Partition-Variant Desferrithiocin Analogues: Organ Targeting and Increased Iron Clearance[1]

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Altering the lipophilicity (log P_{app}) of desferrithiocin analogues can change the organ distribution of the chelators and lead to enhanced iron clearance. For example, alkylation of (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-4-methyl-4-thiazolecarboxylic acid [(*S*)-4′-(HO)-DADFT] and its analogues to more lipophilic compounds, such as (*S*)-4,5-dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-methyl-4-thiazolecarboxylic acid [(*S*)-4′-(CH_3O)-DADFT], provides ligands that achieved between a 3- and 8-fold increase in chelator concentrations in the heart, liver, and pancreas (the organs most at risk in iron-overload disease) of treated rodents. The 4′-*O*-methylated compounds are demethylated to their hydroxylated counterparts in rodents; furthermore, this *O*-demethylation takes place in both rodent and human liver microsomes. The relationship between chelator lipophilicity and iron-clearing efficacy in the iron-overloaded *Cebus apella* primate is further underscored by a comparison of the iron-clearing efficiency of (*S*)-2-(2,3-dihydroxyphenyl)-4,5-dihydro-4-methyl-4-thiazolecarboxylic acid [(*S*)-3′-(HO)-DADFT] and its 3′-(CH_3O) counterpart. Finally, these DFT analogues are shown to be both inhibitors of the iron-mediated oxidation of ascorbate as well as effective radical scavengers.

Introduction

Essentially all prokaryotes and eukaryotes have a strict requirement for iron. Although this element comprises 5% of the earth's crust, living systems have great difficulty in accessing and managing this vital micronutrient. The low solubility of Fe(III) hydroxide ($K_{sp} = 1 \times 10^{-39}$),[2] the predominant form of the metal in the biosphere, has led to the development of sophisticated iron storage and transport systems in nature. Microorganisms utilize low molecular weight, virtually ferric-iron-specific, ligands, siderophores;[3] higher eukaryotes tend to employ proteins to transport and store iron (e.g., transferrin and ferritin, respectively).[4−6]

Humans have evolved a highly efficient iron management system in which we absorb and excrete only about 1 mg of the metal daily; there is no mechanism for the excretion of excess iron.[7] Whether derived from transfused red blood cells[8−10] or from increased absorption of dietary iron,[11,12] without effective treatment, body iron progressively increases with deposition in the liver, heart, pancreas, and elsewhere. Iron accumulation eventually produces (i) liver disease that may progress to cirrhosis,[13−15] (ii) diabetes related both to iron-induced decreases in pancreatic β-cell secretion and increases in hepatic insulin resistance,[16,17] and (iii) heart disease, still the leading cause of death in thalassemia major and related forms of transfusional iron overload.[7,18,19] The precise pathways of iron deposition and its regulation are incompletely understood, but the available evidence suggests that these differ in the liver, physiologically a major systemic iron depot, and in the pancreas and heart, which normally do not serve as iron storage sites. *Nontransferrin-bound plasma iron*, a heterogeneous pool of iron in the circulation that is not bound to the physiological iron transporter, transferrin, seems to be a principal source of the abnormal tissue distribution of iron that develops with chronic iron overload. Nontransferrin-bound plasma iron is rapidly taken up by hepatocytes in the liver, the major iron storage organ, perhaps through the divalent metal transporter 1[20] and other pathways that remain poorly characterized. Nontransferrin-bound plasma iron also seems to gain entry into cardiomyocytes, pancreatic β-cells, and anterior pituitary cells specifically via L-type voltage-dependent Ca^{2+} channels not found in hepatocytes or Kupffer cells.[21] These differences in modes of iron uptake also may underlie clinical observations of differences in the course and pace of iron deposition and damage to the liver, pancreas, and heart in patients with all forms of iron overload.[7]

The toxicity associated with excess iron, whether a systemic or a focal problem, derives from its interaction with reactive oxygen species, for instance, endogenous hydrogen peroxide (H_2O_2).[22−25] In the presence of Fe(II), H_2O_2 is reduced to the hydroxyl radical (HO•), a very reactive species, and HO^-, a process known as the Fenton reaction. The Fe(III) liberated can be reduced back to Fe(II) via a variety of biological reductants (e.g., ascorbate), a problematic cycle. The hydroxyl radical reacts very quickly with a variety of cellular constituents and can initiate free radicals and radical-mediated chain processes that damage DNA and membranes, as well as produce carcinogens.[23,26,27] Radical traps can help to attenuate the already ongoing free-radical-

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mediated damage; for example, *N,N′*-bis(2-hydroxybenzyl)ethylenediamine-*N,N′*-diacetic acid (HBED)[28] and desferrioxamine B (DFO)[29,30] are excellent radical traps.

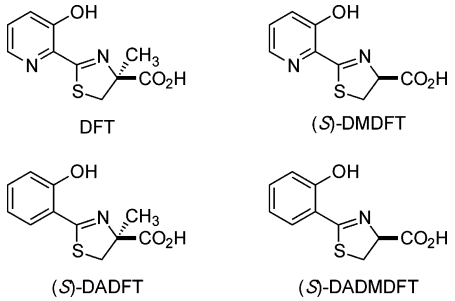
A major concern in the design of iron chelators for therapeutic use is related to their potential for facilitating this reduction of Fe(III) to Fe(II), thus promoting toxic Fenton chemistry.[22,31] Some agents inhibit the Fenton reaction (e.g., DFO[24]); paradoxically, certain ligands, such as ethylenediaminetetraacetic acid (EDTA),[31] nitrilotriacetic acid (NTA),[22] 5-aminosalicylic acid (5-ASA),[32,33] and 1,2-dimethyl-3-hydroxypyridin-4-one (deferiprone, L1),[31,34] actually enhance the reduction of Fe(III) to Fe(II). Although the precise nature of iron-mediated damage is complex[24] and remains somewhat elusive, the solution is clear: identify chelators that will sequester this transition metal and lead to its excretion without promoting Fenton chemistry.

In the majority of patients with thalassemia major or other transfusion-dependent refractory anemias, the severity of the anemia precludes phlebotomy therapy as a means of removing toxic accumulations of iron. Treatment with a chelating agent capable of sequestering iron and permitting its excretion from the body is then the only therapeutic approach available. The iron-chelating agents now in use or under clinical evaluation[35] include Desferal (DFO, mesylate salt), deferiprone, ICL670A (4-[3,5-bis(2-hydroxyphenyl)-1,2,4-triazol-1-yl]benzoic acid), and our desferrithiocin (DFT) analogue, (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-4-methyl-4-thiazolecarboxylic acid [(*S*)-4′-(HO)-DADFT].

Each of these agents chelate iron derived predominantly from systemic storage sites, either from iron released by macrophages after catabolism of senescent red blood cells, from iron in hepatic pools, or from both.[35] Small but detectable amounts of intracellular desferrioxamine and ferrioxamine are seen within a single hour of incubation with these compounds in vitro.[36] In patients, treatment with DFO can sometimes improve cardiomyopathy within days, before the total iron burden could have changed appreciably.[37] Nonetheless, the amounts removed from the heart are small; daily 24-h infusions of DFO are then required for periods of years to reverse iron-induced heart disease completely.[37] The efficacy of deferiprone in patients with heart disease remains uncertain, and ICL670A likely does not enter the heart in appreciable amounts. In any event, almost daily administration of near maximal tolerated doses of the first three agents is required to keep pace with rates of transfusional iron loading in patients with thalassemia major and other refractory anemias[38] and thereby minimize the amounts of circulating nontransferrin-bound iron. With increasing body iron, deposits develop in the liver, heart, pancreas, and other endocrine organs. Prolonged, intensive chelation therapy is then needed to arrest progression of the complications of iron overload or, in the case of established heart disease, to reverse cardiac dysfunction.

