Effects of C-4 Stereochemistry and C-4′ Hydroxylation on the Iron Clearing Efficiency and Toxicity of Desferrithiocin Analogues

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Additional structure−activity studies of desferrithiocin analogues are carried out. The effects of stereochemistry at C-4 on the ligands' iron clearing efficiency are reviewed and assessed using the enantiomers 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*R*)-carboxylic acid and 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*S*)-carboxylic acid. The utility of 4′-hydroxylation as a method of reducing the toxicity of desazadesferrithiocin analogues is also examined further with the synthesis and in vivo comparison of 4,5-dihydro-2-(2-hydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic acid, which is the natural product 4-methylaeruginoic acid, and 4,5-dihydro-2-(2,4-dihydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic acid. The stereochemistry at C-4 is shown to have a substantial effect on the iron clearing efficiency of desferrithiocin analogues, as does C-4′-hydroxylation on the toxicity profile. All of the compounds are evaluated in a bile-duct-cannulated rodent model to determine iron clearance efficiency and are carried forward to the iron-overloaded primate for iron clearing measurements. On the basis of the results of the present work, although 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*S*)-carboxylic acid is still the most promising candidate for clinical evaluation, 4,5-dihydro-2-(2,4-dihydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic acid (4′-hydroxydesazadesferrithiocin) also merits further preclinical assessment.

Introduction

Iron is required for life as we know it. Although it is one of the most abundant elements in the earth's crust,[1] it exists in the biosphere largely as insoluble ferric hydroxide ($K_{sp} = 1 \times 10^{-38}$).[2] Microorganisms have evolved low molecular weight, virtually ferric ion specific ligands, siderophores, to transport and store iron which would otherwise be unavailable.[3] For example, *Streptomyces pilosus* assembles and excretes desferrioxamine, a hydroxamate iron chelator[4] that forms a 1:1 hexacoordinate octahedral complex with Fe(III); the formation constant is 3×10^{30} M^{-1}.[5] The microorganism can utilize this complex as an iron source.

In fact, desferrioxamine B (Desferal) remains the drug of choice for the treatment of transfusional iron overload.[6−10] Although considerable effort has been invested in the development of new therapeutics for managing iron overload diseases, treatments have remained the same for over a generation. Patients with primary hemochromatosis are still treated by frequent phlebotomy; patients suffering from iron overload secondary to blood transfusions (e.g., Cooley's anemia) must be maintained on chelation therapy. Desferrioxamine's efficacy and long-term tolerability are well documented, but there are still problems associated with its use. If one assumes that the stoichiometry described above applies when this ligand is administered to an animal, 5% or less of the calculated iron excretion is observed.[11] This chelator also has a very short half-life in the body; it must therefore be administered by continuous infusion over long periods of time. Patient compliance can become a major problem over a lifetime of chelation therapy. Furthermore, the cost of desferrioxamine treatment renders the population in areas with the greatest need untreatable. There are two possible solutions to these problems: the development of an orally effective chelator or the identification of more efficient parenteral chelators. This report focuses on the former option.

Earlier structure−activity analyses of the desferrithiocin pharmacophore revealed that the three ligating centers—the thiazoline nitrogen, the carboxyl group, and the aromatic hydroxyl—are critical to the compounds' iron clearing capabilities. Modification of some of the nonchelating fragments, such as removal of the thiazoline methyl or the aromatic nitrogen, had little impact on the deferration properties of the resulting molecules. However, either replacement of the sulfur with oxygen, nitrogen, or a methylene or expansion of the five-membered thiazoline to a six-membered dihydro-Δ^2-thiazine resulted in compounds with little iron clearing activity.[12−14] Investigation of benz-fused desazadesmethyldesferrithiocins, i.e., the (*R*)- and (*S*)-pairs of naphthyl analogues 2-(2-hydroxynaphth-1-yl)-Δ^2-thiazoline-4-carboxylic acid and 2-(3-hydroxynaphth-2-yl)-Δ^2-thiazoline-4-carboxylic acid, demonstrated that although the former enantiomers were not effective iron clearing agents after oral administration to the rats, their positional isomers were.[15]

In a recent study utilizing (*S*)-desazadesmethyldesferrithiocin as the parent compound,[14] we determined that introduction of a hydroxyl in the 4-position of the aromatic ring resulted in a ligand which was orally

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active in both rodents and primates, yet was profoundly less toxic than the parent compound. The current series of experiments further explores 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*S*)-carboxylic acid and how the stereochemistry at C-4 affects its activity. In addition, the impact of 4′-hydroxylation of the aromatic ring on desazadesferrithiocin's toxicity is assessed.

Design Concepts

In the course of our recent structure−activity study focused on the biological effects of changing the redox potential of the aromatic ring of desferrithiocin by introducing electron-donating or -withdrawing groups,[14] we found that hydroxylation at the 4′-position had a pronounced effect on the molecule's toxicity profile. 4,5-Dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*S*)-carboxylic acid was indeed far less toxic than the 4′-dehydroxy parent chelator. This decreased toxicity was not related to a difference in octanol−water partition coefficients as the log *P* values between the parent and hydroxylated analogues were similar. The remarkable difference in toxicity between these two analogues has prompted us to further examine the utility of 4′-hydroxylation as a method of reducing the toxicity of other desferrithiocin analogues.

In the course of most of our previous investigations we principally explored desferrithiocin analogues in the (*S*)-configuration. This was based on earlier evidence that the (*S*)-enantiomers were more active iron clearing agents in primates.[16] In the current series of experiments we reviewed and further assessed the effects of stereochemistry at C-4 on the ligands' iron clearing efficiency.

To determine the importance of 4′-hydroxylation and C-4 stereochemistry, three systems were synthesized and evaluated in both a non-iron-overloaded, bile-duct-cannulated rat model and an iron-overloaded *Cebus apella* monkey model. The desferrithiocin analogues included 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*R*)-carboxylic acid (**6**), 4,5-dihydro-2-(2-hydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic acid (desazadesferrithiocin, **7**), and 4,5-dihydro-2-(2,4-dihydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic acid (**8**) (Table 1).

At the drug discovery level, the fact that the (*S*)-enantiomers tend to outperform the (*R*)-enantiomers in a given set of desferrrithiocin analogues is more than an interesting observation. The amino acid reagent required to assemble the (*S*)-desferrithiocin analogues (e.g., D-cysteine) is much more expensive than the naturally occurring L-cysteine. This issue compelled us to synthesize and assess the iron clearing properties of 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*R*)-carboxylic acid (**6**), the (*S*)-enantiomer of which (**5**) was already shown to be a very active iron chelator.

