

# Chelator-facilitated removal of iron from transferrin: relevance to combined chelation therapy

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Current iron chelation therapy consists primarily of DFO (desferrioxamine), which has to be administered via intravenous infusion, together with deferiprone and deferasirox, which are orally-active chelators. These chelators, although effective at decreasing the iron load, are associated with a number of side effects. Grady suggested that the combined administration of a smaller bidentate chelator and a larger hexadentate chelator, such as DFO, would result in greater iron removal than either chelator alone [Grady, Bardoukas and Giardina (1998) *Blood* **92**, 16b]. This in turn could lead to a decrease in the chelator dose required. To test this hypothesis, the rate of iron transfer from a range of bidentate HPO (hydroxypyridin-4-one) chelators to DFO was monitored. Spectroscopic methods were utilized to monitor the decrease in the concentration of the Fe–HPO complex. Having established

that the shuttling of iron from the bidentate chelator to DFO does occur under clinically relevant concentrations of chelator, studies were undertaken to evaluate whether this mechanism of transfer would apply to iron removal from transferrin. Again, the simultaneous presence of both a bidentate chelator and DFO was found to enhance the rate of iron chelation from transferrin at clinically relevant chelator levels. Deferiprone was found to be particularly effective at 'shuttling' iron from transferrin to DFO, probably as a result of its small size and relative low affinity for iron compared with other analogous HPO chelators.

**Key words:** deferiprone; desferrioxamine (DFO); hydroxypyridin-4-one (HPO), iron chelation, scavenging activity, transferrin.

## INTRODUCTION

Although current chelation therapy is effective at improving the life expectancy of individuals with transfusion-dependent anaemias, its use causes numerous disadvantages. DFO (desferrioxamine) is not orally active and must be administered by subcutaneous infusion over 8 h periods up to five times per week. This property decreases compliance with the drug. Two orally active iron chelators have also been approved for clinical use: deferasirox [1] and deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one) [2]. A comparative study of the safety and efficacy of deferiprone and DFO in  $\beta$ -thalassaemia major patients demonstrated that both chelators have a similar ability to remove iron from the liver, but a greater increase in cardiac function was observed with deferiprone treatment [3]. Interest in the application of iron chelators in the treatment of non-iron overload diseases has also increased over the last 5 years, with considerable effort concentrated in two important fields, the treatment of cancer [4] and the treatment of neurodegeneration [5,6].

It has been suggested previously that the combined administration of a bidentate and a hexadentate chelator can lead to enhanced scavenging of iron from certain iron-containing proteins [7]. In principle, the smaller, kinetically labile bidentate chelator can rapidly access intracellular iron pools. The scavenged iron is subsequently transferred to the kinetically inert hexadentate chelator, forming a more stable complex, which is excreted. In this way, the bidentate chelator acts as a 'shuttle' molecule and the hexadentate chelator as a 'sink'. The concept of combined chelation therapy has been utilized previously to remove excess metals from the body in the cases of both plutonium and cad-

mium poisoning [8]. For instance, a combination of DTPA (diethylenetriaminepentacetic acid) and salicylic acid was utilized in the removal of plutonium. Combined administration of these two chelators had a greater effect than either DTPA or salicylic acid alone.

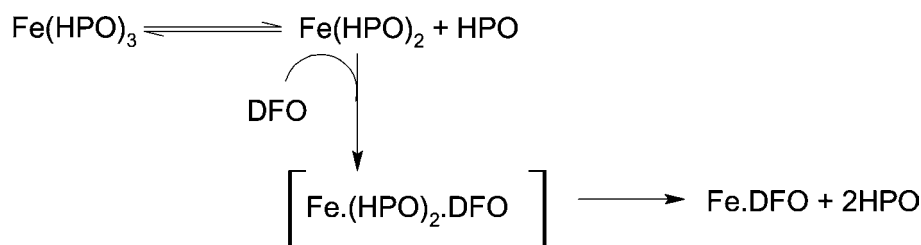
Enhanced scavenging, if it occurs, would be dependent on the transfer of iron from the bidentate to the hexadentate chelator (Scheme 1). Therefore it is of interest to investigate whether appreciable rates of transfer can occur at clinically relevant chelator concentrations.

