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ORIGINAL ARTICLE

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Fast biological iron chelators: kinetics of iron removal from human diferric transferrin by multidentate hydroxypyridonates

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Abstract For decades, desferrioxamine B (Desferal) has been the therapeutic iron chelator of choice for iron-overload treatment, despite numerous problems associated with its use. Consequently, there is a continuous search for new iron chelating agents with improved properties, particularly oral activity. We have studied new potential therapeutic iron sequestering agents: multidentate ligands containing the hydroxypyridonate (HOPO) moiety. The ligands TRENCAM-3,2-HOPO, TRPN-3,2-HOPO, TREN-Me-3,2-HOPO, TREN-1,2,3-HOPO, 5LIO-3,2-HOPO, and BU-O-3,4-HOPO have been examined for their ability to remove iron from human diferric transferrin. The iron removal ability of the HOPO ligands is compared with that of the hydroxamate desferrioxamine B, the catecholates TRENCAM and enterobactin, as well as the bidentate hydroxypyridonate deferiprone, a proposed therapeutic substitute for Desferal. All the tested HOPO ligands efficiently remove iron from diferric transferrin at millimolar concentrations, with a hyperbolic dependence on ligand concentration. At high ligand concentrations, the fastest rates are found with the tetra- and bidentate hydroxypyridonates 5LIO-3,2-HOPO and deferiprone, and the slowest rates with the catecholate ligands. At low concentrations, closer to therapeutic dosage, hexadentate ligands which possess high pM values have the fastest rates of iron removal. TRENCAM-3,2-HOPO and TREN-Me-3,2-HOPO are the most efficient at lower doses and are regarded as having high potential as therapeutic agents. The kinetics of removal of Ga(III) from transferrin [in place of the redox active Fe(III)] were performed with TRENCAM and TREN-Me-3,2-HOPO to determine that there is no catalytic reduction step involved in iron removal.

Key words Iron chelation · Hydroxypyridonates · Multidentate ligands · Human diferric transferrin · Kinetics

Paper number 28 in the series "Ferric ion sequestering agents". For the previous paper, see [60]

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Introduction

With the apparent exception of *Lactobacillus* [1], iron is essential for all living organisms [2]. It is involved in a variety of biological mechanisms and reactions, including oxygen transport, electron transfer, photosynthesis, nitrogen fixation, and DNA synthesis [2]. Serum transferrin is a member of a family of iron-binding and transporting proteins which includes lactoferrin, ovotransferrin, and melanotransferrin [3, 4]. These proteins are responsible for controlling the level of free iron in body fluids [5]. Serum iron is scavenged by transferrin, resulting in a free serum iron concentration of 10^{-24} M [6]. Iron concentrations in secretions are also low because free iron is sequestered by lactoferrin. In addition to their iron scavenging ability, transferrin proteins provide an efficient iron transport and delivery system for the host [5, 7, 8]. As an essential nutrient, iron is absorbed by the intestinal mucous membrane, transferred to transferrin by an as-yet uncharacterized process, and then delivered through the bloodstream to cells such as hepatocytes and immature erythrocytes [2, 3]. Ferric transferrin binds to specific cell membrane receptors and is internalized in endosomes, after which the iron is released as a result of a low intraendosomal pH of 5.5 and apparent active participation of the receptor [3]. Finally, transferrin is transported back into the serum without degradation.

Iron is lost almost exclusively by blood loss; there is no mechanism to excrete excess iron [9]. Hence, when absorption exceeds excretion, iron overload results. The primary iron overload disorders include genetically induced iron overload (hemochromatosis) as well as iron overload induced by long-term transfusion therapy (e.g. thalassemia, sickle cell anemia). As a result of iron overload, iron is deposited in organs, and tissues are damaged [10] due to the ability of ferrous iron to catalyze the formation of free radical species (hydroxyl and superoxide radicals and hydrogen peroxide). This leads to organ failure (usually of the heart or liver) and eventually to death [11, 12].