The choice of systems to further investigate the effects of 4′-hydroxylation on the toxicity profile of desferrithiocin analogues was also fairly clear. The parent ligand in this case was 4,5-dihydro-2-(2-hydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic acid (desazadesferrithiocin, **7**). As with desazadesmethyldesferrithiocin in the previous study, desazadesferrithiocin does not have a nitrogen in the aromatic ring. This choice also makes it possible to determine whether with **7**, similar to desazadesmethyldesferrithiocin,[14] removal of the aromatic nitrogen alters the toxicity profile from renal to gastrointestinal (GI) and then if 4′-hydroxylation [4,5-dihydro-2-(2,4-dihydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic acid, **8**] helps ameliorate the GI problems.

Synthetic Methods

Chelator **6** was synthesized by condensation of 2,4-dihydroxybenzonitrile (**10**) with L-cysteine in phosphate buffer and methanol (Scheme 1). Nitrile **10** was prepared from 2,4-dihydroxybenzaldehyde with nitroethane in sodium acetate and acetic acid.[17]

The production of desazadesferrithiocin (**7**) was accomplished by cyclocondensation of 2-cyanophenol (**11**) with (*S*)-α-methyl cysteine (**12**) in buffered aqueous CH_3-OH (Scheme 2). The high-field NMR spectra of synthetic **7** and the siderophore 4-methylaeruginoic acid, which has recently been isolated from the *Streptomyces* species KCTC 9303, were virtually identical; however, the stereochemistry at C-4 of the natural product was not specified.[18] Therefore, a circular dichroism measurement (CD) of **7** was run. While wavelengths of the maximum and two minima of the CD of **7** matched closely with literature values of the natural chelator and the molar ellipicities ([θ]) at 320 nm were within 4% of each other, the [θ] values at 280 and 260 nm of **7** were approximately 50% of the reported values.[18] There is no plausible explanation for this discrepancy, since (*S*)-α-methyl cysteine (**12**) used to generate **7** is derived from hydrolysis of desferrithiocin (DFT, **9**) and, possessing no α-proton, cannot racemize. Thus the first total synthesis of the natural product 4-methylaeruginoic acid proves that, like DFT, it possesses the (*S*)-configuration.

The preparation of 4′-hydroxydesazadesferrithiocin **8** also employed the unusual amino acid (*S*)-α-methyl cysteine (**12**), prepared from hydrolysis[19] of DFT (**9**) in 6 N HCl (Scheme 3). The mixture of **12** and the byproduct, 3-hydroxypicolinic acid (**13**), was reacted with 2,4-dihydroxybenzonitrile (**10**) under weakly acidic conditions. Filtration of the solid and recrystallization from aqueous ethanol resulted in crystalline tridentate ligand **8**.

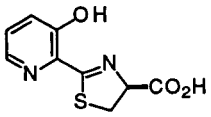
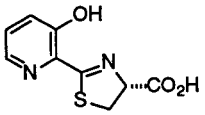
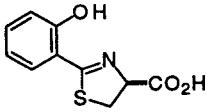
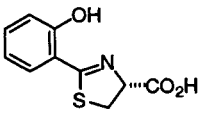
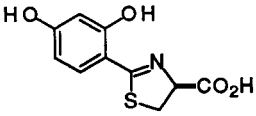
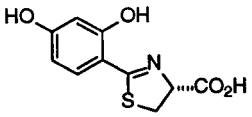
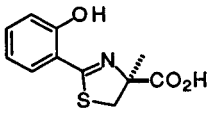
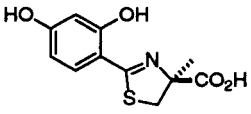
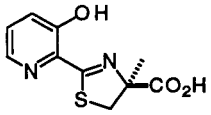
Metal Complex Stoichiometry

In an attempt to determine whether there was anything unusual about the nature of the Fe(III) complexes of analogues **7** or **8**, we ran Job's plots on these ligands. Not surprisingly, like **9**, these chelators formed 2:1 complexes with Fe(III) (Figure 1). We are currently investigating both the thermodynamics of formation and the structure of the complexes.

Racemization

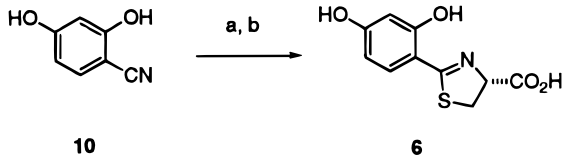
One of the interesting observations regarding the significance of stereochemistry in the biological activity of the desferrithiocin analogues is that the (*S*)-enantiomers are more active in primates than are their (*R*)-counterparts. It was thus of interest to evaluate the potential for racemization of the 4-carboxyl methine under physiological conditions. A study on the lability of the C-4 methine of 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*S*)-carboxylic acid (**5**) was conducted at pD 7.2 in D_2O. Interestingly, whereas there was little, if any, exchange at H-4 during the 5-h course of the

Table 1. Comparison of Efficiencies of the Desferrithiocins

compound no.	structure	efficiency % (rat)[a]	efficiency % (monkey)[b]	*P*-value (*S*) vs. (*R*)
1		2.4 ± 0.6 (po)[20] [82 bile, 18 urine]	150 μmol/kg 4.8 ± 2.7 (po)[20] [48 stool, 52 urine] 300 μmol/kg 8.0 ± 2.5 (po)[20] [42 stool, 58 urine]	
2		3.9 ± 1.8 (po)[13] [99 bile, 1 urine]	300 μmol/kg 0.5 ± 2.0 (po)[16] [54 stool, 46 urine]	Rat > 0.2 Monkey < 0.002
3		1.4 ± 0.6 (po) [100 bile, 0 urine]	300 μmol/kg 12.4 ± 7.6 (po) [90 stool, 10 urine]	
4		4.2 ± 1.6 (po)[20] [96 bile, 4 urine]	300 μmol/kg 8.2 ± 3.2 (po)[20] [80 stool, 20 urine]	Rat < 0.02 Monkey > 0.44
5		2.4 ± 0.9 (po)[14] [100 bile, 0 urine]	150 μmol/kg[14] 4.2 ± 1.4 (po) [70 stool, 30 urine] 150 μmol/kg[c] 5.6 ± 0.9 (sc) [92 stool, 8 urine] 300 μmol/kg 5.3 ± 1.7 (po) [90 stool, 10 urine] 300 μmol/kg[c] 4.8 ± 1.4 (po) [85 stool, 15 urine]	
6		≤ 0.5 (po)	150 μmol/kg 1.7 ± 0.8 (po) [76 stool, 24 urine]	Rat < 0.02 Monkey[d] < 0.03
7		2.7 ± 0.5 (po) [100 bile, 0 urine]	75 μmol/kg 21.5 ± 12.0 (po) [76 stool, 24 urine] 300 μmol/kg 13.1 ± 4.0 (po) [86 stool, 14 urine]	
8		≤ 0.5 (po)	75 μmol/kg 17.7 ± 3.9 (po) [79 stool, 21 urine] 150 μmol/kg 13.4 ± 5.8 (po) [86 stool, 14 urine]	
9		5.5 ± 3.2 [93 bile, 7 urine]	150 μmol/kg 16.1 ± 8.5 (po) [78 stool, 22 urine]	