It has been established previously that DFO cannot remove iron from transferrin at physiological concentrations, probably as a result of its relatively large size [9]. However, it is hypothesized that in the presence of a smaller bidentate chelator, iron could be transferred from transferrin to DFO. Experiments investigating the possible synergistic effects of combined administration of chelators in the removal of iron from transferrin have been carried out previously [10,11]. However, these studies were undertaken using high chelator concentrations. The observation that deferiprone is able to chelate transferrin-bound iron [9] indicates that it may also be able to access other iron pools that are inaccessible to DFO. Thus the combination of deferiprone with DFO may permit the chelation of iron, which would not normally be removed by DFO therapy alone. As deferiprone has been shown to be able to access and remove iron from cardiac tissue [3], the combination of deferiprone and DFO, for instance, might be expected to facilitate the removal of cardiac iron more efficiently.

This paper investigates the transfer of iron from bidentate chelators to DFO in an attempt to establish whether appreciable

Abbreviations used: DFO, desferrioxamine; DTPA, diethylenetriaminepentacetic acid; FO, ferrioxamine; HPO, hydroxypyridin-4-one; NTA, nitrilotriacetic acid.

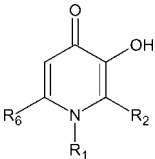
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**Scheme 1** The proposed mechanism of iron transfer from Fe–HPO to DFO

The fully co-ordinated  $\text{Fe(HPO)}_3$  complex exists in equilibrium with the partially co-ordinated  $\text{Fe(HPO)}_2$  complex in solution. DFO is able to form a ternary complex with  $\text{Fe(HPO)}_2$  and subsequently displace the remaining HPO molecules from iron.

**Table 1** Structure of hydroxypyridin-4-ones

Chelator				Reference
	R <sub>1</sub>	R <sub>2</sub>	R <sub>6</sub>	
Deferiprone	CH <sub>3</sub>	CH <sub>3</sub>	H	[12]
CP40	CH <sub>2</sub> CH <sub>2</sub> OH	CH <sub>3</sub>	H	[12]
CP95	CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	C <sub>2</sub> H <sub>5</sub>	H	[12]
CP357	CH <sub>3</sub>	CH <sub>2</sub> NHCOCH <sub>2</sub> CH <sub>2</sub> NHCOCH <sub>3</sub>	CH <sub>3</sub>	[13]
CP358	CH <sub>3</sub>	CH <sub>2</sub> NHCOCH <sub>2</sub> CH <sub>2</sub> CONHC <sub>3</sub> H <sub>7</sub>	CH <sub>3</sub>	[13]
CP363	CH <sub>3</sub>	CH(CH <sub>3</sub> )OCH <sub>3</sub>	CH <sub>3</sub>	[14]
CP375	CH <sub>3</sub>	CH(C <sub>2</sub> H <sub>5</sub> )OCH <sub>3</sub>	CH <sub>3</sub>	[15]
CP502	CH <sub>3</sub>	CONHCH <sub>3</sub>	CH <sub>3</sub>	[12]
CP511	H	CONHCH <sub>3</sub>	CH <sub>3</sub>	[16]

rates are achievable under clinical conditions, and whether this phenomenon will lead to an increase in the rates of iron removal from transferrin when compared with either chelator alone.

## EXPERIMENTAL

### Materials

Deferiprone was purchased from Sigma. Nine HPO (hydroxypyridin-4-one) chelators were used in this study and each was prepared as described previously [12–16]. These chelators fall into three main categories: the low-affinity chelators (deferiprone, CP95 and CP40:  $\text{pFe}^{3+} < 20$ ), chelators with a large ring substituent (CP357 and CP358) and the high-affinity chelators (CP363, CP375, CP502 and CP511:  $\text{pFe}^{3+} > 20$ ). The  $\text{pFe}^{3+}$  value is defined as  $-\log [\text{Fe}^{3+}]$  with  $1 \mu\text{M}$  total iron and  $10 \mu\text{M}$  total ligand at pH 7.4. The structures of these chelators are presented in Table 1. All chemicals required for urea gel electrophoresis were purchased from National Diagnostics (Atlanta, GA, U.S.A.). All other chemicals were purchased from Sigma.