Consequently, there is a pressing need for the continued development of new iron-chelating agents that are more effective that can selectively remove iron from organs and tissues especially vulnerable to iron-induced toxicity, or both. Ligands that are more effective would be able to more rapidly reduce dangerous body iron burdens to safer levels and forestall the development or progression of complications. Iron-chelating agents that could selectively enter cardiac, pancreatic, or hepatic cells could help immediately reduce toxic iron pools and provide prompt protection against the progression of injury. Especially with respect to heart disease, still the cause of death in 70% or more of patients with thalassemia major,[8,18,19] the development of such agents could be life-saving.

Chart 1. Structures of Desferrithiocin (DFT, Top Left) and Historical Analogues (*S*)-Desmethyldesferrithiocin [(*S*)-DMDFT, Top Right], (*S*)-Desazadesferrithiocin [(*S*)-DADFT, Bottom Left], and Desazadesmethyldesferrithiocin [(*S*)-DADMDFT, Bottom Right]

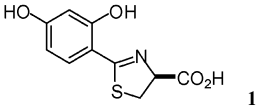
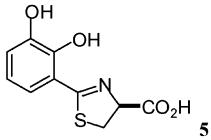
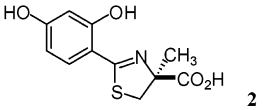
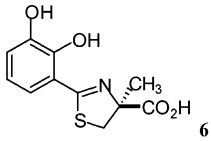
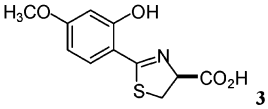
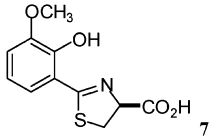
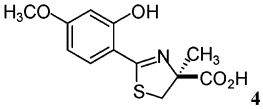
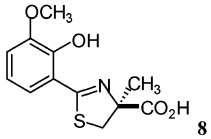


Iron-clearing efficiency (ICE) is used as a measure of the amount of iron excretion induced by a chelator. The ICE, expressed as a percent, is calculated as (ligand-induced iron excretion/theoretical iron excretion) × 100. To illustrate, the theoretical iron excretion after administration of 1 mmol of DFO, a hexadentate chelator which forms a 1:1 complex with Fe(III), is 1 mg-atom of iron. In reality, DFO in clinical use is only about 5−7% efficient.[39]

Desferrithiocin. (*S*)-4,5-Dihydro-2-(3-hydroxy-2-pyridinyl)-4-methyl-4-thiazolecarboxylic acid (DFT, Chart 1) is a tridentate siderophore[40] that forms a stable 2:1 complex with Fe(III); the cumulative formation constant is 4×10^{29} M^{-1}.[41,42] The donor groups include a phenolic oxygen, a thiazoline nitrogen, and a carboxyl. Desferrithiocin was one of the first iron chelators that was shown to be orally active.[43] It performed well in both the bile duct-cannulated rodent model (ICE, 5.5%)[44] and in the iron-overloaded *Cebus apella* primate (ICE, 16%).[45,46] Unfortunately, DFT was severely nephrotoxic.[46] Nevertheless, the outstanding oral activity spurred a structure−activity study to identify an orally active and safe DFT analogue. Our initial goal was to define the minimal structural platform compatible with iron clearance upon po administration.

Our approach entailed simplifying the platform. The thiazoline methyl of DFT was deleted to produce (*S*)-4,5-dihydro-2-(3-hydroxy-2-pyridinyl)-4-thiazolecarboxylic acid [(*S*)-DMDFT, Chart 1], reducing the ICE (4.8%) by two-thirds in the primate model.[46] Removal of DFT's aromatic nitrogen left (*S*)-4,5-dihydro-2-(2-hydroxyphenyl)-4-methyl-4-thiazolecarboxylic acid [(*S*)-DADFT, Chart 1], modestly diminishing the compound's ICE to 13% in *C. apella*.[47] Abstraction of the thiazoline methyl from (*S*)-DADFT, leaving (*S*)-4,5-dihydro-2-(2-hydroxyphenyl)-4-thiazolecarboxylic acid [(*S*)-DADMDFT, Chart 1], had little effect on efficacy, 12% vs 13% ICE.[46,48] These observations suggested that more apparently lipophilic chelators are more active, for example, DFT, (*S*)-DADFT, or (*S*)-DADMDFT vs (*S*)-DMDFT.

Table 1. Desferrithiocin Analogues' Iron-Clearing Activity When Administered Orally to *C. apella* Primates and the Partition Coefficients of the Compounds

4'-Substituted Compounds			3'-Substituted Compounds		
Desferrithiocin Analogue (compd. no.)	Iron Clearing Efficiency (%)[a]	log P_{app}[b]	Desferrithiocin Analogue (compd. no.)	Iron Clearing Efficiency (%)[a]	log P_{app}[b]
 **1**	4.2 ± 1.4[c] [70/30]	–1.33	 **5**	5.8 ± 3.4 [91/9]	–1.67
 **2**	13.4 ± 5.8[d] [86/14]	–1.05	 **6**	23.1 ± 5.9 [83/17]	–1.17
 **3**	16.2 ± 3.2[e] [81/19]	–0.89	 **7**	15.5 ± 7.3 [87/13]	–1.52
 **4**	24.4 ± 10.8[e] [91/9]	–0.70	 **8**	22.5 ± 7.1 [91/9]	–1.12

[a] In the monkeys [$n = 4$ (**1**, **2**, **3**, **6**), 7 (**4**), 5 (**8**), 6 (**7**), or 8 (**5**)], the dose was 150 μmol/kg. The efficiency of each compound was calculated by averaging the iron output for 4 days before the administration of the drug, subtracting these numbers from the 2-day iron clearance after the administration of the drug, and then dividing by the theoretical output; the result is expressed as a percent. The relative percentages of the iron excreted in the stool and urine are in brackets. [b] Data are expressed as the log of the fraction in the octanol layer (log P_{app}); measurements were done in TRIS buffer, pH 7.4, using a "shake flask" direct method. The values obtained for compounds **1**–**4** are from ref 53. [c] Data are from ref 48. [d] Data are from ref 47. [e] Data are from ref 53.

Few further structural changes could be made to the (*S*)-DADMDFT framework without an adverse effect on ICE. Alterations of the distances between the donor centers results in loss of activity.[48] Thiazoline ring modifications, that is, expansion (dihydrothiazine), oxidation (thiazole), or reduction (thiazolidine), abrogated iron-clearing activity.[48,49] Likewise, replacement of the sulfur with oxygen (oxazolines), with nitrogen (dihydroimidazole), or with a methylene (dihydropyrrole) resulted in significant loss of efficacy.[48,49] Changes in configuration at C-4 also had a profound effect on ICE in the *C. apella* model,[47,48,50,51] demonstrating a potential stereoselective barrier in iron clearance; an (*S*)-configuration at the C-4 carbon is optimal.[51,52] Benzofusions, designed to improve the ligands' tissue residence time and possibly ICE, were ineffective; both the naphthyl analogues and the quinoline systems performed poorly.[48,50]

Finally, the (*S*)-DADMDFT framework was subjected to a structure–activity study aimed at ameliorating its toxicity. Both DADFT analogues, (*S*)-DADFT and (*S*)-DADMDFT, were quite toxic: severe GI toxicity was prominent, rather than nephrotoxicity, as with DFT.[46–48] This structure–activity analysis was based on the idea that by altering the lipophilicity (i.e., partition properties, log P_{app}) and/or the redox potential of the aromatic ring, the drug's toxicity profile, organ distribution properties, and potential metabolic disposition could change.