[a] In the rats, doses were 150 μmol/kg via the route indicated. The net iron excretion was calculated by subtracting the iron excretion of control animals from the iron excretion of treated animals. Efficiency of chelation is defined as net iron excretion/total iron-binding capacity of chelator administered, expressed as a percent. Directly beneath the efficiency calculation is the percentage breakdown of iron excretion in the bile and urine, respectively. [b] In the monkeys, the doses and routes were as shown in the table. 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. Directly beneath the efficiency calculation is the percentage breakdown of iron excretion in the stool and urine, respectively. [c] The compound was given as a suspension in dH_2O rather than in 60% H_2O, 40% Cremophor. [d] The comparison was made at the 150 μmol/kg po dose.

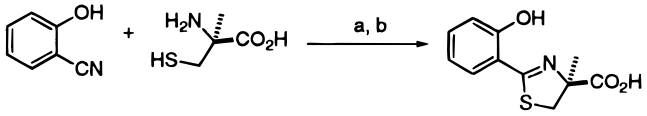
Scheme 1. Synthesis of 4,5-Dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*R*)-carboxylic Acid (**6**)[a]



10 **6**

[a] Reagents: (a) L-cysteine/CH_3OH/phosphate buffer/reflux/6 h, (b) 1 N HCl.

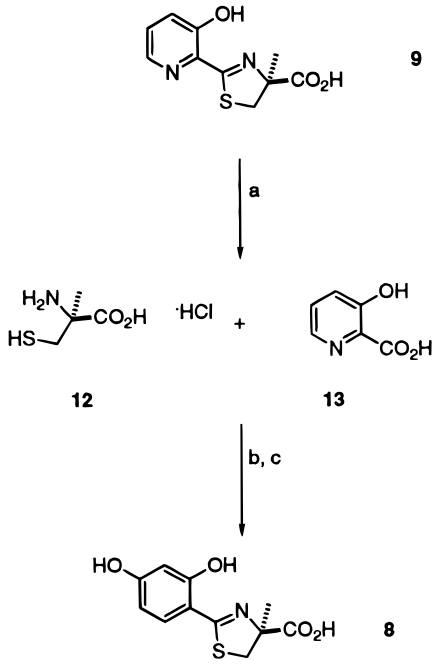
Scheme 2. Synthesis of 4,5-Dihydro-2-(2-hydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic Acid (**7**)[a]



11 **12** **7**

[a] Reagents: (a) phosphate buffer/CH_3OH/34 °C/5 days, (b) citric acid.

Scheme 3. Synthesis of 4,5-Dihydro-2-(2,4-dihydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic Acid (**8**)[a]



[a] Reagents: (a) 6 N HCl/100 °C/80 min, (b) **10**/CH_3OH/71 °C/3.5 days/phosphate buffer/$NaHCO_3$, (c) 1 N HCl.

experiment, the H-3′ aromatic proton between the two hydroxyls was very labile. The rate constant for the exchange was first-order in chelator with $k = 4.6 \pm 0.6 \times 10^{-4}$ s^{-1}. Identical experiments did not indicate any deuterium exchange of carbon-bound protons with either the parent desazadesmethyldesferrithiocin (**3**) or 2,4-dihydroxybenzoic acid. In a preliminary study with the Ga(III) complex of **5** at a 2:1 ligand:metal ion ratio, although the spectra were somewhat more complicated owing to the likely presence of diastereomeric mixtures, it was nevertheless possible to identify the H-3′ proton. Again, the rate constant was first-order in substrate, but it was about 7 times slower, $k = 7.5 \times 10^{-5}$ s^{-1}. We were also able to identify the H-4 methine in the Ga(III) complex, and there was no exchange of this proton.

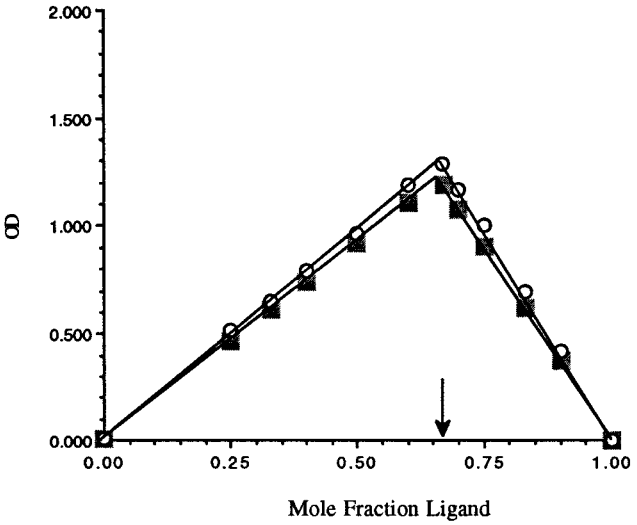


Figure 1. Job's plots of ligands **7** and **8**. The plots are superimposed on each other to show the similarity between the two graphs. Solutions containing different ligand/Fe(III) ratios were prepared so that [ligand] + [Fe(III)] = 1.0 mM. Compound **7** is indicated by the open circles, and analogue **8** is shown by the filled squares. The data points were fitted to the mole fractions (1) from 0−0.667 and (2) from 0.667−1.000; $r^2 = 0.993-1.000$. Theoretical mole fraction maximum for a 2:1 ligand:Fe complex is 0.667 (arrow).