### Purification of transferrin

Commercially available transferrin was not utilized for the iron removal studies since these preparations are frequently contaminated with other proteins and low molecular-mass molecules, which could interfere with the iron removal process. The presence of such low molecular-mass contaminants, such as citrate, could facilitate the transfer of iron from transferrin to DFO,

thereby resulting in artefacts. For these reasons, transferrin was purified from human serum. Solid ammonium sulfate was added to the serum sample (approx. 500 ml) to give a 45% (w/v) saturated solution. The amount of ammonium sulfate required was calculated as described previously [17]. The solution was left stirring overnight at 4°C and subsequently centrifuged at 8000 g for 30 min at 4°C. Further ammonium sulfate was added to the supernatant to increase the saturation to 70% (w/v), and the solution was left to equilibrate by stirring for 3 h at 4°C. The solution was then centrifuged at 16000 g for 1 h at 4°C, after which the supernatant was discarded and the pellet was redissolved in 50 mM sodium bicarbonate solution. The sample was then dialysed using 12 kDa molecular-mass cut-off tubing (Medicell International, London, U.K.) overnight against deionized water at 4°C to remove the ammonium sulfate. Fe–NTA (nitrilotriacetic acid) solution (0.1 M) was added to completely saturate the transferrin present in the sample. The volume of 0.1 M Fe–NTA required for saturation was calculated as stated in the following equation:

$$\text{Fe-NTA vol.} = \frac{(2 \times \text{transferrin mol.}) \times \text{transferrin vol.}}{\text{Fe-NTA mol.}} \quad (1)$$

where mol. is the molarity and vol. is the volume in  $\mu\text{l}$ . In all cases, Fe–NTA was prepared by the addition of the free acid to ferric chloride in a 1:1 ratio. This solution was freshly prepared at the time of use. Sodium bicarbonate was then added to the solution to a final concentration of 5 mM, together with Tris/HCl (pH 8.0), which was added to a final concentration of 10 mM.

The partially purified transferrin was applied to a DEAE–Sephacrose column (30 cm × 4 cm radius) equilibrated with 500 ml 20 mM Tris/HCl (pH 8.0). Both albumin and caeruloplasmin have a greater net negative charge than transferrin, and therefore displace it from the DEAE–Sephacrose column. As the solution was passed through the column, albumin and caeruloplasmin were found to bind more tightly and transferrin was collected in the eluate. Albumin and caeruloplasmin were displaced from the column by elution with 2 M NaCl. The column was re-equilibrated with 20 mM Tris/HCl (pH 8.0) and the process repeated twice until transferrin bound to the column without being displaced, indicating that the other proteins had been removed. Once bound to the column, transferrin was eluted using a salt gradient of 20 mM Tris/HCl and 0.2 M NaCl. Fractions were pooled and samples resolved by SDS/PAGE (8% gels) to confirm the purity of the purified transferrin. Selected fractions were then dialysed against deionized water overnight at 4 °C and subsequently freeze-dried.

### Preparation of diferric transferrin

Although an excess of Fe–NTA was added to the solution to completely saturate transferrin during the purification process, a mixture of all four forms of transferrin are usually present in the final product. In order to completely saturate the transferrin, the freeze-dried product was dissolved in 0.1 M sodium bicarbonate and 0.1 M Fe–NTA was added, with the volume required calculated using eqn (1). A Sephadex G-25 column (30 cm × 3 cm radius) was equilibrated with 25 mM ammonium bicarbonate and the transferrin solution was applied. This process removed excess Fe–NTA. The transferrin sample was again freeze-dried and stored at –20 °C.

### Transfer of iron from hydroxypyridinones to DFO

A 1 mM stock solution of HPO was prepared in water and treated with atomic absorption standard iron, to which 50 mM Mops buffer (pH 7.4) was subsequently added. The final total concentrations of iron and HPO in the solution were 10 μM and 30 μM respectively. The HPOs are bidentate chelators and therefore a 3-fold molar excess of HPO over iron is required to fully complex the iron in solution. When additional citrate (300 μM) or HPO (100 μM) were needed, the required quantity of either citric acid or HPO was subsequently added to the preformed iron(III) complex.