The redox potential of the analogues was modified by addition of several different substituents to the aromatic ring;[48] log P_{app} was also changed by addition of these same substituents and/or the presence or absence of the thiazoline methyl. It was determined that hydroxylation, as in the systems (*S*)-2-(2,4-dihydroxyphenyl)-4,5-dihydro-4-thiazolecarboxylic acid [(*S*)-4′-(HO)-DADMDFT, **1**] or (*S*)-4′-(HO)-DADFT (**2**) (Table 1), was compatible with iron clearance in the primate model and also profoundly reduced toxicity in rodents.[47,48,53] For example, rats that were treated with (*S*)-DADFT or (*S*)-DADMDFT were dead by day 5 of a planned 10-day dosing regimen;[46] those administered **1** and **2** at the same dose did not display any frank toxicity.[47] In fact, (*S*)-4′-(HO)-DADFT (**2**) is now our lead compound in clinical trials. These initial results suggested an inverse correlation between toxicity and lipophilicity. In the *C. apella* model, the hydroxylated compounds were generally less active than their corresponding parent drugs, e.g., a 5.3% ICE for (*S*)-4′-(HO)-DADMDFT[47] [vs 12.4% for (*S*)-DADMDFT when administered po at a dose of 300 μmol/kg]. This result further underscores the importance of lipophilicity in the chelator's iron-clearing efficiency.

The challenge was to increase lipophilicity and ICE without increasing toxicity. In the current study, consistent with our previous results,[53] we demonstrate that this goal can be achieved by methoxylation of (*S*)-DADMDFT or (*S*)-DADFT. Either 3′- or 4′-methoxylation increases lipophilicity, altering the octanol–water partition coefficients (log P_{app}), and improving ICE in the iron-overloaded *C. apella* primate. Further, we show that the more lipophilic compounds, as exemplified by (*S*)-4′-(CH_3O)-DADFT (**4**), have better access to the organs most affected by iron overload, that is, the liver, heart, and pancreas. Our studies also measured the ability of representative systems to prevent ascorbate-

mediated reduction of Fe(III) to Fe(II) and to act as radical scavengers. Finally, we verified that the methoxy group, while increasing the ligand's lipophilicity and ICE, did not appreciably alter the compound's toxicity profile.

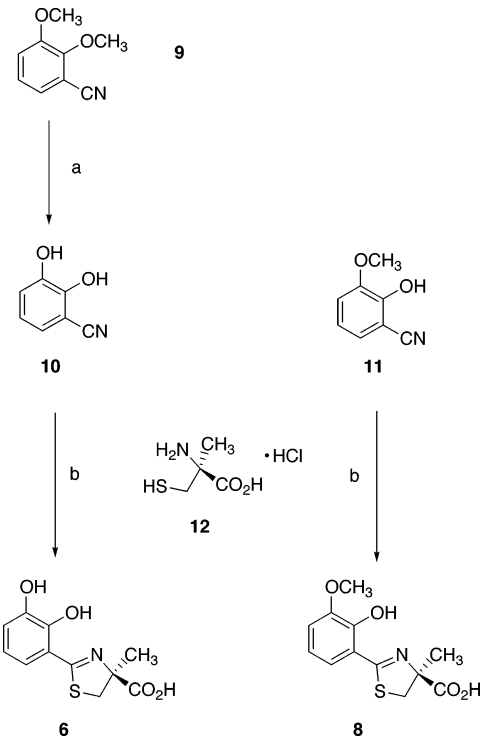
Results and Discussion

Design Concept. Initial considerations[48] suggested that the correlation between chelator lipophilicity, that is, octanol−water partition coefficients (log P_{app}), and ICEs exists principally within "families" of ligands. For example, although the octanol−water partition coefficients of DFT and (*S*)-DMDFT are very similar (−1.77 and −1.87, respectively), their ICEs in primates were quite different, 16.1 ± 8.5% and 4.8 ± 2.7%, respectively. However, considerations within the same structural group afforded very different results. For example, a comparison of the partition properties of DADMDFT and (*S*)-4′-(HO)-DADMDFT (**1**) (−0.93 and −1.2, respectively) and their corresponding ICEs (12.4 ± 7.6% vs 5.3 ± 1.7%) does imply a relationship between lipophilicity and iron-clearing efficiency. Accordingly, we have assembled a group of structurally similar desferrrithiocins that have graded variations in their lipophilic properties. On the basis of the substitutions in the aromatic ring, two different core systems were considered, 2,4-dihydroxyphenyl (**1**−**4**) from earlier studies[53] and 2,3-dihydroxyphenyl (**5**−**8**) compounds in the present work (Table 1). The 4′-substituted systems included (*S*)-4′-(HO)-DADMDFT (**1**), (*S*)-4′-(HO)-DADFT (**2**), (*S*)-4,5-dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-thiazolecarboxylic acid [(*S*)-4′-(CH_3O)-DADMDFT, **3**], and (*S*)-4,5-dihydro-2-(2-hydroxy-4-methoxyphenyl)-4-methyl-4-thiazolecarboxylic acid [(*S*)-4′-(CH_3O)-DADFT, **4**]. The 3′-substituted systems encompassed (*S*)-2-(2,3-dihydroxyphenyl)-4,5-dihydro-4-thiazolecarboxylic acid [(*S*)-3′-(HO)-DADMDFT, **5**], (*S*)-2-(2,3-dihydroxyphenyl)-4,5-dihydro-4-methyl-4-thiazolecarboxylic acid [(*S*)-3′-(HO)-DADFT, **6**], (*S*)-4,5-dihydro-2-(2-hydroxy-3-methoxyphenyl)-4-thiazolecarboxylic acid [(*S*)-3′-(CH_3O)-DADMDFT, **7**], and (*S*)-4,5-dihydro-2-(2-hydroxy-3-methoxyphenyl)-4-methyl-4-thiazolecarboxylic acid [(*S*)-3′-(CH_3O)-DADFT, **8**].

Synthetic Methods. (*S*)-4′-(HO)-DADMDFT (**1**),[48] (*S*)-4′-(CH_3O)-DADMDFT (**3**),[53] and (*S*)-3′-(CH_3O)-DADMDFT (**7**)[48] were synthesized in these laboratories by cyclization of D-cysteine with the requisite nitrile. The reaction of the unusual amino acid D-α-methylcysteine[40] under these conditions provided (*S*)-4′-(HO)-DADFT (**2**)[47] and (*S*)-4′-(CH_3O)-DADFT (**4**).[53]

(*S*)-2-(2,3-Dihydroxyphenyl)-4,5-dihydro-4-thiazolecarboxylic acid (**5**), the desmethyl analogue of (*S*)-3′-(HO)-DADFT (**6**), had been prepared by heating D-cysteine with 2,3-dihydroxybenzimidate[54] at a pH of 6.[48] However, when D-α-methylcysteine was treated with this imidate, none of the chelator **6** resulted. Thus, 2,3-dihydroxybenzonitrile (**10**) was generated by unmasking the chemically sensitive catechol group of 2,3-dimethoxybenzonitrile (**9**) with boron tribromide in CH_2Cl_2[55,56] in 84% yield (Scheme 1). Cyclocondensation of D-α-methylcysteine (**12**) onto the cyano group of **10** in heated phosphate-buffered methanol at pH 6 afforded (*S*)-3′-(HO)-DADFT (**6**) in 88% yield. Analogously, condensation of 3-methoxy-2-hydroxybenzonitrile (**11**)[57,58] with amino acid **12** gave (*S*)-3′-(CH_3O)-DADFT (**8**) in 39% yield.

Scheme 1. Synthesis of Desferrithiocin Analogues **6** and **8**[a]



[a] Reagents: (a) BBr_3, CH_2Cl_2, 84%; (b) $NaHCO_3$, pH = 6.0 phosphate buffer, CH_3OH, 72−75 °C, 18 h, 88% (**6**); 39% (**8**).

