ± 2	75 ± 3	93 ± 4
+ Fe^{3+} (10 μM)	53 ± 3	72 ± 4	96 ± 4
+ Deferoxamine (20 μM)	44 ± 3	72 ± 3	86 ± 2
+ [Deferoxamine:Fe^{3+}] (20 μM:10 μM)	41 ± 2	69 ± 2	88 ± 3*
+ Deferiprone (20 μM)	54 ± 3	64 ± 4	100
+ [Deferiprone:Fe^{3+}] (20 μM:10 μM)	27 ± 5	28 ± 2	33 ± 4
+ Gallic acid (20 μM)	47 ± 4	56 ± 3	69 ± 3
+ [Gallic acid:Fe^{3+}] (20 μM:10 μM)	36 ± 4	37 ± 5	38 ± 4
+ Dihydrocaffeic acid (20 μM)	52 ± 5	76 ± 4	97 ± 4
+ [Dihydrocaffeic acid:Fe^{3+}] (20 μM:10 μM)	34 ± 3	41 ± 4	42 ± 3*
+ Chlorogenic acid:(30 μM)	52 ± 3	63 ± 4	100
+ [Chlorogenic acid:Fe^{3+}] (20 μM:10 μM)	35 ± 2	36 ± 3	54 ± 5*
+ Caffeic acid (20 μM)	43 ± 4	68 ± 5	92 ± 9
+ [Caffeic acid: Fe^{3+}] (20 μM:10 μM)	30 ± 3	54 ± 5	57 ± 3*

Isolated rat hepatocytes (10^6 cells/mL) (10 mL) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating round-bottomed 50 mL flasks, under an atmosphere of 95% O_2 and 5% CO_2 for 30 min at 37°. Then the ligands and their iron complexes were introduced to the hepatocytes and incubated under an atmosphere of 95% N_2 and 5% CO_2 for 90 min. Before re-oxygenation with a 1% O_2, 94% N_2, and 5% CO_2 atmosphere, the first asessment (90 min) of the viability of the cells was carried out by determining trypan blue (0.1%, w/v) exclusion from hepatocytes. Results are expressed as means ± SD for 3 independent determinations.

* Significantly different with lower toxicity than form hypoxia–reoxygenated cells, $P < 0.05$.

Table 3
Reduction of the deferiprone:ferric complex by the hepatocyte ferrireductase

Treatment	An increase in optical density at 535 nm			
	Deferiprone		Deferoxamine	
	Hepatocyte	Buffer pH 7.4	Hepatocyte	Buffer pH 7.4
Control	0	0	0	0
+ [BPS]	0	0	0	0
+ [Ligand:Fe^{3+}]	0	0.065 ± 0.007	0.051 ± 0.012[a]	0.036 ± 0.008[a]
+ "+ [BPS][b]	0.079 ± 0.003*		0.033 ± 0.007	
+ " + [BPS]	0.131 ± 0.008*	0.007 ± 0.003	0.024 ± 0.006[a]	0.056 ± 0.009[a]
+ [BPS:Fe^{3+}]	0.205 ± 0.017*	0.029 ± 0.001	0.205 ± 0.017*	0.029 ± 0.001
+ [BPS:Fe^{2+}]	0.288 ± 0.023*,[a]	0.313 ± 0.024[a]	0.288 ± 0.023[a]	0.313 ± 0.024
+ [Ligand:Fe^{2+}]	0.014 ± 0.002	0.054 ± 0.004	0.033 ± 0.006[b]	0.062 ± 0.013[a]
+ " + [ascorbic acid]		0.032 ± 0.006		0.031 ± 0.014
+ " + [BPS][b]	0.085 ± 0.003*		0	
+ "+ [BPS]	0.146 ± 0.0012*,[a]	0.118 ± 0.019[a]	0.053 ± 0.013[a]	0.061 ± 0.011[a]
+ [BPS:ligand] + [Fe^{2+}]		0.293 ± 0.026		0.286 ± 0.022

Iron complexes were premixed before addition to the hepatocytes (1×10^6 cell/mL). All other treatments were added at the same time. The hepatocytes were incubated at 37° for 15 min. The cells were pelleted by centrifugation (500 *g* for 1 min at 45°) before monitoring the absorbance of the supernatant. Phosphate buffer (0.1 M, pH 7.4) was used to prepare the metal complexes as controls. Values are means ± SD; a minimum of three independent samples was used. [BPS] and [ligand] = 40 μM, [Fe^{3+}] and [Fe^{2+}] = 20 μM, and ascorbic acid = 10 mg/mL.

[a] No difference in absorbance.

[b] Added after centrifuging the sample and before monitoring the absorbance at 535 nm.

* Significantly different from the controls ($P \leq 0.05$).