A solution of DFO was prepared in water and added to the Fe/HPO solution, to give a final concentration of 10 μM DFO. The transfer of iron was monitored by spectroscopy at room temperature (20 °C). The Fe–DFO complex has an absorbance maximum at 430 nm and the Fe–HPO complexes have maxima in the region of 450 nm–460 nm, therefore the transfer of iron from one chelator to the other can be monitored by UV–visible spectroscopy. In most cases, the absorbance was monitored at a wavelength of 455 nm. The rates of iron transfer were calculated using the method of Rose [18].

### Iron removal from transferrin: urea polyacrylamide gels

Diferric transferrin (32.5 μM) was prepared in 50 mM Mops buffer (pH 7.4). A combination of bidentate chelators, followed by DFO, were added and the sample was incubated for 2 h at 37 °C. The sample was then resolved on a urea polyacrylamide gel. Urea gels (6 M) were prepared as described previously [19]. Transferrin samples were dissolved in sample buffer [0.01%

(w/v) bromophenol blue and 10% (w/v) glycerol in 0.089 M Tris base, 0.089 M boric acid and 2 mM EDTA (pH 8.3)] prior to loading on to the gel. The gel was run at 80 V overnight. The bands were visualized by the addition of Coomassie Blue stain [0.25% (w/v) Coomassie Blue, 9% (v/v) acetic acid and 45.5% (v/v) methanol]. After de-staining [10% (v/v) acetic acid and 10% (v/v) methanol], the relative percentages of each of the forms of transferrin were calculated using densitometry scanning.

When iron removal from transferrin was investigated in the presence of serum, the sample buffer was replaced with Rivanol solution [0.375% (w/v) Rivanol and 10% (w/v) glycerol in 0.089 M Tris base, 0.089 M boric acid and 2 mM EDTA (pH 8.3)]. Rivanol is a dye which precipitates out γ-globulins, leaving transferrin in the supernatant [19]. A 25 μl aliquot was removed from the serum mixture, and 200 μl of Rivanol solution added. This mixture was centrifuged at 2000 g for 3 min, and 20 μl of the supernatant was loaded on to the gel. The gel was then run at 80 V overnight. Normal serum samples were iron-loaded with Fe–NTA. The amount of Fe–NTA added was calculated so that the transferrin would not be completely saturated, thereby decreasing the likelihood of the presence of NTBI (non-transferrin-bound iron). The saturation of transferrin in these experiments was approx. 80%, as determined by densitometry scanning of urea gels.

The percentage iron saturation of transferrin was calculated by determining the percentage of each of the four forms of transferrin using densitometry scanning of urea gels and calculated using the following equation:

$$\text{Iron sat.} = \text{diferric Tf} + \left( \frac{\text{N-Tf} + \text{C-Tf}}{2} \right) \quad (2)$$

where Tf is transferrin, C-Tf is C-monoferric transferrin, N-Tf is N-monoferric transferrin and sat. is saturation.

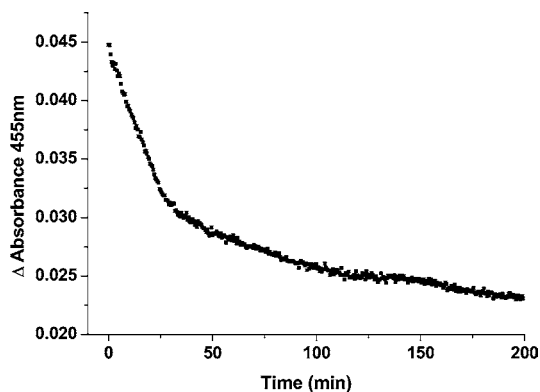
### Iron removal from transferrin: FO (ferrioxamine) assay

Diferric transferrin (32.5 μM) in 20 mM Mops buffer (pH 7.0) was incubated with a combination of bidentate chelators and DFO at various concentrations at 37 °C. Aliquots were removed at regular time intervals and ultrafiltered through 30 kDa filters (Whatman) by centrifugation at 15 000 g for 5 min to remove the protein. The filtrate (50 μl) was then injected on to the HPLC column for analysis.