Thus, it appears that racemization of this ligand in its free or complexed form is unlikely at physiological pH.

Biological Evaluations

The iron clearing properties of the compounds were tested both in a non-iron-overloaded, bile-duct-cannulated rat model and in an iron-overloaded *C. apella* monkey model. The non-iron-overloaded, bile-duct-cannulated rat model[12,20,21] represents a very rapid, functional initial screen of potential iron chelators. We can measure the total amount of iron cleared, the relative biliary versus urinary iron excretion, and the rate at which various chelators induce iron clearance in the bile and urine. If the ligand of interest does not induce iron clearance in the bile or the urine, then additional experimentation is generally regarded as unnecessary. Many chelators have appeared promising in rodents, but these compounds have failed at the clinical level. The iron-overloaded *C. apella* monkey serves as an intermediate screen for evaluating iron chelators before human studies; there are many similarities in this model to humans.[11,22]

Chelator-Induced Iron Clearance in Rodents. In Table 1, the iron clearing efficiencies of the compounds in rodents are shown, along with the relative fractions excreted in the bile and in the urine. We have included historical data (compounds **1**−**4** and **9**)[13,16,20] for the sake of discussion. A comparison of the historical enantiomeric pairs (**1,2**; **3,4**) suggests that, in general, the issue of C-4 stereochemistry is not profoundly significant in the bile-duct-cannulated rat model at a dose of 150 μmol/kg. However, the (*S*)-enantiomer of 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4-carboxylic acid (**5**) was more effective in terms of efficiency, 2.4 ± 0.9% vs ≤0.5% for the (*R*)-enantiomer **6** ($P < 0.02$, Table 1).

A comparison of (*S*)-desazadesferrithiocin (**7**) with the corresponding 4′-hydroxylated analogue **8** reveals that

these two ligands behaved very differently. Desazadesferrithiocin **7** was moderately effective in the rodents with an efficiency of 2.7 ± 0.5% under normal experimental conditions, (i.e., bile and urine were collected for 24 h post drug administration). Under these conditions, no chelator-induced iron excretion was observed with ligand **8**; the efficiency was calculated to be ≤0.5%. However, because of our interest in how C-4′ hydroxylation might affect the toxicity profile of **7**, a toxicity study was initiated in spite of the apparent lack of activity. After 2 days of treatment with **8** at a dose of 384 μmol/kg/day, the stools of non-iron-overloaded rodents were red, although guaiac tests were negative. This compelled us to initiate additional experiments with this drug in primates, shown below. The explanation for the apparent lack of iron clearing activity in the rodents under normal experimental conditions is not clear. It is possible that either the higher dose or the multiple doses used in the toxicity study led to the greater iron clearance. Neither can it be dismissed that uninterrupted enterohepatic circulation in these animals would result in the recirculation of free ligand and thus delayed and increased iron clearance. These issues are currently under consideration.

Chelator-Induced Iron Clearance in Primates. Inspection of Table 1 reveals some interesting differences in the iron clearance properties of the enantiomeric pairs of desferrithiocin analogues. We have included historical data (compounds **1**−**4** and **9**)[13,16,20] for the sake of discussion; the differences between enantiomers are greater in the primate model. Of the three enantiomeric pairs shown (**1,2**; **3,4**; **5,6**), it seems clear that the (*S*)-enantiomer is consistently better (compounds **1**, **3**, and **5**). The comparisons between compounds **1,2** and **3,4** were made at the 300 μmol/kg dose; the comparison between **5** and **6** was made at the 150 μmol/kg dose. Between compounds **1** and **2**, the (*S*)-enantiomer **1** was about 16 times better than the (*R*)-enantiomer **2**, $P < 0.002$. Ligand **1** at 300 μmol/kg had an efficiency of 8.0 ± 2.5% with 42% of the iron in the stool and 58% in the urine, whereas **2** had an efficiency of only 0.5 ± 2.0% with 54% of the iron in the stool and 46% in the urine.[16] The (*S*)-enantiomer **3**, with an efficiency of 12.4 ± 7.6%, generated 90% of the iron in the stool and 10% in the urine; (*R*)-enantiomer **4** as its sodium salt had an efficiency of 8.2 ± 3.2% with 80% of the iron in the stool and 20% in the urine. However, this difference is not significant ($P > 0.44$), possibly due to the high variability among the animals in the former set.

Interestingly, a pharmacokinetic study of the initial set (**1,2**)[16] clearly demonstrated that the peak plasma concentrations over time curves as well as areas under the curve (AUCs) of **1** and Fe(III):(**1**)$_2$ were equal to or lower than those of the corresponding **2** and Fe(III):(**2**)$_2$ curves. At 8 h posttreatment, the plasma concentrations of **1** and Fe(III):(**1**)$_2$ had declined to levels approaching the limits of detection, whereas substantial plasma concentrations of **2** and Fe(III):(**2**)$_2$ still remained. In every instance Fe(III):(**2**)$_2$ in the plasma exceeded 25 mg/L (50 μM) for several hours and remained above 10 mg/L (20 μM) at 8 h. The ratios for $AUC_{0-4}/AUC_{0-\infty}$ are 0.89 and 0.81 for **1** and Fe(III):(**1**)$_2$, respectively, compared to 0.50 and 0.44 for **2** and Fe(III):(**2**)$_2$, reflecting the prolonged residence times of the latter enantiomer. The most noteworthy finding from the pharmacokinetic experiments was the marked enantioselectivity of the renal clearance observed with **1**, a clearance that was 3.5 times greater than that of **2**, and an Fe(III):(**1**)$_2$ clearance that was 6.8 times greater than that of Fe(III):(**2**)$_2$. Owing to the death of one of the primates given **2**, we were unwilling to perform intravenous pharmacokinetic studies. These observations, coupled with the very low toxicity of the 4′-hydroxy ligand **5**,[14] encouraged us to consider an additional enantiomeric set of compounds, **5,6**.