Siderophores are powerful iron chelators produced by microorganisms, and present unique chemical and physiochemical properties [13]. They can be differentiated based on the chemical nature of their chelating functionalities: catecholate, hydroxamate, or hydroxypyridonate [14, 15]. Of the siderophores, the tris(catecholate) enterobactin is the most powerful ferric iron sequestering agent at neutral pH, with a pM value of 35.5 [16], and was used as a model for the synthesis of several catecholate chelating agents [17–20], as well as the hydroxypyridonates (HOPO) used in this study. For several decades, patients with iron overload diseases have been treated with desferrioxamine B (Desferal), a natural hydroxamate-containing siderophore produced by *Streptomyces pilosus* [21–24]. However, its retention time in the body is quite short and its oral activity is very poor [25]. It must be administered intravenously on a continuous basis, and consequently, problems arise with poor patient compliance [26]. A continuing search for orally active and highly efficient iron chelators has led to the development of some bi- and tridentate hydroxypyridonate ligands as possible new therapeutic agents [27, 28], including deferiprone, also known as L1 [29, 30].

Since transferrin is the major iron transport agent within the body, it is a suitable target for drugs intended to treat iron overload. Serum transferrin is known to be the primary mammalian iron transport protein, but also acts as an iron buffer, maintaining free ferric ion concentration in the body at a very low value. While, in healthy humans, transferrin is only one third saturated, it is usually completely saturated in patients with iron overload. Therefore transferrin may be a good iron source in chelation therapy for chronic iron overload. Indeed, ferric transferrin has been shown to be an iron source for chelation by deferiprone (a widely used therapeutic chelator) [31]. Studies of iron removal from human diferric transferrin have been previously performed with several classes of ligands, including catecholates [32, 33], phosphonic acids [34], hydroxamates [35, 36], and pyrophosphates [37–39]. Even though Desferal (DFO) has a high affinity for ferric ion, part of its ineffectiveness resides in its inability to remove iron from diferric transferrin at a significant rate, both in vitro and in vivo [12, 40, 41]. The slow rate of iron removal by DFO is also common for other hydroxamate-based sequestering agents [35, 36]. Until now, the most rapid kinetics of iron removal have been observed with catecholate ligands [33, 42]. Catechol ligands, either covalently attached to the hydroxamate sequestering agent or added separately, catalyze the rate at which DFO removes iron from transferrin [43]. This capacity of catechol functionalities to catalyze iron removal from transferrin explains why the fastest ligands so far have been synthetic catecholates [33].

The potential of hydroxypyridonates as orally active iron chelators has been reviewed and is exemplified in the widely studied bidentate ligand deferiprone [26, 44]. However, in vivo, deferiprone is rapidly glucuronidated, causing a reduction in its iron removal efficiency [45]. In addition, this bidentate 3,4-HOPO ligand is highly toxic at therapeutic doses [46, 47]. Because of the desired low concentration of any therapeutic ligand when administered for iron chelation therapy, new compounds with a higher iron scavenging efficiency are needed.

We present here an evaluation of the ability of some hydroxypyridonate (HOPO) ligands to mobilize iron, as well as their kinetics of iron removal from human diferric transferrin. These HOPO ligands are unusually fast at removing iron from transferrin, faster than the catecholate-based ligands. The ligands studied include bi-, tetra-, and hexadentate chelators, some of which are promising orally active therapeutic iron sequestering agents.

Materials and methods

Iron chelators

The chelators used in this work are either hexa-, tetra-, or bidentate ligands, containing catecholate or hydroxypyridonate [48] moieties (Fig. 1). Desferrioxamine B was obtained as a gift from Salutar, and enterobactin was purified according to literature procedures [49].

Preparation of ferritransferrin

Human apotransferrin (98% iron free, Sigma) was dissolved in 50 mM Tris (pH 7.4), 150 mM NaCl, 20 mM $NaHCO_3$ buffer to give a final protein concentration of 200 μM. Iron nitrilotriacetate was prepared as described by Harris and Aisen [50]. Briefly, 15 μmol of $FeCl_3$ were dissolved in a solution of 6 M HCl and 30 μmol of nitrilotriacetic acid before adjusting the pH to 4.0 with KOH. This solution was diluted to 10 mL with water for a final concentration of 1.5 mM of iron nitrilotriacetate. The transferrin solution was saturated with iron by the addition of 400 μM of freshly prepared iron nitrilotriacetate and incubated at 37 °C for 2–3 h. Unbound iron was removed by passing the resultant red ferritransferrin solution through a Sephadex G25-column (PD-10 column, Pharmacia Biotech) equilibrated with the buffer described above, and eluted with the same buffer. The ferritransferrin solution was filter sterilized and stored at –20 °C until use. Protein concentration was determined by UV-Vis spectroscopy by measuring the absorbance at 280 nm and calculating the concentration using an extinction coefficient