A metal-free HPLC system (Waters Bio-system 625 with 911 Photodiode Array detector) with PEEK (polyether-ethyl ketone) tubing was used in order to prevent iron contamination of the samples. A Chrompak ODS (octadecylsilane) glass column was used, and the flow rate was set at 0.4 ml/min. The temperature of the injector was maintained at 4 °C during the analysis to minimize any changes in the rate of reaction while the samples were in the autosampler. The detection wavelength was set at 430 nm to detect FO. The mobile phase consisted of 20 mM phosphate buffer, pH 7.0, with 6% (v/v) acetonitrile (Rathburn Chemicals, Walkersburn, Peebleshire, U.K.), yielding a retention time of 6 min for FO. All samples were prepared in 20 mM Mops buffer (pH 7.0). The concentration of FO in the samples was determined by use of a standard concentration curve of FO.

### Speciation plots

Speciation plots of the iron complexes of deferiprone and CP511 were generated using the computer program Hyperquad Simulation and Speciation (HySS) [20]. The parameters



**Figure 1** Rate profile of iron transfer from CP358 to DFO

Iron transfer from CP358 to DFO measured as a decrease in absorbance at 455 nm in 50 mM Mops buffer (pH 7.4) at room temperature.  $[Fe] = 10 \mu M$ ,  $[CP358] = 30 \mu M$  and  $[DFO] = 10 \mu M$ .

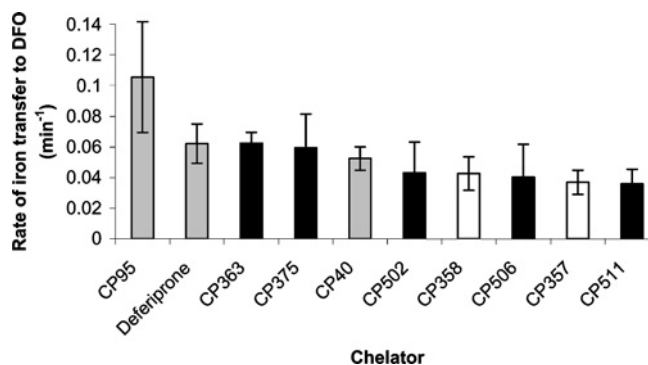
incorporated for speciation plot generation were  $\log K(FeOH) = 2.563$ ,  $\log K[Fe(OH)_2] = 6.205$ ,  $\log K[Fe(OH)_4] = 21.883$ ,  $\log K[Fe_2(OH)_2] = 2.843$  and  $\log K[Fe_3(OH)_4] = 6.054$  [21]. For deferiprone:  $\log K_1 = 15.1$ ,  $\log K_2 = 11.51$ ,  $\log K_3 = 9.27$  [22],  $pK_{a1} = 3.68$  and  $pK_{a2} = 9.77$  [23]. For CP511:  $\log K_1 = 14.5$ ,  $\log K_2 = 10.49$ ,  $\log K_3 = 7.47$ ,  $pK_{a1} = 2.32$  and  $pK_{a2} = 6.66$  [16].