(*S*)-Enantiomer **5** performed well in the primate model; the efficiency was 4.2 ± 1.4% when given po at a dose of 150 μmol/kg[14] and 5.3 ± 1.7% when given at a dose of 300 μmol/kg (Table 1). Iron clearance was largely in the stool at both 150 and 300 μmol/kg doses, 70% and 90%, respectively; the urinary percentages were 30% and 10%, respectively. These results were encouraging; in an effort to more closely mimic clinical applications, the ligand was given as a suspension in H_2O both po and sc. The iron clearing properties were not altered; the efficiency was 5.6 ± 0.9% at a dose of 150 μmol/kg sc and 4.8 ± 1.4% at a dose of 300 μmol/kg po. Again, iron clearance was overwhelmingly in the stool, 92% and 85%, respectively; 8% and 15% of the iron, respectively, was excreted in the urine. However, (*R*)-enantiomer **6** was a poor ligand at this dose with an efficiency of 1.7 ± 0.8%. Iron clearance was mostly fecal, 76% vs 24% in the urine.

In the case of (*S*)-desazadesferrithiocin **7** and the corresponding 4′-hydroxylated analogue **8**, the iron clearing efficiencies and modes of iron excretion of both compounds were identical. The efficiency of **7** at a dose of 300 μmol/kg was 13.1 ± 4.0%; that of **8** at a dose of 150 μmol/kg was 13.4 ± 5.8%. In both instances, 86% of the iron was in the stool and 14% in the urine. The efficiencies of these compounds were comparable, 21.5 ± 12% (76% stool, 24% urine) and 17.7 ± 3.9% (79% stool, 21% urine), respectively, at a dose of 75 μmol/kg (Table 1). However, as will be discussed below, the differences in toxicity profiles between these compounds are remarkable. Furthermore, the efficiency of these analogues is not substantially less than that of the natural product **9**, with its 16.1 ± 8.5% efficiency and 78% fecal and 22% urinary iron excretion at a dose of 150 μmol/kg.

Toxicity. If the ligands were shown to be effective (>3% efficiency) in the primates, a 10-day toxicity trial was initiated in rodents. The drugs were administered po to normal rats by gavage or sc once a day for 10 days, or until the animals expired, as indicated in Table 2. The data for **9**, although historical,[20] are included in Table 2 for the reader's convenience. Desferrithiocin **9**, the natural product, was a very effective iron chelator when given orally to rats or monkeys. However, the compound also elicited significant side effects, particularly nephrotoxicity, when administered chronically to rats. At a dose of 384 μmol/kg/day (equivalent to 100 mg/kg of the sodium salt), five out of five animals were dead within 10 days (Table 2). Histologically, the kidneys presented with vacuolar degeneration and acute, diffuse epithelial necrosis throughout the cortex; severe intralobular granular hyaline casts and severe multi-

Table 2. Chronic Toxicity Evaluations of Desferrithiocin and Desazadesferrithiocin Analogues

compd no.	dose/day (μmol/kg) × no. of days[a]	route	no. of deaths	comments
7	384 × 10	po	3/3	all animals dead by day 5: severe GI toxicity
	384 × 10	sc	3/3	all animals dead by day 5: severe GI toxicity
8	384 × 10	po	0/5	well-tolerated for the 10-day test period; histopathologies normal except for mild nephrotoxicity
9	384 × 10	po	5/5	all animals dead by day 5: severe nephrotoxicity[20]

[a] The compounds were given to rats at the doses, test periods, and routes shown.

focal tubular ectasia were also noted. Abstraction of the pyridine nitrogen from **9** to yield **7** resulted in a compound that presented a different toxicity profile; the primary toxicity was GI in nature. At a daily dose of 384 μmol/kg either po or sc, all of the animals were dead by day 5. At necropsy, the stomachs of all of the animals were hemorrhagic and grossly distended with gas and fluid; the stomach walls were translucent. The intestines were also hemorrhagic, and pressure necrosis of the spleen, from the grossly distended stomach, was noted in one of the animals dosed sc.

Interestingly, when an electron-donating hydroxyl group was added to position 4 of the aromatic ring of **7** to give **8**, the GI toxicity problem found with **7** was absent; no deaths occurred when this compound was given at 384 μmol/kg/day po for 10 days, greater than twice the time period for **7** (Table 2). Gross inspection of organs 1 day after the final dose revealed no drug-related abnormalities. However, histopathological analysis of tissues (see Experimental Section for a list) showed that this compound was associated with some nephrotoxicity, but nothing comparable to what was observed with the natural product **9**. Histologically, the kidneys presented with some vacuolar degeneration in the proximal tubular epithelium, with a pathology considered to be reversible; however, one animal showed possible early necrosis.

It is also notable that with **7**, **8**, and **9** in the primates the fecal iron clearance figures were similar, 86%, 86%, and 78% of the total, respectively; in the rodents the biliary iron clearance figures were 100% and 93%, respectively, for **7** and **9**. Thus, the differences in toxicity in the rodents cannot be readily attributed to the mode of iron clearance. Finally, it is critical to point out that (1) these were not iron-overloaded animals and (2) only a single dosing schedule, i.e., 384 μmol/kg/day, was evaluated. Dose-ranging studies with **8** are underway, along with an investigation of the impact of iron load in test animals on the drug's toxicity profile.

Conclusion

Our earlier structure−activity studies on the impact of alteration in the aromatic ring on the pharmacological properties of desazadesmethyldesferrithiocin were predicated on the idea that changing the redox potential of this fragment of the molecule would change its metabolic profile and potentially its toxicity. Indeed, this proved to be the case; introduction of an electron-donating hydroxyl group at the C-4′ position in both desazadesmethyldesferrithiocin (**3**) and desazadesferrithiocin (**7**) resulted in compounds (**5** and **8**, respectively) which were remarkably less toxic than the corresponding parent drugs.

Whereas in the case of **3**, C-4′-hydroxylation did reduce toxicity (**5**), it also diminished the compound's iron clearing efficiency by about 60%. Nevertheless, with very low toxicity this ligand is a promising candidate. However, with **7**, C-4′ hydroxylation had little, if any, impact on the iron clearing properties of the molecule, in addition to substantially reducing its toxicity (**8**). Although **8** elicited some renal toxicity, because of its impressive iron clearing efficiency even at lower doses, further toxicity analyses at lower doses and with iron-overloaded animals are warranted.

At the level of C-4 stereochemistry, the results obtained from **5** and **6** confirm that overall the (*S*)-enantiomers of the desferrithiocin analogues are indeed more effective iron clearing agents in primates than their (*R*)-counterparts. It further seems clear that racemization at C-4 under physiological conditions is unlikely.