Iron Clearance. The animal model selected for this series of experiments was the iron-overloaded *C. apella* monkey. In this model, we are able to measure both total iron excretion, expressed as ICE, and the modes of iron clearance, that is, biliary (stool) and renal. The results for compounds **1**−**4** are historical[47,48,53] and are included in Table 1 for comparison with the present findings. Data are presented as the mean ± standard error of the mean. For comparison of the means between two compounds, a two-sample *t*-test (without the assumption of equality of variances) was performed. All tests were two-tailed, and a significance level of $P < 0.05$ was used.

We had observed that increasing the lipophilicity of 4′-hydroxylated DADFT analogues increased their ICE;[53] the situation was similar with the 2′,3′-dihydroxy derivatives. When a methyl group was introduced into the 4-position of the thiazoline ring of (*S*)-3′-(HO)-DADMDFT (**5**) to afford (*S*)-3′-(HO)-DADFT (**6**), the iron-clearing efficiency changed. The efficiency of compound **5** was 5.8 ± 3.4%; the stool:urine ratio was 91:9. Upon methylation of the thiazoline ring to produce (**6**), the efficiency increased to 23.1 ± 5.9% ($P < 0.01$ vs **5**). Eighty-three percent of the iron was in the stool, and 17% was in the urine. Although an increase in iron-clearing efficiency was also observed upon addition of a 4-methyl to the 2′-hydroxy-3′-methoxy system (analogues **7** and **8**, Table 1), the difference between these two ligands was not statistically significant, 15.5 ± 7.3% for derivative **7** vs 22.5 ± 7.1% for 4-methyl compound **8** ($P > 0.05$). 4-Methylation had no significant effect on the proportion of the iron in the stool, 91% for **8** versus 87% for **7**. Although methylation of the 3′-hydroxyl also increased the iron-clearing efficiency (**7** vs **5**, $P = 0.02$),

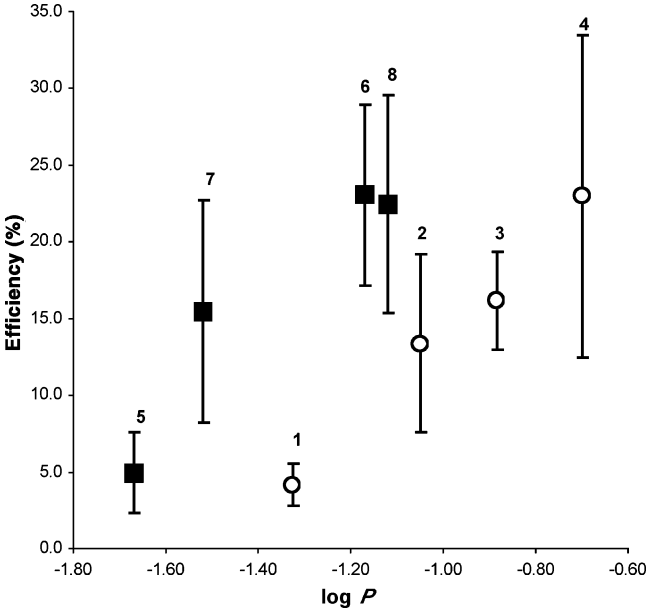


Figure 1. Iron-clearing efficiency (percent) of 4′-substituted ligands **1**−**4** (open circles) and 3′-substituted analogues **5**−**8** (filled squares) plotted versus the respective partition coefficients (log P_{app}) of the compounds. The plot of ligands **1**−**4** is from ref 53 and is included for comparison.

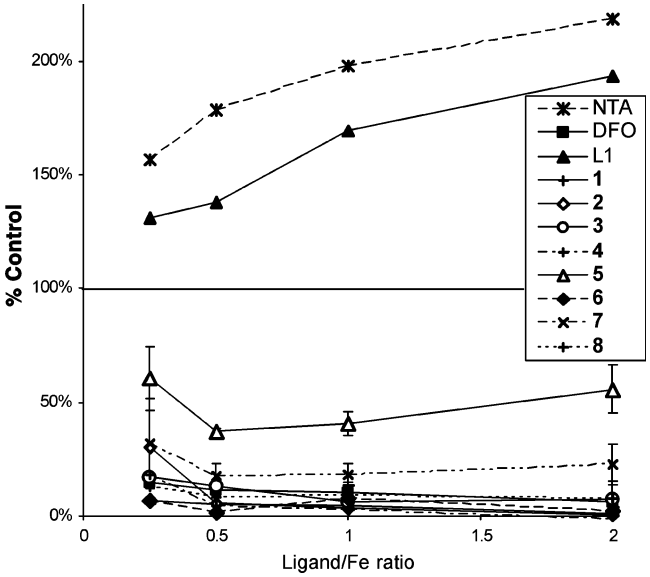


Figure 2. Effect of various chelators on the iron-mediated oxidation of ascorbate (Fenton chemistry) (percent of control, *y*-axis): nitrilotriacetic acid (NTA), 1,2-dimethyl-3-hydroxypyridin-4-one (L1), desferrioxamine (DFO), and DFT analogues **1**−**8** at several ligand:metal ratios (*x*-axis). Typically, each assay contained three controls with only ascorbate and $FeCl_3$; these varied less than 5% (±SD). Each assay also usually included a "negative control" containing ascorbate, $FeCl_3$, and L1 at a ligand:iron ratio of 2:1; this value was 174 ± 27%.

methylation of the thiazoline ring exerted a more profound effect (**6** vs **5**, $P < 0.01$).

Relationship between Partition Coefficients and Iron-Clearing Efficacy. The partition coefficients of the analogues were evaluated in buffered octanol−water. The log P_{app} values (Table 1) ranged from −0.70 for **4** to −1.67 for **5**. Attaching alkyl groups to the ligands increases a compound's lipophilicity (solubility in octanol); furthermore, when the iron-clearing efficiency is plotted versus partition coefficient (Figure 1), it is obvious that increasing the lipophilicity also augments the ICE within a particular family. Weighted regression analyses of the log P_{app}/ICE data for the 2′,4′-disubstituted analogues demonstrated that there was sufficient evidence to conclude a significant relationship between efficiency and log P_{app}. Furthermore, methylation of the 4′-hydroxyl of the parent **1**, as in **3**, seemed to have a more dramatic effect on lipophilicity and iron-clearing efficiency than did appending a methyl to the 4-position of the thiazoline ring (**2**).[53]

In the case of the 2′,3′-dihydroxy compounds (**5**−**8**), there was also an apparent relationship between log P_{app} and iron-clearing efficiency. However, what is most interesting is the difference in the impact that changing log P_{app} has on the efficiency of the 2′,3′-substituted derivatives vs the effect on that of the 2′,4′-substituted analogues. Linear regression analyses were conducted to find whether the relationship between log P_{app} and iron-clearing efficiency was the same for both series of compounds. The data consisted of the log P_{app} for all of the drug groups within both families. A preliminary inspection of the data indicated that ordinary least squares estimation could be applied to test for the relationship between iron-clearing efficiency and log P_{app}. Although the data do not appear to be perfectly linear (Figure 1), any curvature was negligible so that ordinary least squares could still be applied. A group × log P_{app} interaction was tested to determine whether the slopes of the lines for both families were equal (i.e., H_o: group × log P_{app} = 0), that is, that the relationship between iron-clearing efficiency and log P_{app} was the same for both groups. The results indicated that the interaction was not significant, suggesting that the slopes for the two groups were not significantly different from each other ($P = 0.815$). The interaction term (group × log P_{app}) was then dropped from the model. The effect for the group was tested to indicate for which group iron-clearing efficacy was the greater (i.e., H_o: groups = 0). These results clearly show a significant group effect with the 2′,3′-disubstituted set of analogues having a greater overall efficiency (Figure 1).