As shown in Table 3, the isolated rat hepatocytes reduced 50% of the [deferiprone:Fe^{3+}] (40 μM:20 μM) complex to the [deferiprone:Fe^{2+}] complex. The amount of ferrous formed was measured using BPS, an iron(II) specific chelator. Hepatocytes were also able to reduce 70% of the [BPS:Fe^{3+}] (40 μM:20 μM) complex to the [BPS:Fe^{2+}] complex. However, the deferoximine:Fe^{3+} complex was not reduced to the deferoxamine:Fe^{2+} complex by hepatocytes.

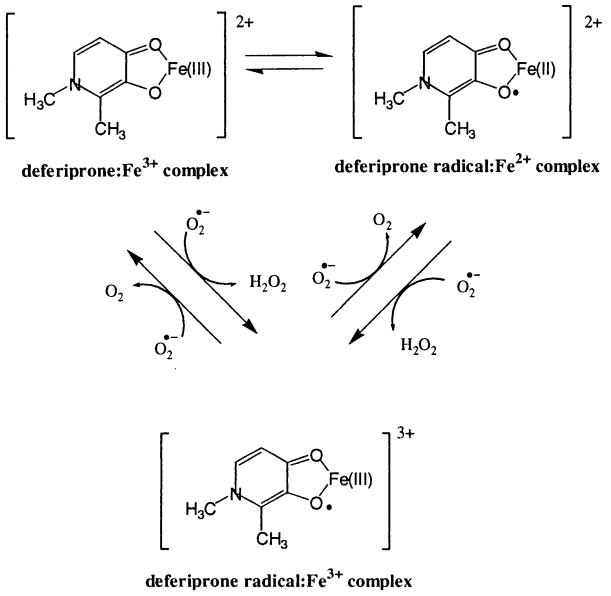
4. Discussion

Previously, we showed that the iron complexes of catechol antioxidants, such as quercetin, were much more effective than the uncomplexed ligands at preventing hypoxia–reoxygenation injury, as well as inhibiting superoxide formation [19]. SOD also protected the hepatocytes against hypoxic injury (particularly when added with catalase). This suggested that cytotoxicity could be attributed to extracellular ROS. The cytoprotectiveness of the tested iron complexes was similar, despite the differences in the partition coefficient of the corresponding catecholic compounds, and this in turn suggested that the iron complexes may protect the hepatocyte cell membrane extracellularly [19]. Afanas'ev *et al.* [23,24] also reported that a copper rutin chloride complex was much more effective than uncomplexed rutin as an antioxidant in preventing microsomal lipid peroxidation, which may be due to the presence of the copper superoxide dismutating centre.

In the present work, we also compared two clinically available iron chelators, deferoxamine and deferiprone, in order to investigate their SRS properties and their abilities to prevent hepatocyte injury from hypoxia in comparison to catechols. Spectral evidence suggests that deferiprone, deferoxamine, 4-*tert*-butylcatechol, caffeic acid, dihydrocaffeic acid, and chlorogenic acid form complexes with Fe^{2+}, Fe^{3+}, or Cu^{2+} that can be reversed with EDTA. However, EDTA did not reverse the Cu^{2+} complexes of gallic acid, which suggests that Cu^{2+}, but not Fe^{2+} or Fe^{3+}, partially oxidises gallic acid.

It was also discovered that the iron complexes of the catechols when incubated with the isolated rat hepatocytes were much more effective than the uncomplexed catechols at preventing hypoxia–reoxygenation injury. However, the 2:1 complex of deferiprone with ferrous and ferric metals was less effective at scavenging enzymically generated superoxide radicals, even though the deferiprone–iron complexes were effective at preventing hepatocyte hypoxia–reoxygenation injury. On the other hand, deferoxamine and its ferric complex demonstrated zero SRS activity and did not prevent hypoxia–reoxygenation injury in isolated rat hepatocytes. This was probably due to the strong sequestering property of the hexadentate deferoxamine towards the ferric ion with an affinity constant of 31 [25,26].

At a physiological pH, most of the catecholic compounds form 2:1 complexes with Fe^{3+}, whereas catechols with lower pK_a values bind Fe^{3+} in a 3:1 fashion [10,27,28]. Deferiprone also forms a 3:1 complex with Fe^{3+} [26]. Previously, it was suggested by Graf *et al.* [29] that the availability of an iron coordinate site that is open or occupied by a readily displaceable ligand, such as water, facilitates the reaction of iron chelates with superoxide radicals.



Scheme 1. Mechanisms of the SOD by the deferiprone–iron complexes. The 1:1 [deferiprone:iron] complex was used for simplicity.

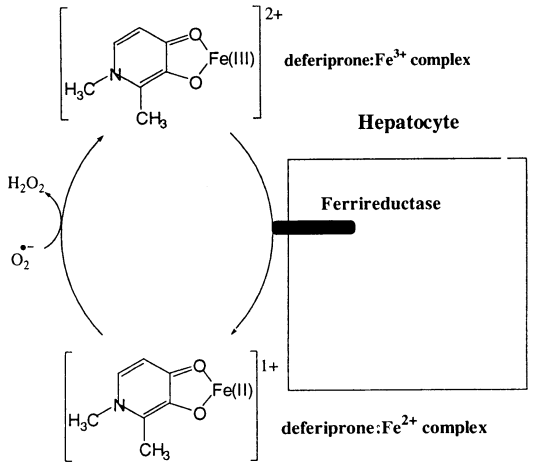


Fig. 2. Proposed mechanism for the SRS of the deferiprone: Fe^{3+} complex *in vitro*. Deferiprone: Fe^{3+} complex is reduced by a ferrireductase activity on the hepatocyte membrane surface. Once in its reduced form, deferiprone: Fe^{2+} complex can scavenge superoxide radicals extracellularly and protect the hepatocytes against hypoxia–reoxygenation injury.