## RESULTS

### Iron transfer between hydroxyppyridinones and DFO

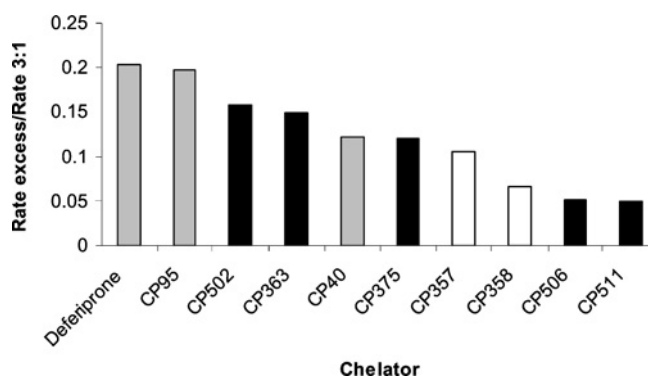
An absorption trace recording iron transfer from CP358 to DFO is shown in Figure 1. It can be seen that the transfer of iron from the bidentate chelator to DFO follows a biphasic pattern, with a relatively fast initial transfer followed by a slower phase. A similar pattern was observed for the rate of iron transfer from all the HPO chelators tested in this study. This observation can best be explained by a two-step iron transfer mechanism, where the initial fast phase corresponded to the formation of a ternary Fe/HPO/DFO mixed-ligand complex, followed by the slower formation of the Fe–DFO complex (Scheme 1). The initial rates of transfer of iron from various Fe–HPO complexes ( $10 \mu M$  Fe and  $30 \mu M$  HPO) to  $10 \mu M$  DFO are presented in Figure 2. It can be seen that there is an appreciable variation in the rate of iron transfer. CP95 was the most effective chelator at transferring iron under the experimental conditions used. The rate of iron transfer from deferiprone and CP40 to DFO was approx. 1.5-fold slower than that observed from CP95 to DFO. CP363 and CP375, which are higher-affinity chelators, had rates of iron transfer similar to that of deferiprone. The slowest rates of iron transfer to DFO were observed with CP357, CP358, CP506 and CP511. These iron transfer experiments were repeated in the presence of  $300 \mu M$  citrate, a physiological iron chelator present in plasma, and the rate of transfer of iron from the majority of the HPO chelators to DFO was reduced by approx. 1.5-fold. However, the converse was noted in the case of iron transfer from CP357 and CP358 to DFO, with the addition of citrate resulting in a 2-fold increase in the rate of iron transfer to DFO (results not shown).

Under *in vivo* conditions, a major proportion of the administered dose of a chelator would be present in the unchelated form. It was therefore decided to examine the rate of iron transfer from HPO to DFO in the presence of  $100 \mu M$  excess HPO chelator (Figure 3). The results are presented as the ratio of the rate of iron



**Figure 2** Rate of iron transfer from Fe–HPO to DFO

Initial rates of iron transfer from Fe–HPO ( $10 \mu M$  Fe and  $30 \mu M$  HPO) complexes to  $10 \mu M$  DFO in 50 mM Mops buffer (pH 7.4) at room temperature. Results are means  $\pm$  S.D. ( $n = 3$ ). The black bars represent higher-affinity chelators, the grey bars represent lower-affinity chelators and the white bars represent chelators possessing large ring substituents.

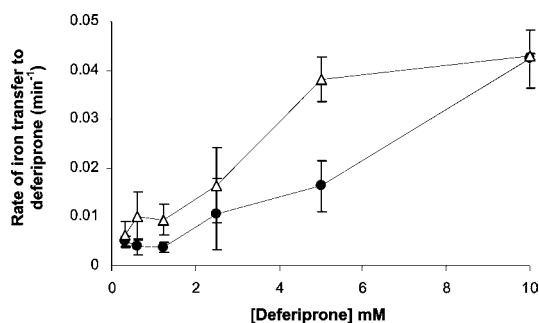


**Figure 3** Ratio of the rate of iron transfer from Fe–HPO in the presence of excess HPO to DFO compared with the rate of iron transfer from a 1:3 molar concentration of Fe:HPO to DFO

Rate of iron transfer from the Fe–HPO ( $10 \mu M$ : $30 \mu M$ ) complex in the presence of  $100 \mu M$  excess HPO to  $10 \mu M$  DFO in 50 mM Mops buffer (pH 7.4) at room temperature. Results are the ratio of the rate of iron transfer in the presence of excess HPO compared with the rate of transfer in the presence of a 1:3 molar ratio of Fe/HPO. The black bars represent higher-affinity chelators, the grey bars represent lower-affinity chelators and the white bars represent chelators possessing large ring substituents.

transfer to DFO in the presence of excess HPO chelator against the rate of iron transfer to DFO in the presence of a 1:3 ratio of iron to HPO (with no excess chelator). All of the rates were markedly reduced in the presence of excess chelator. Significantly, the rates of iron transfer from the lower-affinity chelators CP95 and deferiprone were the least affected by the presence of excess HPO. The presence of excess HPO had the greatest effect on the transfer of iron from the chelators CP357, CP358, CP506 and CP511.