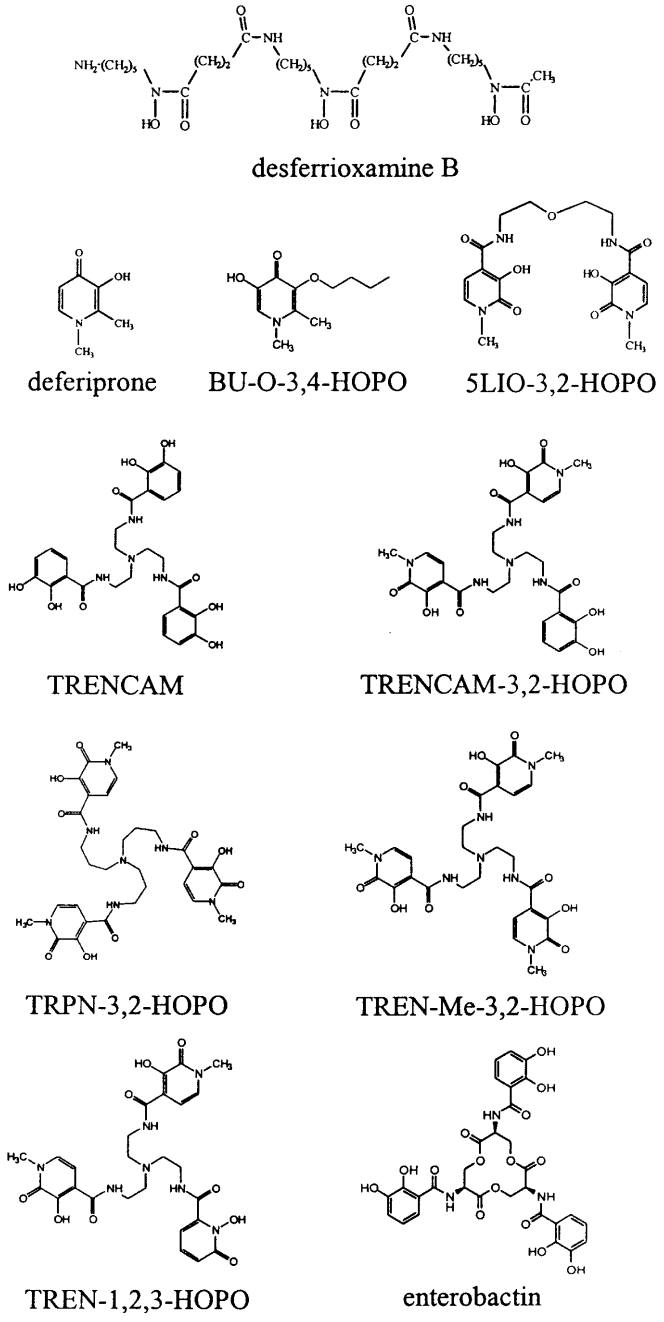


Fig. 1 Chemical structures of the ligands discussed in this paper

ε=8.72×10^4 M^{-1} cm^{-1} and a molecular weight of 81000 Da. The percentage of protein iron saturation was estimated by measuring UV-Vis absorbance at 470 nm using the extinction coefficient ε=4.86×10^3 M^{-1} cm^{-1} [51].

Gallium transferrin complex preparation

Gallic transferrin was prepared from a solution of 200 μM apotransferrin by adding a stoichiometric amount of gallium from a gallium atomic absorption standard solution (Aldrich). Complexation was monitored by UV difference spectroscopy at 280 nm; the complex required several hours to stabilize. Non-specifically bound and unbound metal was removed by passing the gallium transferrin solution through a G-25 sephadex column (Pharmacia Biotech) using a procedure identical to the one previously described.