Antioxidant and Free-Radical-Scavenging Activity. The principal reason for removing excess iron from tissue is to prevent iron-mediated oxidative damage. Thus, one of the issues of major concern is related to the effect of any potential therapeutic ligand on the reduction of Fe(III) to Fe(II). In the +2 oxidation state, iron drives Fenton chemistry. Thus, a situation in which the reduction of Fe(III) to Fe(II) is promoted by a chelator would worsen any Fenton chemistry-mediated cell damage. Because some iron chelators are known to catalyze this reduction,[22,31] a determination of whether a ligand promotes, prevents, or has no effect on Fe(III) reduction is critical in designing therapeutic chelators for clinical use. Of the many physiological reductants that can reduce Fe(III) to Fe(II), ascorbate is a frequently used model. Ascorbate oxidation is easy to follow qualitatively by its disappearance spectrophotometrically.[31,34] The data in Figure 2 are plotted as the change in ascorbate concentration versus the ligand/metal ratio. Diminished Fe(III)-mediated ascorbate oxidation is indicated by a value less than 100% of control. Because the equilibria unfolding during the reaction are quite complicated, these results should not be overinterpreted.

Table 2. ABTS Radical Cation Quenching Activity of Desferrithiocin Derivatives

compd	slope × 10^3 OD units/μM[a]
DFT	−0.9[b]
7	−35 (−33[c,d])
Trolox	−37[b]
8	−39 (−36[d])
6	−58 (−106[b])
5	−62 (−102[b])

[a] The slope was derived from A_{734} versus concentration data after a 6-min reaction period between the chelator of interest and the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS•$^+$), which was formed from the reaction between ABTS and persulfate. A negative slope represents a decrease in the amount of highly colored radical cation over the time interval from an initial A_{734} of about 0.900. Trolox, an analogue of vitamin E, served as a positive control. [b] Data are from ref 30. [c] Numbers in parentheses are the slopes obtained with the corresponding 4′-substituted compound, e.g., the results for compound **3** are shown in parentheses next to the slope for analogue **7**. [d] Data are from ref 53.

The question addressed is, simply, in the presence of a test ligand, is the rate of ascorbate oxidation/iron reduction increased, decreased, or the same relative to controls containing iron(III) and ascorbate without the compound? Desferrioxamine functions as a positive control, as it decreases ascorbate loss;[31] L1, which increases ascorbate disappearance at ratios less than 3:1, serves as a negative control. Recall that the desferrithiocin analogues form 2:1 complexes with Fe(III); accordingly, the ligand-to-metal ratios from 0.5 to 2 are measured for ligands **1**−**8** (Figure 2). The 4′-substituted (**1**−**4**),[53] 3′-hydroxy (**5** and **6**), and 3′-methoxy (**7** and **8**) analogues slowed Fe(III) reduction considerably, even at a 0.5:1 ratio, as did the parent compound DFT (data not shown).[30,59] The results with the parent ligand are consistent with those reported in this same assay[31,34] and in a cultured cell system.[34] These results indicate that an enhancement of Fenton chemistry is unlikely to account for the toxicity of desferrithiocin and some of its derivatives.

Radical traps can help to attenuate Fenton chemistry-induced, free-radical-mediated damage; thus, the radical-scavenging properties of a particular ligand are of importance. Two hexacoordinate iron chelators, HBED[28] and DFO,[29,30] are excellent radical traps. The issue of Fenton chemistry and chelator design has a dimension beyond the prevention of the reduction of Fe(III). The liberated HO• molecules are very short-lived, reacting with most surrounding molecules at a diffusion-controlled rate. Ultimately, less active, more selective radicals, which can initiate a radical chain process, are produced and can cause significant cell damage.[60]

We have evaluated the 3′-hydroxylated compounds (**5** and **6**) and their respective *O*-methylated derivatives (**7** and **8**) in a free-radical-scavenging assay; the previously reported results obtained for the 4′-substituted compounds (**1**−**4**)[53] are included in Table 2 for comparison. The capacity of each of these ligands to function as a one-electron donor to the preformed radical monocation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•$^+$) was compared with that of Trolox, an analogue of vitamin E.[61] Table 2 shows the calculated slopes of the decrease in A_{734} versus ligand concentration line for each compound; a more negative slope indicates a more effective radical scavenger. As anticipated, the 3′-methoxylated compounds were less effective radical scavengers than were the corresponding 3′-hydroxylated molecules; nevertheless, **7** and **8** were as effective as Trolox and their corresponding positional analogues (**3** and **4**, respectively) at trapping free radicals and much better than the parent DFT. Compounds **5** and **6** scavenged the model radical cation at a level considerably less than those of the corresponding 4′-hydroxy analogues **1** and **2**.

Disposition of Desferrithiocin Analogues. From a drug design perspective, the relationship between ICE and lipophilicity (log P_{app}) described above was very useful. Nonetheless, the pharmacology behind this phenomenon needed clarification. Why, for example, should (*S*)-4′-(CH_3O)-DADMDFT (**3**) be nearly 4 times more efficient than (*S*)-4′-(HO)-DADMDFT (**1**), or (*S*)-4′-(CH_3O)-DADFT (**4**) be nearly twice as efficient as (*S*)-4′-(HO)-DADFT (**2**), beyond the fact that both methoxylated ligands partition into octanol to a greater degree than do their hydroxylated counterparts?

To explore the possible relationship between tissue disposition and ICE, we studied the behavior of the 4′-methoxylated compounds (**3** and **4**) as representatives. To avoid potential bioavailability problems associated with GI absorption, we first examined tissues from rodents given these compounds sc. The liver, heart, and pancreas were removed from the animals 0.5, 1, 2, 4, 6, and 8 h after single-dose administration of **4** or **2** (300 μmol/kg); compounds **4** and **2** were detected in tissue homogenates by HPLC (Figure 3). Liver tissue taken from animals given **4** sc revealed two peaks in the chromatogram, one corresponding to the drug administered, **4**, and the second corresponding to the 4′-demethylated metabolite **2**.

The differences in total chelator (either methoxylated derivative **4** plus putative metabolite **2** or **2** when administered alone) tissue concentrations seen were profound (Figure 3). In the liver after sc administration (panel A), the difference at 0.5 h was greater than 2-fold—275 ± 85 nmol/g wet wt of tissue for [**4**] plus [**2**] vs 123 ± 17 nmol/g for [**2**] ($P < 0.05$). The contrast became more prominent, roughly 9-fold, at 8 h after dosing, 106 ± 19 vs 12 ± 0.8 nmol/g, respectively ($P < 0.007$). A different, and more interesting, scenario is unfolding in the heart (panel C). Although the total chelator concentration ([**4**] + [**2**]) in the hearts of animals treated with **4** is about one-half of that measured in the liver of the same animals, for example, 119 ± 20 nmol/g at 0.5 h, this total is almost 5-fold that of **2** (27 ± 7 nmol/g) in animals treated with **2** ($P < 0.005$, panel C). By 4 h, whereas the concentration of **4** in the heart has dropped nearly 5-fold and **2** as the metabolite of **4** is near detectable limits, in **2**-treated animals, the compound is below detectable limits (<4.8 nmol/g). Similar proportions of **4** to **2** are found in the pancreas (panel E), although the levels of compound achieved are lower than those in the liver and heart. Excellent oral bioavailability of the methoxy compound is suggested by the comparable concentrations of methoxylated compound and hydroxylated metabolite attained when po administration is employed and the same time course is examined (Figure 3, panels B, D, and F).