Given the iron clearing efficacy at low doses, 4,5-dihydro-2-(2,4-dihydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic acid (**8**) is certainly worth further preclinical assessments. However, the encouraging results obtained with ligand **5** in our earlier study[14] and the iron clearance observed when this analogue was administered to the primates in a more clinically applicable matrix indicate that 4,5-dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*S*)-carboxylic acid (**5**) remains the most promising desferrithiocin analogue for continued preclinical development as an orally active iron chelator.

Experimental Section

Chelators **1**−**5** were prepared by methodology developed in these laboratories.[12,14] All reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification. Distilled solvents and glassware that had been presoaked in 3 N HCl for 15 min were employed for reactions involving chelators. Fisher Optima grade solvents were routinely used, and DMF was distilled. Organic extracts were dried with sodium sulfate. Phosphate buffer was made up to 0.1 M at a pH of 5.95.[23] Lipophilic Sephadex LH-20 from Sigma Chemical Co. (St. Louis, MO) was used for column chromatography, and melting points are uncorrected. Proton NMR spectra were obtained on a Varian Unity 300 (300 MHz) or Varian Unity 600 (600 MHz) spectrometer in the specified deuterated organic solvent or in D_2O; chemical shifts are expressed in parts per million downfield from tetramethylsilane or sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$, respectively. Coupling constants (*J*) are in hertz. Optical rotations were run at 589 nm (sodium D line) utilizing a Perkin-Elmer 341 polarimeter with *c* as grams of compound per 100 mL of solution. Circular dichroism (CD) spectra were obtained with a Jasco J500 spectropolarimeter. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA). Cremophor RH-40 was obtained from BASF (Parsippany, NJ). Sprague−Dawley rats were purchased from Charles River (Wilmington, MA). Nalgene metabolic cages, rat jackets, and fluid swivels were purchased from Harvard Bioscience (South Natick, MA). Intramedic polyethylene tubing (PE 50) was obtained from Fisher Scientific (Pittsburgh, PA). Rodent bedding (Beta-Chips) was purchased from Northeastern Products Corp. (Warrensburg, NY). *C. apella* monkeys were obtained from World Wide Primates (Miami, FL). Ultrapure salts were obtained from Johnson Matthey Electronics (Royston, U.K.). Imferon, an iron dextran solution, was obtained from Fisons (Bedford, MA).

4,5-Dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*R*)-carboxylic Acid (6).[24] L-Cysteine hydrochloride (4.38 g, 27.8

mmol), phosphate buffer (50 mL), and $NaHCO_3$ (2.41 g, 28.7 mmol) were added to a solution of **10**[14] (2.51 g, 18.5 mmol) in CH_3OH (74 mL). The solution was degassed with nitrogen and was refluxed with stirring for 6 h under nitrogen. The reaction mixture was cooled to room temperature and was stirred overnight. After the mixture was acidified to pH 2 with 1 N HCl, the dark yellow precipitate was filtered, washed with distilled water (3 × 30 mL) and cold EtOH (2 × 30 mL), and dried in vacuo, producing 3.065 g of **6** (69%) as a yellow powder, mp (sealed capillary) 268−270 °C dec (lit. mp 261−262 °C[24]): $[\alpha]^{23}_D$ −30.4° (*c* 1.00, DMF); ^{1}H NMR (DMSO-d_6) δ 3.57 (dd, 1 H, J = 11.3, 7.2), 3.65 (dd, 1 H, J = 11.3, 9.4), 5.37 (dd, 1 H, J = 9.4, 7.2), 6.31 (d, 1 H, J = 2.3), 6.38 (dd, 1 H, J = 8.5, 2.3), 7.25 (d, 1 H, J = 8.5), 10.26 (s, 1 H), 12.3−13.6 (m, 2 H); ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 33.2, 76.1, 102.3, 107.9, 108.1, 132.0, 160.5, 162.3, 171.6, 171.8. Anal. ($C_{10}H_9NO_4S$): C, H, N, S.

4,5-Dihydro-2-(2-hydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic Acid (4-Methylaeruginoic Acid) (7). Phosphate buffer (140 mL) and CH_3OH (200 mL) were degassed with nitrogen, and **12** (5.22 g, 38.6 mmol) and then **11** (4.56 g, 38.3 mmol) were added. The reactants, which dissolved after brief sonication, were heated at 34 °C for 5 days with stirring under nitrogen. The reaction mixture was cooled to room temperature, and the bulk of the CH_3OH was removed by rotary evaporation. The concentrate was cooled in ice water, and citric acid (10.4 g, 54.1 mmol) was added with swirling. The mixture was extracted with EtOAc (100 mL, 4 × 50 mL). The combined organic extracts were washed with water (2 × 50 mL) and brine (50 mL) and were concentrated in vacuo. Purification of the residue on a Sephadex LH-20 column, eluting with 2% EtOH/toluene, gave 2.89 g of **7** (32%) as a pale green solid, mp 133−134 °C: $[\alpha]^{23}_D$ +42.0° (*c* 1.58, CH_3OH); 600 MHz ^{1}H NMR (CD_3OD) δ 1.67 (s, 3 H), 3.34 (d, 1 H, J = 11.4), 3.88 (d, 1 H, J = 11.5), 6.89 (ddd, 1 H, J = 7.9, 7.9, 1.0), 6.94 (dd, 1 H, J = 8.3, 1.0), 7.37 (ddd, 1 H, J = 8.3, 7.9, 1.5), 7.44 (dd, 1 H, J = 7.9, 1.5); 600 MHz ^{1}H NMR[18] (CD_3OD) δ 1.65 (s, 3 H), 3.33 (d, 1 H, J = 12.0), 3.85 (d, 1 H, J = 12.0), 6.88 (ddd, 1 H, J = 8.0, 8.0, 0.8), 6.94 (dd, 1 H, J = 8.3, 0.8), 7.35 (ddd, 1 H, J = 8.3, 8.0, 1.5), 7.44 (dd, 1 H, J = 8.0, 1.5). Anal. ($C_{11}H_{11}NO_3S$): C, H, N.

Circular dichroism measurements were run in CH_3OH with the cell path length of 1 cm at 24 °C. Values could not be obtained for the UV maxima at 218 and 206 nm since the instrument was not sensitive below about 255 nm due to the strong UV absorption of **7** below 260 nm.