Visible spectroscopy

To perform kinetic studies of iron removal from human diferric transferrin, a solution of 100 μM ferritransferrin in 50 mM Tris (pH 7.4), 150 mM NaCl, 20 mM $NaHCO_3$ buffer was pre-incubated at 37 °C to equilibrate the solution temperature. Iron chelators were added to the solution at a final concentration of 0.1–3 mM in a final volume of 100 μL. This represents a range of ligand:transferrin ratios from 1:1 to 30:1. The spectra were monitored immediately after the addition of the ligands, at 466 nm every 20 s for 30–60 min using the UV-Vis Chemstation software from Hewlett-Packard. Experiments were repeated three times at each ligand concentration. These measurements were performed in 1 cm quartz cuvettes maintained at constant temperature (37 °C) using a Hewlett-Packard spectrophotometer equipped with a thermostated cell.

Determination of A_∞ values

The A_∞ values were determined experimentally for each ligand and correspond to the absorbance recorded when it reached a plateau. Kinetics were performed under the same conditions as described above, but were monitored for several hours until a plateau occurred. The maximum stable absorbance recorded was designated as A_∞. Mineral oil was layered on top of the solution in the cuvettes at the beginning of each reaction to prevent evaporation of the samples.

Kinetic data analysis

Pseudo-first-order rate constants (k_{obs}) were calculated as described previously [33, 43] from linear fits of Eq. 1:

$$k_{obs} = \frac{\ln[(A_t - A_\infty)/(A_{t0} - A_\infty)]}{\text{time}} \quad (1)$$

where A_t, $A_{t0,}$ and A_∞ are the absorbances at any time, initial time at which ligands were added to the transferrin solution, and the infinite time measured experimentally (see previous paragraph), respectively. The average of the three experiments was calculated and designated as the observed rate constant (k_{obs}). Plots of k_{obs} versus ligand concentration were obtained for each ligand. A hyperbolic relationship between k_{obs} and ligand concentrations was observed, indicating saturation behavior with respect to the concentration of ligand. Under pseudo-first-order conditions, the saturation kinetics are governed by Eq. 2 [37]:

$$k_{obs} = \frac{k_2[L]}{1 + (k_2/k_{max})[L]} \quad (2)$$

where [L] is the ligand concentration. The data set for each ligand was fit to Eq. 2 using a nonlinear least-squares program, and values of k_2 and k_{max} were calculated. As the rate reaches a plateau as a function of ligand saturation, k_{obs} is equal to k_{max}. When the ligand concentration is very small, the rate is first order in ligand concentration and k_{obs} is equal to k_2. The second-order rate constant (k_2) allows direct comparison between the efficacy of various ligands to remove iron from diferric transferrin.

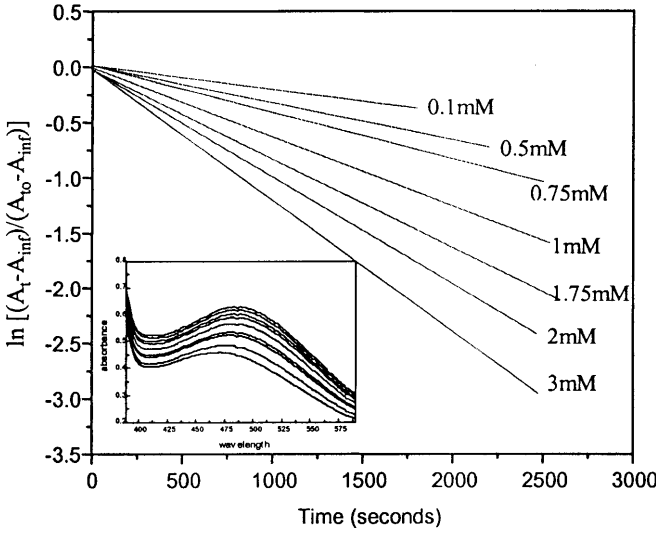


Fig. 2 A plot of $\ln[(A_t-A_\infty)/(A_{t0}-A_\infty)]$ versus time for iron removal from 100 μM human diferric transferrin by various concentrations of 5LIO-3,2-HOPO at 37 °C in Tris buffer. *Insert*: absorbance spectra for the reaction of 0.1 mM diferric transferrin and 0.5 mM TREN-Me-3,2-HOPO under the same conditions