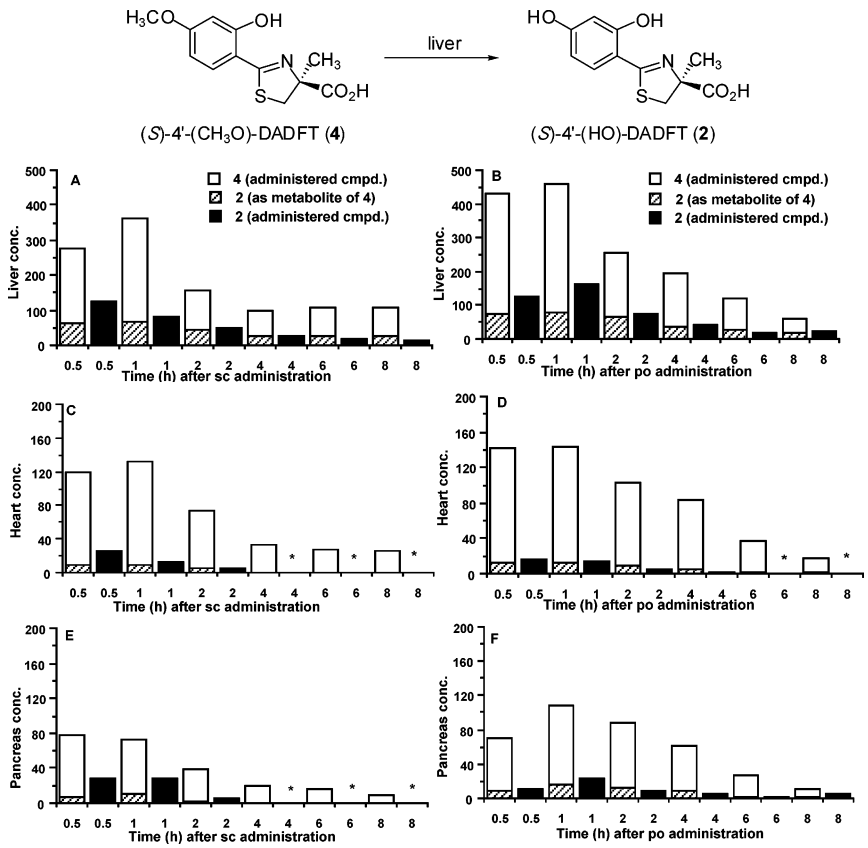


Figure 3. Tissue distribution in liver (panels A and B), heart (panels C and D), and pancreas (panels E and F) of (*S*)-4′-(CH_3O)-DADFT (**4**) vs that of (*S*)-4′-(HO)-DADFT (**2**) upon sc administration (panels A, C, and E) and those of **4** upon po administration (panels B, D, and F). The concentrations (*y*-axis) are reported as nmol compound per g wet wt of tissue. For all time points, $n = 3$. The asterisks indicate a level below detectable limits (approx. 5 nmol/g wet wt).

A similar measurement was made comparing (*S*)-4′-(CH_3O)-DADMDFT (**3**) vs (*S*)-4′-(HO)-DADMDFT (**1**) at times from 2 to 8 h postdosing. The rats were given either compound sc at 300 μmol/kg. Only the 2- and 4-h time points were remarkable; the levels of both ligands in all tissues were below detectable limits by 6 h postdosing. In like fashion to **4**, **3** was demethylated to **1**. Further, the total chelator concentration ([**3**] + [**1**]) in the liver of animals treated with **3** was 6-fold (2 h postdosing: 203 ± 62 vs 35 ± 29 nmol/g, $P < 0.02$) and almost 10-fold (4 h postdosing: 45 ± 8 vs < 4.8 nmol/g) higher than that of **1** when given alone. In the heart and pancreas, the only measurable concentrations of ligands were found at 2 h. In the heart, the total chelator concentration ([**3**] + [**1**]) was approximately 10-fold higher than that found when **1** was given alone, 62 ± 31 vs < 4.8 nmol/g, respectively ($P < 0.05$).

Apparently, more total chelator gets into the tissues of animals treated with **4** than of those treated with **3**. Clearly, much more total ligand is present in the tissues of animals treated with the methoxy compounds than those treated with their hydroxylated counterparts. The metabolites found in the heart and pancreas are probably derived from metabolism of the methoxylated compounds by the liver, but we have not yet examined this pathway. Moreover, the total chelator tissue residence time is longer for (*S*)-4′-(CH_3O)-DADFT (**4**) than for (*S*)-4′-(CH_3O)-DADMDFT (**3**). Taken collectively, this is in keeping with the increased ICE (i.e., **3** vs **1**; **4** vs **2**). Overall, we emphasize that the methoxylated ligands achieve these concentrations in the very organs that are most adversely affected by iron overload, that is, liver, heart, and pancreas.

Metabolism of (*S*)-4′-(CH_3O)-DADFT by Microsomes in Vitro. Although the details of the demethylation shown in Figure 3 remained uncharacterized, such a demethylation is not without precedent. Demethylation of codeine, catalyzed by an isoform of cytochrome P450, is responsible for its conversion to morphine in the liver.[62−64] It seemed likely that a similar process was occurring with the 4′-methoxylated compounds (**3** and **4**) to their hydroxylated counterparts (**1** and **2**, respectively) in the rodents. It is critical to note that when a putative rodent-*de*activated metabolite of DADFT, (*S*)-5′-(HO)-DADFT, was given to primates, it was quite active (unpublished observations), underscoring our view that hydroxylated DFT analogues can be handled differently by primates vs rodents.

Unwilling to sacrifice a primate at this stage, we compared the metabolism of **4** in rat and human liver microsomes.[65] In brief, pooled human or rat liver microsomes were used as the source of cytochrome P450. The viability of the microsomes was evaluated using resorufin benzyl ether, a substrate of cytochrome P450 3A4 (CYP3A4).[65] The reaction mixture contained microsomes and an NADPH-generating system in buffer. The reaction was initiated by the addition of compound **4** and was incubated for up to 2 h. The results (Figure 4) are presented as concentration of either the starting drug (**4**) or metabolite (**2**) (as quantified by HPLC of the trichloroacetic acid supernatant) in the mixture, normalized to microsomal protein concentration. This sug-

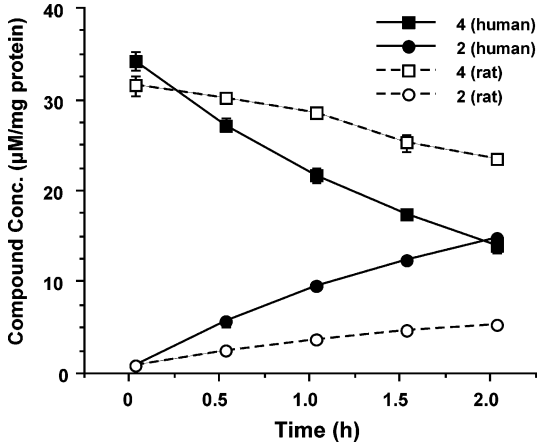


Figure 4. Metabolism of (*S*)-4′-(CH_3O)-DADFT (**4**) by rat and human liver microsomes. The reaction mixture contained microsomes and an NADPH-generating system in buffer. The reaction was initiated by the addition of compound **4** and was incubated up to 2 h (times shown on *x*-axis). The results are shown as concentration of compound, normalized to protein concentration present (*y*-axis, mean ± SD), as quantified by HPLC of triplicate reaction mixtures after the incubation period indicated.

gests that, in a human liver, roughly 50% of ligand **4** is demethylated to **2** in 2 h. On the basis of these findings, further in vivo measurement, i.e., following the metabolism of selected compounds in primates via liver biopsies, is warranted. This will allow us to correlate the in vivo rodent data with the in vivo primate data as they relate to the respective microsomal numbers.