The 2:1 [bidentate:Fe^{3+}] complexes have at least two coordinate sites that are available to a superoxide radical (Scheme 1), whereas in the 3:1 [catechol:Fe^{3+}] and 1:1 [deferoxamine:Fe^{3+}] complexes all the coordinate sites are occupied by the ligands, which, in principle, hinder a free electron transfer between the complex and superoxide radical.

It is predicted that deferiprone and its iron complexes have higher redox potential values than catechol analogues and, therefore, are expected to be slightly less effective than catechols in scavenging superoxide radicals, which was in accordance with our findings. The order of SRS activity in descending order of effectiveness was: [catechols:Fe^{3+}/Fe^{2+}] > [catechols] > [deferiprone:Fe^{3+}/Fe^{2+}] > [deferiprone] ≥ [deferoxamine] > [deferoxamine:Fe^{3+}/Fe^{2+}] complexes. It is hypothesised that as a result of the reaction with a superoxide radical, the [catechol:Fe^{3+}] complexes are oxidised to a [semiquinone:Fe^{3+}] complex, which either disproportionates to a quinone and free Fe^{3+} or redox cycles back to a [catechol:Fe^{3+}] complex by reacting with a second superoxide radical. [Catechol:Fe^{2+}] complexes are oxidised to [catechol:Fe^{3+}] complexes readily, whereas uncomplexed catechols are oxidised to quinones. As a result of reacting with a superoxide radical, the [deferiprone:Fe^{3+}] complex is oxidised to a [deferiprone radical:Fe^{3+}] complex, which is then reduced back to a [deferiprone:Fe^{3+}] complex by reacting with a second superoxide radical (Scheme 1).

In addition, we were able to show that the isolated rat hepatocytes reduced the [deferiprone:Fe^{3+}] complex to the [deferiprone:Fe^{2+}] complex by using BPS, a specific ferrous ion chelator. However, the hepatocytes were not able to facilitate reduction of Fe^{3+} bound to deferoxamine. Han *et al.* [30] previously demonstrated the presence of such a ferrireductase in Caco-2 cells and have proposed that dietary nonheme ferric ions must be converted to the ferrous state either by dietary or endogenous reductants (e.g. ascorbic acid) in the lumen or by a ferrireductase activity on the brush membrane surface. Trinder and Morgan [31] also suggested a similar mechanism for ferric citrate uptake by human hepatoma HuH7 cells.

As illustrated in Fig. 2, the deferiprone:Fe^{3+} complex but not the deferoxamine:Fe^{3+} complex is reduced by a ferrireductase present on the surface of the hepatocyte membrane. Like the catechol:Fe^{2+} complexes, the reduced deferiprone:Fe^{2+} product readily scavenges the superoxide radicals (Scheme 1).

In summary, our findings suggest that the iron complexes of catecholic compounds and deferiprone, a hydroxypyridinone, scavenged superoxide radicals generated extracellularly. In the absence of iron, a lower degree of cytoprotection against hypoxic injury was achieved when the ligand was incubated alone. The Fe^{3+} complexes of catecholic agents and deferiprone (L1) were also much more effective than the uncomplexed chelating agents in protecting isolated hepatocytes against hypoxia–reoxygenation injury. The 2:1 complexes of deferiprone with $Fe^{2+/3+}$ were less effective than expected at scavenging enzymically generated superoxide radicals. Surprisingly, when the Fe^{3+} complexes of the clinically used iron-chelating agents were employed, the 2:1 [deferiprone:Fe^{3+}] complex was found to be much more cytoprotective than deferiprone, while the 2:1 [deferoxamine:Fe^{3+}] complex was less cytoprotective than deferoxamine in preventing hypoxia–reoxygenation-induced injury. These results suggest that the initial step in SRS was the formation of a redox complex between Fe^{3+} and the catechols or deferiprone, which, in part, could scavenge superoxide radicals directly. Alternatively, the subse-

quent reduction of the deferiprone:Fe^{3+} complex by a ferrireductase present on hepatocyte cell membrane [30,31] could result in much more efficient SRS, although this reduction was not observed for the deferoxamine:Fe^{3+} complex. Therefore, deferiprone should have a therapeutic advantage over deferoxamine when it chelates iron to alleviate iron overload pathology, as the deferiprone–iron complex formed can scavenge superoxide radicals. These results further suggest that deferiprone should be tested *in vivo* as a novel therapy for ischemia–reperfusion injury.

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