Results and discussion

Kinetics of iron removal from diferric transferrin by the HOPO ligands

The kinetics of iron removal from human diferric transferrin were studied for several HOPO ligands (Fig. 1): two monomeric HOPOs (deferiprone and BU-O-3,4-HOPO), one dimeric HOPO (5LIO-3,2-HOPO), three trimeric HOPOs (TREN-Me-3,2-HOPO, TREN-1,2,3-HOPO, and TRPN-3,2-HOPO), and one mixed HOPO-catecholate (TRENCAM-3,2-HOPO). The addition of each HOPO ligand to a solution of transferrin in Tris buffer resulted in a spectral change, as shown in the insert of Fig. 2. The band maximum at 466 nm, which corresponds to the diferric transferrin, shifts to 490 nm, which is characteristic of a HOPO ligand complexed with iron. This change clearly indicates that the ligands are removing the metal from the protein.

Kinetics were performed at constant temperature (37 °C) and monitored at 466 nm. Pseudo-first-order rate constants (k_{obs}) were calculated from nonlinear least-square fits of the absorbance versus time, as described in Materials and methods. Plots of $\ln[(A_t-A_\infty)/(A_{t0}-A_\infty)]$ versus time are shown in Fig. 2 and represent linear behavior. The slope was then calculated for each of these plots and the average was designated as the observed rate constant (k_{obs}). It is clear that all the HOPO ligands successfully remove iron from diferric transferrin. The k_{max} of all the ligands tested are also reported in Table 1. Among the HOPO chelators, the highest maximum rate constants are obtained with the tetradentate 5LIO-3,2-HOPO (8.34×10^{-2} min^{-1}) as well as the bidentate ligands deferiprone (8.12×10^{-2} min^{-1}) and BU-O-3,4-HOPO (7.05×10^{-2} min^{-1}), while the lowest maximum rate is obtained by the hexadentate HOPO-catecholate TRENCAM-3,2-HOPO (2.66×10^{-2} min^{-1}).

The pseudo-first-order rate constants, k_{obs}, were also plotted against the ligand concentrations, as shown in Fig. 3. Most of these ligands rapidly reach saturation when at a concentration of 2–3 mM. Some monomeric hydroxypyridonate ligands, including deferiprone, have been previously reported to be kinetically effective at removing iron from transferrin [52, 53]. Our experimental results show that deferiprone, at high concentration, is the fastest of all the ligands tested in this study (Fig. 3). Interestingly, the tetradentate 5LIO-3,2-HOPO demonstrates comparable rates, which could reveal it as a good potential iron chelator. However, at the lowest ligand concentration tested (between 0.1 and 0.5 mM), which is closest to an expected concentration range in patient plasma, TREN-Me-3,2-HOPO and TRENCAM-3,2-HOPO show the fastest iron removal.

The second order rate constants (k_2) are listed in Table 1 and represent the ability of the ligands to remove iron from ferric transferrin at very low ligand

Table 1 Comparison of ligand ability to remove iron from transferrin

Competing ligand	k_{max} (10^{-2} min^{-1})	k_2 (M^{-1} min^{-1})	pM[b]	Ref.
Enterobactin	2.1	8.1	35.5	[64]
TRENCAM	1.5	10	27.8	[65]
Desferrioxamine B	~0	~0	26.6	[66]
TRENCAM-3,2-HOPO	2.7	23	–	–
TRPN-3,2-HOPO	3.1	5.9	24.2	–[c]
TREN-1,2,3-HOPO	3.7	6.3	–	–
TREN-Me-3,2-HOPO	3.2	16	26.7	[44]
BU-O-3,4-HOPO	7.0	4.5	20.8	–[d]
5LIO-3,2-HOPO	8.3	7.2	–	–
Deferiprone	8.1	7.8	21.1	[56]

[a]Reported in all cases for 0.1 mM of transferrin

[b]As described in the text, $k_{obs} = \frac{k_2[L]}{1+(k_2/k_{max})[L]}$ for pM values only