7 (*c* = 0.014)		lit.[18] (*c* = 0.01)	
λ (nm)	[θ] (deg)	λ (nm)	[θ] (deg)
318	+2530	321	+2631
280	−3610	280	−7416
258	−5330	260	−10058
		218	+4268
		206	+9326

4,5-Dihydro-2-(2,4-dihydroxyphenyl)-4-methylthiazole-4(*S*)-carboxylic Acid (8). Degassed 6 N HCl (500 mL) was added to **9** (11.50 g, 48.3 mmol), and the mixture was heated at 100 °C for 80 min under nitrogen.[19] The solution was concentrated under high vacuum, and the residue was dissolved in distilled water (70 mL). Evaporation was repeated to give 18.77 g of **12** and **13** (quantitative) as a white solid, to which a solution of **10** (5.72 g, 42.3 mmol) in degassed CH_3OH (250 mL) was added. Degassed phosphate buffer (250 mL) and $NaHCO_3$ (14.1 g, 0.168 mol) were introduced to the reaction to maintain a pH of 6. The reactants were heated at 71 °C for 3.5 days with stirring under nitrogen. The reaction mixture was cooled to room temperature, and the bulk of the CH_3OH was removed by rotary evaporation. Cold 1 N HCl (150 mL) was slowly poured into the residue with stirring. Solid was filtered, washed with water (2 × 15 mL), recrystallized from aqueous EtOH, and dried under high vacuum for 1 day at 111 °C, affording 6.084 g of **8** (57%) as pale tan needles, mp 281−283 °C dec: $[\alpha]^{25}_D$ +63.9° (*c* 1.12, DMF); ^{1}H NMR (DMSO-d_6) δ 1.56 (s, 3 H), 3.33 (d, 1 H, J = 11.4), 3.76 (d, 1 H, J = 11.5), 6.29 (d, 1 H, J = 2.1), 6.37 (dd, 1 H, J = 8.6, 2.2), 7.24 (d, 1 H, J = 8.4), 10.2 (s, 1 H), 12.6 (br s, 1 H), 13.1 (br s, 1 H). Anal. ($C_{11}H_{11}NO_4S$): C, H, N.

Determination of Stoichiometry of Ligand−Fe(III) Complexes. The stoichiometry of ligand−Fe(III) complexes was determined spectrophotometrically from Job's plots for ligands **7** and **8**. Desferrithiocin, known to form a 2:1 ligand:Fe(III) complex,[25] was run as a positive control. Solutions were monitored at the visible λ_{max} (482 nm and 481 nm, respectively). Solutions containing different ligand/Fe(III) ratios were prepared by mixing appropriate volumes of 0.5 mM ligand in 100 mM TRIS Cl, pH 7.4, and 0.5 mM Fe(III) nitrilotriacetate (NTA) in 100 mM TRIS Cl, pH 7.4, so that [ligand] + [Fe(III)] = 1.0 mM. The Fe(III)−NTA solution was prepared immediately prior to use by dilution of a 50 mM Fe(III)−NTA stock solution with TRIS buffer. The stock solution was prepared as described previously,[14] and the iron content was verified by atomic absorption spectroscopy.

^{1}H NMR Sample Preparation and Experimental Conditions. Ligand **5** and 2,4-dihydroxybenzoic acid were analyzed by ^{1}H NMR (300 MHz) in DMSO-d_6 and in D_2O/phosphate buffer (μ = 0.25, pD = 7.2, 25 °C). The sodium salt of **3** and the Ga(III) chelate of ligand **5** were analyzed in D_2O/phosphate buffer only. The Ga(III) chelate of **5** was prepared by dissolving **5** in a $Ga(NO_3)_3$ suspension in D_2O/phosphate buffer in a 2:1 **5**:Ga(III) molar ratio. The resulting solution cleared immediately upon addition of ligand. Solutions prepared for the kinetic runs were allowed to temperature equilibrate at 25 °C for 10 min after buffer addition; spectra were then collected at approximately 15-min intervals over 2−3 h. Chemical shifts (δ) in D_2O/phosphate buffer are reported in ppm downfield from an external sodium-3-trimethylsilylpropionate-*2,2,3,3-d*$_4$ reference.

^{1}H NMR of 4,5-Dihydro-2-(2,4-dihydroxyphenyl)thiazole-4(*S*)-carboxylic Acid (5) and Ga(III):(5) Chelate. The proton resonances for ligand **5** in DMSO-d_6 were assigned as follows: δ 6.31 (d, 1 H, J = 2.1, H-3′), 6.38 (dd, 1 H, J = 8.5, 2.1, H-5′), 7.25 (d, 1 H, J = 8.5, H-6′), 5.38 (dd, 1 H, J = 9.6, 7.1, H-4), 3.57 (dd, 1 H, J = 11.3, 7.1, H-5), 3.65 (dd, 1 H, J = 11.3, 9.6, H-5), 10.26 (s, OH), 12.62 (s, OH), 13.14 (br s, OH). For **5** in D_2O/phosphate buffer, observed resonances were assigned as δ 6.32 (t, 1 H, J = 2.0, H-3′), 6.38 (dd, 1 H, J = 9.9, 2.0, H-5′), 7.38 (d, 1 H, J = 9.9, H-6′), 5.14 (dd, 1 H, J = 9.5, 7.3, H-4), 3.55 (dd, 1 H, J = 11.2, 7.3, H-5), 3.72 (dd, 1 H, J = 11.2, 9.5, H-5). The resonance intensity for H-3′ decreased with time, relative to the other observed resonances, indicative of slow exchange. Over the range of 1.0−10.0 mM, plots of concentration of **5** vs time demonstrated that above approximately 4 mM (in unexchanged **5**) the kinetics appeared as zero-order. As the concentration of unexchanged substrate fell below 4 mM, the kinetics were first-order; plots of the natural log of the concentration vs time were linear, with k = 4.7 ± 0.6 × 10^{-4} s^{-1}.