Iron transfer from the Fe–DFO complex, FO to deferiprone was investigated at both pH 5.5 and pH 7.4 in order to verify whether FO could act as an efficient sink and prevent the redistribution of iron under both extracellular conditions and lysosomal conditions. At deferiprone concentrations less than  $300 \mu M$ , there was no detectable transfer of iron to deferiprone at either pH 5.5 or 7.4. As the concentration of deferiprone was increased above  $300 \mu M$ , transfer of iron occurred, with the transfer at pH 5.5 greater than transfer at pH 7.4 (Figure 4). However, these latter



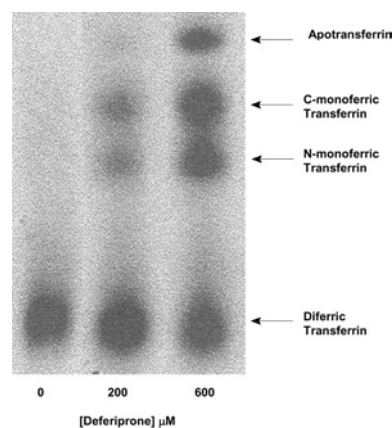
**Figure 4** Rate of iron transfer from DFO to deferiprone

Iron transfer from the FO (10  $\mu\text{M}$ ) complex to various concentrations of deferiprone at pH 5.5 [20 mM acetate buffer (20 mM sodium acetate/acetic acid)] ( $\Delta$ ) and pH 7.4 (20 mM Mops buffer) ( $\bullet$ ). Incubations were performed at room temperature. Results are means  $\pm$  S.D. ( $n = 3$ ).

deferiprone concentrations would not be observed under clinical conditions.

### Iron exchange between transferrin and chelating agents

Having established that the transfer of iron from the bidentate chelators to DFO occurred under clinically relevant chelator concentrations, it was of interest to examine whether this transfer of iron would result in enhanced scavenging of iron from transferrin. This was examined by urea PAGE, and a representative gel is shown in Figure 5. Lane 1 shows a single band corresponding to diferric transferrin. Lanes 2 and 3 show the presence of 4 bands, namely apotransferrin, C-monoferric transferrin, N-monoferric transferrin and diferric transferrin, following a 2 h incubation of diferric transferrin with 200  $\mu\text{M}$  and 600  $\mu\text{M}$  deferiprone respectively. The iron saturation of transferrin decreased with increasing chelator concentration. A combination of bidentate chelators and DFO were incubated with transferrin and iron removal was monitored using urea PAGE. Three chelators were chosen for this study: deferiprone, CP358 and CP502; both CP358 and CP502 are analogues of deferiprone (Table 1). CP358 is a larger chelator, which has a bulky side-chain and is therefore not expected to be able to efficiently chelate iron from the iron-binding sites of transferrin. CP502 is a small, higher-affinity iron chelator, which should be more effective at iron removal from transferrin in comparison with deferiprone. No iron removal was observed in the presence of 100  $\mu\text{M}$  DFO alone. Transferrin was incubated with a range of deferiprone concentrations in the presence or absence of 100  $\mu\text{M}$  DFO (results not shown). At deferiprone concentrations greater than 1 mM, a large percentage of the iron was removed from transferrin (approx. 60%). At this concentration of deferiprone, further addition of 100  $\mu\text{M}$  DFO resulted in enhanced iron removal, with approx. 75% of the iron removed from transferrin. As the concentration of deferiprone was increased up to 5 mM, almost all the iron was removed from transferrin, but further addition of DFO had virtually no effect. In humans, following administration of a 25 mg/kg dose of deferiprone, the maximal concentration of the drug in the plasma is approx. 100  $\mu\text{M}$  [24]. The steady-state concentration of DFO during a 40 mg/kg intravenous infusion is 10  $\mu\text{M}$  [25]. Iron removal from transferrin was therefore investigated in the presence of a range of HPO chelators (100  $\mu\text{M}$ ) in the presence or absence of 10  $\mu\text{M}$  DFO (Table 2). In all cases, an increase in iron removal from transferrin was observed when DFO was combined with HPO compared with HPO alone; however, statistical significance was not reached. When the concentration of HPO was further reduced to 50  $\mu\text{M}$  and the DFO concentration