[c]Xu J, O'Sullivan B, Raymond KN (1999) manuscript in preparation

[d]O'Sullivan B (1999) unpublished data

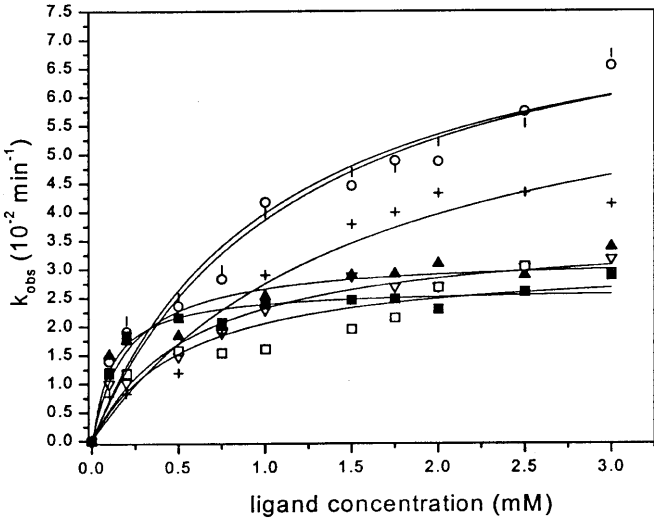


Fig. 3 Observed rate constants as a function of increasing concentration of deferiprone (|), 5LIO-3,2-HOPO (○), BU-O-3,4-HOPO (+), TREN-Me-3,2-HOPO (▲), TREN-1,2,3-HOPO (▽), TRPN-3,2-HOPO (□), and TRENCAM-3,2-HOPO (■). Kinetic studies were performed with 100 μM of human diferric transferrin at 37 °C in Tris buffer in the presence of 1- to 30-fold excess ligand. Reactions were followed by visible spectroscopy at 466 nm and analyzed as described in Materials and methods

concentration. Again, TRENCAM-3,2-HOPO and TREN-Me-3,2-HOPO have the highest k_2 of all the HOPO ligands tested in this study, which suggests that they should be more efficient at low concentration.

The affinity constant, pM, is a mean of the concentration of unchelated hexaaqua-iron(III) in a solution containing 1 μM Fe^{3+} and 10 μM chelator at pH 7.4 [54]. The more effective a chelator is at complexing iron at physiological pH, the larger its pM. Transferrin has a pM of 23.6 [55], which implies that ligands with pM values lower than 23.6 should not be able to compete strongly with it for iron. The pM values for most of the ligands tested in this study are reported in Table 1. The bidentate ligands deferiprone and BU-O-3,4-HOPO are faster at removing iron from diferric transferrin at high ligand concentrations (Fig. 3), even though they have lower iron affinity constants (21.1 and 20.8, respectively) than the other hydroxypyridonates at a realistic therapeutic ligand concentration. It is clear that there is no direct correlation between the pM value and the effectiveness of iron removal for large doses of ligands. However, the second-order rate constants (k_2) shows that HOPO ligands with the highest k_2 values generally have the highest pM values. Consequently, this thermodynamic constant (pM) is a significant indicator of how efficiently (and perhaps rapidly) a ligand can remove iron from ferric transferrin at low concentration. By this measure, the hexadentate TREN-Me-3,2-HOPO is certainly the most promising of the ligands tested.

As a bidentate ligand, deferiprone forms a 3-to-1 ligand-to-metal complex at millimolar concentration and physiological pH, but forms a 2-to-1 complex at micromolar concentration [56]. In the case of a tetradentate ligand such as 5LIO-3,2-HOPO, 1-to-1 complexes will coexist with 3-to-2 complexes at low ligand concentration. Such incomplete coordination of the ferric ion can, following reduction, generate oxygen-based radicals through Fenton-type chemistry, thus damaging biological molecules and contributing to toxic effects [57–59]. Such toxicity has indeed been reported with deferiprone [46, 47]. By contrast, hexadentate ligands such as TREN-Me-3,2-HOPO and TRENCAM-3,2-HOPO chelate the iron completely and avoid free radical formation; thus these ligands may be less toxic than bi- or tetradentate ligands.

Animal tests with TREN-Me-3,2-HOPO have already confirmed the expectation that this multidentate hydroxypyridonate ligand should be more effective at low concentrations than its bidentate analogue, which is only effective at relatively high concentrations [60]. These studies, performed by Yokel et al. [60], demonstrated an appreciable iron decorporation efficiency by this multidentate hydroxypyridonate ligand, even greater than larger dosages of desferrioxamine B. In addition, preliminary studies in an iron-loaded mouse model indicate that 5LIO-3,2-HOPO and DFO have similar effectiveness for alleviating iron overload [Clarke K, Watson G, Raymond K (1999) unpublished data].