Multiple resonances for each carbon-bound proton were observed in the spectrum of the Ga(III) chelate of **5** (3.0 mM in **5**, 1.5 mM in $Ga(NO_3)_3$, D_2O/phosphate buffer). Observed resonances were assigned as H-3′: 5.84 (d, 0.31 H, J = 2.1), 6.16 (d, 0.10 H, J = 2.1), 6.28 (d, 0.53 H, J = 2.1); H-5′: 6.33 (dd, 0.28 H, J = 8.7, 2.1), 6.39 (dd, 0.42 H, J = 9.0, 2.1), 6.41 (dd, 1 H, J = 8.7, 2.1); H-6′: 7.41 (d, 0.21 H, J = 8.7), 7.46 (d, 0.60 H, J = 9.0), 7.47 (d, 0.21 H, J = 8.7); H-4: 5.35 (dd, 0.60 H, J = 9.0, 13.5), 5.40 (dd, 0.40 H, J = 9.0, 13.2); H-5: 3.51−3.67 (m, 1 H), 3.70−3.86 (m, 1 H). As with free ligand **5**, the intensity of the three doublets assigned to H-3′ decreased with time relative to the other observed resonances. Plots of the natural log of the concentration (based on both the 5.84 ppm and 6.28 ppm resonances) vs time were linear, indicative of first-order kinetics (k = 7.5 × 10^{-5} s^{-1}).

^{1}H NMR of Sodium 4,5-Dihydro-2-(2-hydroxyphenyl)-thiazole-4(*S*)-carboxylate (3, Sodium Salt). We have previously reported the ^{1}H NMR spectrum of the sodium salt of **4** in CD_3OD.[12] In D_2O/phosphate buffer, observed resonances

were assigned as follows: 6.96−7.10 (m, 2 H, H-3′ and H-5′), 7.48 (td, 1 H, J = 7.9, 1.5, H-4′), 7.57 (dd, 1 H, J = 7.9, 1.5, H-6′), 5.27 (dd, 1 H, J = 9.5, 7.9, H-4), 3.57 (dd, 1 H, J = 11.2, 7.9, H-5), 3.74 (dd, 1 H, J = 11.2, 9.5, H-5). No exchange of carbon-bound protons was observed.

^{1}H NMR of 2,4-Dihydroxybenzoic Acid. ^{1}H NMR spectra of 2,4-dihydroxybenzoic acid was obtained under the same conditions as ligand **5** for comparison. In DMSO-d_6 the spectrum was assigned as follows: 6.26 (d, 1 H, J = 2.3, H-3), 6.34 (dd, 1 H, J = 8.7, 2.3, H-5), 7.62 (d, 1 H, J = 8.7, H-6), 10.33 (s, OH), 11.40 (br s, OH), 13.3 (br s, OH). In D_2O/phosphate buffer, assignments were as follows: 6.40 (d, 1 H, J = 2.4, H-3), 6.46 (dd, 1 H, J = 2.4, 8.6, H-5), 7.71 (d, 1 H, J = 8.7, H-6). No exchange of carbon-bound protons was observed.

Cannulation of Bile Duct in Rats. The cannulation has been described previously.[12,15,26] Briefly, male Sprague−Dawley rats averaging 450 g were housed in Nalgene plastic metabolic cages during the experimental period and given free access to water. The animals were anesthetized using sodium pentobarbital (55 mg/kg) administered ip. The bile duct was cannulated using 22-gauge polyethylene tubing. The cannula was inserted into the duct about 1 cm from the duodenum and tied snugly in place. After threading through the shoulder, the cannula was passed from the rat to the swivel inside a metal torque-transmitting tether, which was attached to a rodent jacket around the animal's chest. The cannula was directed from the rat to a Gilson microfraction collector (Middleton, WI) by a fluid swivel mounted above the metabolic cage. Bile samples were collected at 3-h intervals for 24 h. The urine sample was taken at 24 h. Sample collection and handling were as previously described.[12]

Iron Loading of *C. apella* Monkeys. *C. apella* monkeys were given iron dextran iv as described in detail in earlier publications.[11,20,26] Briefly, the iron dextran was administered to the animals by slow infusion at a dose of 200−300 mg of iron/kg of body weight over 45−60 min. Infusions were performed as necessary to provide about 500 mg of iron/kg of body weight. After administration of iron dextran, we waited at least 60 days before using any of the animals in experiments evaluating iron chelating agents.

Primate Fecal and Urine Samples. Fecal and urine samples were collected and processed as accounted previously.[11,20,26] 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 described in earlier publications.[11,15]

Drug Preparation and Administration. The iron chelators were solubilized in 40% Cremophor RH-40/water (v/v) and given po to the rats at a dose of 150 μmol/kg. In the primates, the compounds were solubilized in 40% Cremophor RH-40/water (v/v) and given po at a dose of 75, 150, or 300 μmol/kg as indicated in Table 1. In addition, analogue **5** was given po and sc in dH_2O at doses of 300 and 150 μmol/kg, respectively.

Calculation of Iron Chelator Efficiency. The efficiency of each chelator was calculated on the basis of a 2:1 ligand:iron complex. The efficiencies in the rodent model were calculated by subtracting the iron excretion of control animals from the iron excretion of treated animals. This number was then divided by the theoretical output; the result is expressed as a percentage. In the monkeys, the numbers were generated 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 percentage.

Statistical Analysis. Data are presented as the mean ± the standard error of the mean. For comparisons of the means of two groups, the two-sample t-test (without the assumption of equality of variances) was used for analyzing the rodent and primate data. All tests were two-tailed, and a significance level of $P < 0.05$ was used.

Toxicity Evaluations in Rodents. Male Sprague−Dawley rats averaging 450 g were housed in polycarbonate cages. Before the first drug administration, the rats were weighed and evaluated for their general condition. The rats were given analogue **7** or **8** at a dose of 384 μmol/kg once daily for 10 days following an overnight fast. The drugs were solubilized in 40% Cremophor RH-40/water (v/v). Ligand **7** was administered either po by gavage or sc (n = 3/group), whereas **8** was given po by gavage only (n = 5/group). The rats were fed 3 h after drug administration and were allowed access to food for 5 h. The amount of food and water consumed was recorded daily. In addition, the animals were weighed and evaluated for their activity level and general appearance on a daily basis. Control animals were given an equivalent volume of 40% Cremophor/water (v/v) either po by gavage or sc as appropriate and were maintained on the same feeding schedule as the drug-treated animals.

If an animal expired during the course of the evaluation, a necropsy was performed; any gross abnormalities were recorded. After a 10-day exposure to the drug, animals were sacrificed 24 h after the final dose and extensive tissues including adrenal gland, aorta, bone marrow, brain, eye, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, testicle, heart, kidney, liver, lung, lymph node, pancreas, prostate, salivary gland, skeletal muscle, skin, spleen, thymus, thyroid, trachea, and bladder were sent to an outside pathologist for histological evaluation.

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