In Vivo Toxicity Assessment. At this point, it was clear that we had to verify that the methoxylated compounds, although more lipophilic, did not elicit any unexpected toxic effects. Accordingly, 12 male Sprague–Dawley rats, six with normal iron stores and six iron-loaded to a level of 350 mg/kg, were treated with (*S*)-4′-(CH_3O)-DADMDFT (**3**) at a daily po dose of 100 μmol (25 mg)/kg for 30 days. This dose was selected on the basis of primate iron clearance data, that is, this dose represents enough ligand to clear 450 μg Fe/kg of body weight per day from the primates. Histopathological results from extensive tissues, including the heart, liver, kidney, pancreas, stomach, and intestine, were normal in both sets of animals; this was similar to our earlier findings with hydroxylated counterpart **1**.[48] Thus, at least in this series of compounds, lipophilicity and iron clearance can be increased without a corresponding increase in toxicity.

Conclusion

These observations have led to two hypotheses. (1) Increasing the lipophilicity of selected series of DFT analogues should enhance iron removal from the liver, heart and pancreas, the organs at the greatest risk of iron-induced injury. Extrapolating from the extent to which ligands **3** and **4** enter the liver, heart, and pancreas, one would expect excellent iron clearance from these organs. (2) Increasing the lipophilicity of selected series of DFT analogues can substantially augment their systemic iron-clearing efficiency without increasing their toxicity. Implicit in these hypotheses is the question of whether we have the best platform; for example, as shown in Figure 1, there is a significant difference in ICE of the (*S*)-3′-(HO)-DADMDFTs (**5**–**8**) over the (*S*)-4′-(HO)-DADMDFTs (**1**–**4**).

Furthermore, although methylation of the 3′- or 4′-hydroxyl did not alter the ability of the ligand to prevent ascorbate reduction of Fe(III), it did decrease the ligands' free-radical-scavenging capacity relative to their respective parent nonmethylated systems. Nevertheless, these compounds were still at least as active as Trolox.[30]

Experimental Section

D-α-Methylcysteine as its hydrochloride salt (**12**) was obtained from DSM Fine Chemicals, Linz, Austria. Reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), and Fisher Optima-grade solvents were routinely used. Distilled solvents and glassware that had been presoaked in 3 N HCl for 15 min were employed in reactions involving chelators. Phosphate buffer was made up to 0.1 M at a pH of 5.95[66] and was degassed. Organic extracts were dried with sodium sulfate. Silica gel 32–63 from Selecto Scientific, Inc. (Suwanee, GA) was used for flash column chromatography, and Sephadex LH-20 was obtained from Amersham Biosciences (Piscataway, NJ). Melting points are uncorrected. NMR spectra were obtained at 300 MHz (^{1}H) on a Varian Unity 300, with chemical shifts (δ) given in parts per million referenced to tetramethylsilane. Coupling constants (J) are in hertz. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA).

Desferrioxamine B in the form of the methanesulfonate salt, Desferal (Novartis Pharma AG, Basel, Switzerland), was obtained from a hospital pharmacy. 1,2-Dimethyl-3-hydroxypyridin-4-one (L1) was a generous gift from Dr. H. H. Peter (Ciba-Geigy, Basel, Switzerland). Analogues **1**–**5** and **7** were accessed as described in our earlier publications.[47,48,53]

Spectrophotometric readings (A_λ) for the ascorbate and radical cation assays were taken on a Perkin-Elmer Lambda 3B spectrophotometer (Norwalk, CT).

C. apella monkeys were obtained from World Wide Primates (Miami, FL). Male Sprague–Dawley rats were procured from Harlan Sprague–Dawley (Indianapolis, IN). Cremophor RH-40 was obtained from BASF (Parsippany, NJ). Ultrapure salts were obtained from Johnson Matthey Electronics (Royston, UK). All hematological and biochemical studies[46] were performed by Antech Diagnostics (Tampa, FL). Atomic absorption (AA) measurements were made on a Perkin-Elmer model 5100 PC (Norwalk, CT).

(*S*)-2-(2,3-Dihydroxyphenyl)-4,5-dihydro-4-methyl-4-thiazolecarboxylic Acid (6). Compound **12** (2.19 g, 12.8 mmol) was added to a solution of **10** (1.15 g, 8.51 mmol) in degassed CH_3OH (100 mL). Phosphate buffer (50 mL) and $NaHCO_3$ (2.15 g, 25.6 mmol) were added, and the reactants were heated at 70 °C for 18 h with stirring under nitrogen. The reaction mixture was cooled to room temperature, and the solvents were removed by rotary evaporation. The residue was partitioned between 8% $NaHCO_3$ (175 mL) and EtOAc (85 mL). After further extraction with 8% $NaHCO_3$ (35 mL), the combined aqueous portion was shaken with EtOAc (85 mL) and was acidified with cold 1 N HCl (255 mL). Extraction with EtOAc (3 × 175 mL), washing with saturated NaCl (120 mL), and solvent removal in vacuo gave 1.90 g of **6** (88%) as a green foam: $[\alpha]^{24}_D$ +67.0 ° (*c* 0.10, DMF); ^{1}H NMR (CD_3OD) δ 1.67 (s, 3 H), 3.33 (d, 1 H, J = 11.7), 3.87 (d, 1 H, J = 11.4), 6.74 (t, 1 H, J = 7.8), 6.93 (dd, 1 H, J = 3.3, 1.8), 6.96 (dd, 1 H, J = 2.7, 1.5). Anal. ($C_{11}H_{11}NO_4S$) C, H, N.

(*S*)-4,5-Dihydro-2-(2-hydroxy-3-methoxyphenyl)-4-methyl-4-thiazolecarboxylic Acid (8). Compound **12** (0.68 g, 4.0 mmol) was added to a solution of **11**[57,58] (0.5 g, 3.4 mmol) in degassed CH_3OH (10 mL). Phosphate buffer (5 mL) was added, and the pH was adjusted to 6 with $NaHCO_3$ (0.42 g, 5.0 mmol). The reactants were heated at reflux for 12 h with stirring under nitrogen. The reaction mixture was cooled to room temperature and was concentrated by rotary evaporation. The residue was dissolved in $NaHCO_3$ (20 mL), and the pH was adjusted to 2–3 with 1 N HCl. Extraction with ethyl acetate

(2 × 50 mL) was carried out, and the extracts were concentrated in vacuo. LH-20 column chromatography (6% CH_3CH_2OH/toluene) generated 0.350 g of **8** (39%) as a yellow solid: mp 158−160 °C; $[\alpha]^{24}_D$ +61.6 ° (*c* 1.02, DMF); ^{1}H NMR (DMSO-d_6) δ 1.60 (s, 3 H), 3.40 (d, 1 H, J = 11.4), 3.80 (s, 3 H), 3.82 (d, 1 H, J = 11.1), 6.89 (t, 1 H, J = 7.8), 7.03 (dd, 1 H, J = 8.1, 1.4), 7.15 (dd, 1 H, J = 8.0, 1.4). 12.6 (br s, 1 H), 13.3 (br s, 1 H). Anal. ($C_{12}H_{13}NO_4S$) C, H, N.

2,3-Dihydroxybenzonitrile (10). Boron tribromide (1 M in CH_2Cl_2, 300 mL, 0.3 mol) was added dropwise to **9** (12.24 g, 75.00 mmol) in CH_2Cl_2 (30 mL) with dry ice/acetone cooling. The reaction solution was allowed to warm to room temperature and was stirred for 18 h. After quenching with H_2O (50 mL) with ice-bath cooling, solids were filtered, and the layers of the filtrate were separated. The aqueous phase was diluted with H_2O (200 mL), and the pH was adjusted to 2 with 1 N HCl. Extraction with ethyl acetate (3 × 150 mL) was performed, and the extracts were concentrated in vacuo to produce 8.5 g of **10** (84%) as a pale yellow solid: mp 153−157 °C; ^{1}H NMR (CD_3OD) δ 6.74 (t, 1 H, J = 7.9), 6.96 (dd, 1 H, J = 11.7, 1.5), 6.98 (dd, 1 H, J = 11.7, 1.5). Anal. ($C_7H_5NO_2$) C, H, N.