Comparison of iron mobilization from diferric transferrin between catecholate, hydroxamate, and hydroxypyridonate chelators

In order to accurately compare kinetics between catecholate, hydroxamate, and hydroxypyridonate ligands under the same conditions, the same experiments were performed with the natural siderophore enterobactin and the synthetic catecholate TRENCAM, as well as the hydroxamate desferrioxamine B, the actual drug of choice for iron overload treatments. Enterobactin has previously been reported to be kinetically and thermodynamically capable of iron removal from transferrin [33]. As illustrated in Fig. 4, both TRENCAM and enterobactin show similar iron removal rates. At concentrations ranging from 0.5 to 3 mM, all the HOPO ligands are faster than both catecholates at removing iron from 0.1 mM diferric transferrin (Fig. 4). At ligand saturation, enterobactin has a k_{max} of 2.07×10^{-2} min^{-1} and the k_{max} of TRENCAM is 1.48×10^{-2} min^{-1}, while the HOPO ligands have k_{max} ranging from 2.66 to 8.34×10^{-2} min^{-1} (Table 1). The HOPO ligands are also faster at iron removal from transferrin than desferrioxamine B. The fact that desferrioxamine B does not remove iron from transferrin (Fig. 4), even after several hours under the conditions tested, is not surprising, since it has been reported to have very slow iron removal kinetics [40, 41, 61]. In order to be kinetically efficient at iron removal from

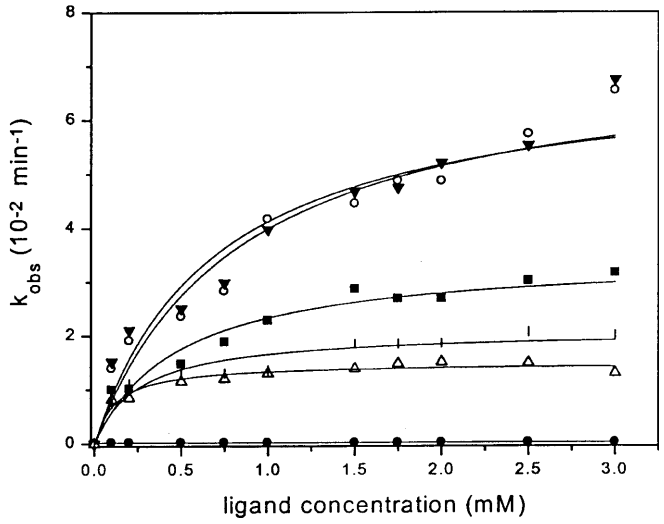


Fig. 4 Observed rate constants as a function of increasing concentration of deferiprone (▾), 5LIO-3,2-HOPO (○), TREN-1,2,3-HOPO (■), enterobactin (|), TRENCAM (△), and desferrioxamine B (●)

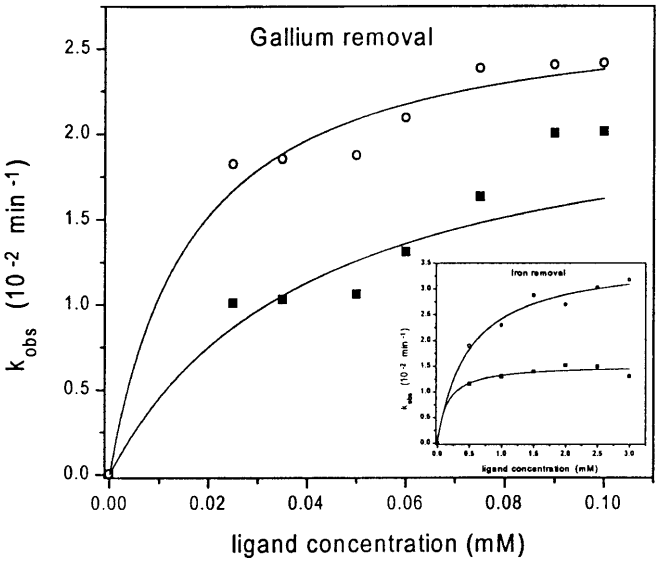


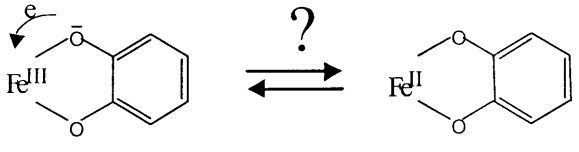
Fig. 5 Observed rate constants as a function of ligand concentration, showing the comparison between iron (*insert*) and gallium removal from human transferrin by TREN-Me-3,2-HOPO (○) and TRENCAM (●)

transferrin, the use of a 100-fold excess of desferrioxamine B or addition of pyrophosphate is required [42].