Prevention of Iron-Mediated Oxidation of Ascorbate. The iron chelators (NTA, L1, DFO, **1**−**8**) were assessed for their ability to diminish the iron-mediated oxidation of ascorbate by a literature method.[31] Briefly, a solution of freshly prepared ascorbate (100 μM) in sodium phosphate buffer (5 mM, pH 7.4) was incubated in the presence of $FeCl_3$ (30 μM) and chelator (ligand/Fe ratios varied from 0 to 2) for 40 min. The A_{265} was read at 10 and 40 min; the ΔA_{265} in the presence of ligand was compared to that in its absence.

Quenching of the ABTS Radical Cation. The iron chelators were tested for their ability to quench the radical cation formed from 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by a published method.[61] Briefly, a stock solution of ABTS radical cation was generated by mixing ABTS (10 mM, 2.10 mL) with $K_2S_2O_8$ (8.17 mM, 0.90 mL) in H_2O and allowing the solution to sit in the dark at room temperature for 18 h. This stock solution of deep blue-green ABTS radical cation was diluted in sufficient sodium phosphate (10 mM, pH 7.4) to give an A_{734} of about 0.900. Test compounds were added to a final concentration ranging from 1.25 to 15 μM, and the decrease in A_{734} was read after 1, 2, 4, and 6 min. Assays were performed in triplicate at each concentration. The reaction was largely complete by 1 min, but the data presented are based on a 6-min reaction time.

Determination of Partition Coefficients. The octanol−H_2O partition data are expressed as distribution coefficients uncorrected for partial ionization of the acids and were all measured at pH 7.4 (50 mM TRIS buffer) using UV spectrometry. The measurements were done using a "shake flask" direct measurement.[67] Quadruplicate samples were vigorously agitated with 1-octanol (HPLC grade 1, Sigma-Aldrich, St. Louis, MO) overnight in a Parr shaker, and the layers were allowed to settle for 1−2 h prior to separation. The experiments were conducted at room temperature (22−24 °C) using a Shimadzu UV-265 spectrophotometer (Columbia, MD).

Iron Loading of *C. apella* Monkeys. The monkeys were iron overloaded with iv iron dextran as specified in earlier publications to provide about 500 mg of iron per kg of body weight;[68] the serum transferrin iron saturation rose to between 70 and 80%. At least 20 half-lives, 60 days,[69] elapsed before any of the animals were used in experiments evaluating iron-chelating agents.

Primate Fecal and Urine Samples. Fecal and urine samples were collected at 24-h intervals and processed as described previously.[45,46,70] Briefly, the collections began 4 days prior to the administration of the test drug and continued for an additional 5 days after the drug was given. Iron concentrations were determined by flame atomic absorption spectroscopy as presented in other publications.[45,50]

Drug Preparation and Administration. For iron clearance experiments, the compounds were administered as a solution or a suspension in 40% Cremophor RH-40/water (v/v) given po to the monkeys at a dose of 150 μmol/kg. In the metabolism studies, the rodents were given the monosodium salt of the compound of interest (prepared by addition of the free acid to 1 equiv of NaOH) either po or sc at a single dose of 300 μmol/kg. In the toxicity trial, the monosodium salt of **3** was administered po by gavage at a daily dose of 100 μmol (25 mg)/kg.

Calculation of Iron Chelator Efficiency. The theoretical iron outputs of the chelators were generated on the basis of a 2:1 complex. The efficiencies in the monkeys were calculated as set forth elsewhere.[47] Data are presented as the mean ± the standard error of the mean; *P*-values were generated via a two-tailed Student's *t*-test, in which the inequality of variances was assumed, and a *P*-value of <0.05 was considered significant.

Collection of Tissue Samples from Rodents. Male Sprague−Dawley rats (250−350 g) were given the compounds prepared as described above either po following an overnight fast or sc. At times 0.5, 1, 2, 4, 6, and 8 h after dosing with compounds **2** and **4** or at times 2, 4, 6, and 8 h after dosing with compounds **1** and **3**, the liver, heart, and pancreas were removed from three animals.

Tissue Analytical Methods. The tissue samples were prepared for HPLC analysis by homogenizing in water at a ratio of 1:2 (w/v). Then, to precipitate proteins, three times the volume of CH_3OH was added, and the mixture was stored at −20 °C for 20 min. This homogenate was centrifuged; the supernatant was filtered with a 0.2 μm membrane. This filtrate was injected directly onto the column or diluted with mobile phase A [95% buffer (25 mM KH_2PO_4, pH 3.0):5% CH_3CN), vortexed, and filtered as above prior to injection.

Analytical separation was performed on a C_{18} reversed-phase HPLC system with UV detection at 310 nm as described previously.[51,52] Mobile phase and chromatographic conditions were as follows: solvent A, 5% CH_3CN:95% buffer; solvent B, 60% CH_3CN:40% buffer. The gradients for each compound were as follows: for **2** and **4**, linear ramp from 100% A to 50% A (9 min), followed by a hold of 10 min; for **3**, linear ramp from 100% A to 20% B (20 min), followed by a hold of 5 min, then a linear ramp to 100% B (15 min), followed by a hold of 20 min; for **1**, linear ramp from 100% A to 20% B (20 min), followed by a hold of 5 min, then a linear ramp to 100% B (8 min), followed by a hold of 5 min.

The concentrations were calculated from the peak area fitted to calibration curves by nonweighted least squares linear regression with Rainin Dynamax HPLC Method Manager software (Rainin Instrument Co.). The method had a detection limit of 0.5 μM and was reproducible and linear over a range of 1−1000 μM.

Toxicity Evaluations in Rodents. Male Sprague−Dawley rats (225−250 g) (n = 12) were iron overloaded by ip administration of iron dextran (Sigma, 100 mg of Fe/mL) to a level of 350 mg/kg. The rats were given four doses of the iron (105 mg/kg/dose) over a two-week period on a Monday, Friday, Monday, Friday schedule. After a two-week equilibration period, the animals were weighed again, and their total iron burden was determined (total mg of iron/final body weight). The rats were then assigned to groups so that the body weight and iron burden between groups were within error of each other.

Additional animals (n = 12) were left unloaded to serve as age-matched counterparts. Both sets of animals were then divided into control (H_2O) and treated (**3**) groups (n = 6 each). The animals were given **3** orally by gavage as described above or an equivalent volume of H_2O for 30 days; the trial was otherwise conducted as described in an earlier publication.[47]

Microsomal Assays.[65] Pooled human liver microsomes or rat liver microsomes (BD Biosciences, Bedford, MA) were used as the source of cytochrome P450. Resorufin benzyl ether (Molecular Probes, Eugene OR), a substrate of CYP3A4, was used to assess the viability of the microsomes.[65] One milliliter of reaction buffer contained microsomes (0.8 mg) and an NADPH-generating system ($NADP^+$, 1.3 mM; glucose-6-phosphate, 3.3 mM; glucose-6-phosphate dehydrogenase, 0.4 U/mL; $MgCl_2$, 3.3 mM) in potassium phosphate buffer (100

mM, pH 7.4). The reaction was initiated by the addition of compound **4** (100 μM) and was incubated for up to 2 h at 37 °C in a shaking water bath. Ice-cold CH_3OH (3 volumes) was added to stop the reaction after either 30, 60, 90, or 120 min; the mixture was kept on ice for 20 min prior to centrifugation at 12 000*g* for 5 min. The collected supernatant was then injected onto a C_{18} column at a 1:10 dilution for quantitation of standard, chelator, and metabolite by HPLC (for resorufin standard, λ_{ex} 550 nm, λ_{em} 585 nm). The results are normalized to protein content and are expressed as μM/mg of protein.

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Supporting Information Available: Elemental analytical data for synthesized compounds **6**, **8**, and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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