These results demonstrate that, at high concentrations, the hydroxypyridonate-based ligands are generally faster at removing iron from human diferric transferrin than iron chelating agents based on other functional groups such as catecholates and hydroxamates. At low concentrations the same relationship between k_2 and the pM values as seen for the HOPO ligands can also be established for the catecholates. Therefore, TRENCAM and enterobactin are probably efficient at low concentration because they are thermodynamically powerful iron sequestering agents. However, they are still less efficient than the hexadentate hydroxypyridonates TREN-Me-3,2-HOPO and TRENCAM-3,2-HOPO.

Mechanism of iron removal from transferrin

What causes the enhanced rate of iron removal by the HOPO and catecholate ligands relative to hydroxamates? Their intense color is due to ligand-to-metal charge transfer; thus one possibility to consider is that they are able to momentarily reduce Fe^{3+} to Fe^{2+} (Scheme 1).



Scheme 1

This question has been explored by examining the kinetics of gallium removal from transferrin, a system in which metal ion reduction cannot occur. Kinetics of gallium removal from human transferrin were performed with TRENCAM and TREN-3,2-HOPO using the same methods as for the iron-loaded transferrin. If a reduction step were necessary in the iron removal mechanism, the plots of k_{obs} as a function of ligand concentration for gallium removal would be significantly slower than those for iron removal.

The same behavior was observed for both ligands for both Fe^{3+} and Ga^{3+} removal. As shown in Fig. 5, they reached approximately the same rate and saturation for both experiments, except that gallium removal occurred 10 times faster than iron removal for the same concentration of ligands used. Indeed, only 0.1 mM of TREN-Me-3,2-HOPO was required for a k_{obs} of approximately 2.5×10^{-2} min^{-1} with gallium while 1 mM of the same ligand was necessary to reach the same rate with iron. This is due to the fact that gallium is more weakly complexed by the protein than iron, and thus 10 times less ligand is required to remove it at the same rate as iron [62]. These results clearly suggest that there is no reduction involved in this iron removal process, and that a reduction step does not contribute to the fast rate of iron removal by the HOPO ligands. The same behavior was previously observed for catecholate ligands [63].

Explanations of the faster rate of iron removal by the HOPO ligands compared to catecholate ligands include a greater ability of the HOPO ligands: (1) to reach the metal inside the transferrin lobes, (2) to displace the bicarbonate synergistic ion, which would

destabilize the metal coordination sphere in transferrin, (3) to break the hydrogen bonds which maintain the protein in a "closed" conformation, or (4) to induce a conformational change leading to the opening of the lobes. Although the reasons for the fast rate of iron removal from transferrin by HOPO ligands are not known, these kinetics studies, combined with the animal studies, show that these ligands have great promise as chelators for iron overload therapy.

Conclusions

In this study, kinetics of iron removal from human diferric transferrin by several hydroxypyridonate ligands have been performed at physiological pH and temperature. At concentrations close to therapeutic dosage, the hexadentate ligands are more efficient at removing iron than the bi- and tetradentates, even if the latter are faster at high concentrations. The hexadentate hydroxypyridonates TRENCAM-3,2-HOPO and TREN-Me-3,2-HOPO are more efficient at removing iron from ferric transferrin than all of the other ligands tested, including catecholate and hydroxamate ligands, which indicates their promise as therapeutic iron sequestering agents. Preliminary studies of TREN-Me-3,2-HOPO in a rat [60] model supports this hypothesis. In addition, ligands with a high effective iron affinity constant (pM) generally have a high second-order rate constant (k_2), which means that they have high affinity for iron and are able to remove iron quickly from transferrin at low concentrations. This correlation could be a useful indicator for the characterization of promising iron chelators in the future. Finally, the kinetics of gallium removal from transferrin show that no catalytic reduction step is involved in transferrin iron release by the HOPO ligands.

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