**Figure 5** Urea gel showing the four forms of transferrin

A urea gel showing the effect of high deferiprone concentrations on the saturation of transferrin. Lane 1 depicts diferric transferrin. Lane 2 depicts apotransferrin, C-monoferric transferrin, N-monoferric transferrin and diferric transferrin, obtained following the incubation of diferric transferrin with 200  $\mu\text{M}$  deferiprone for 2 h at room temperature. Lane 3 depicts apotransferrin, C-monoferric transferrin, N-monoferric transferrin and diferric transferrin, obtained following the incubation of diferric transferrin with 600  $\mu\text{M}$  deferiprone for 2 h at room temperature.

**Table 2** Percentage iron removal from transferrin in the presence of 50  $\mu\text{M}$  HPO  $\pm$  10  $\mu\text{M}$  DFO and 100  $\mu\text{M}$  HPO  $\pm$  10  $\mu\text{M}$  DFO

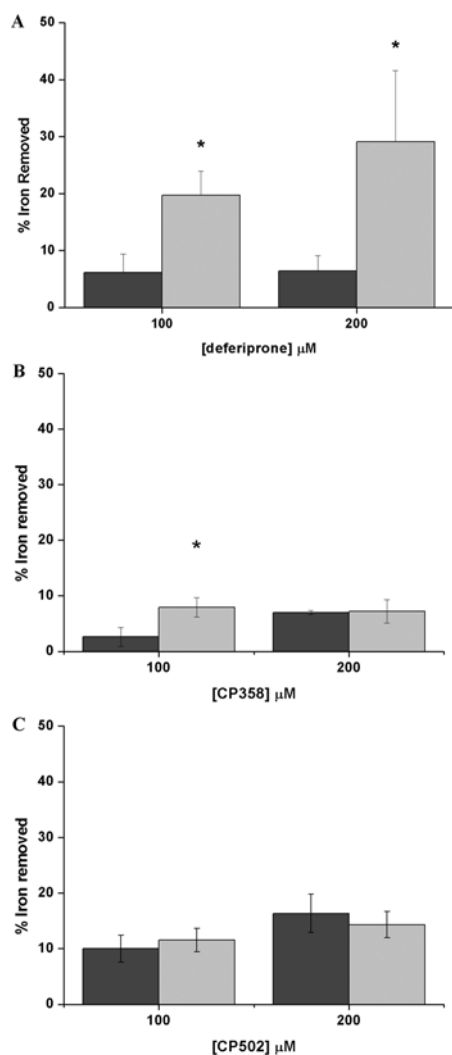
Diferric transferrin (32.5  $\mu\text{M}$ ) was incubated with 50 or 100  $\mu\text{M}$  HPO in the presence or absence of 10  $\mu\text{M}$  DFO for 2 h at 37  $^{\circ}\text{C}$  in 50 mM Mops buffer (pH 7.4). Results are means  $\pm$  S.D. ( $n = 3$ ). No statistical significance was observed between the values for iron removal from HPO alone compared with HPO and DFO using the Student's *t* test. The percentage of iron removed was calculated using densitometry scanning.

HPO	Iron removal from transferrin (%)			
	50 $\mu\text{M}$ HPO	50 $\mu\text{M}$ HPO + 10 $\mu\text{M}$ DFO	100 $\mu\text{M}$ HPO	100 $\mu\text{M}$ HPO + 10 $\mu\text{M}$ DFO
Deferiprone	4.7 $\pm$ 2.6	6.0 $\pm$ 2.1	5.8 $\pm$ 1.9	13.9 $\pm$ 5.2
CP502	5.5 $\pm$ 1.5	5.7 $\pm$ 1.1	13.5 $\pm$ 5.2	17.5 $\pm$ 8.0
CP358	2.8 $\pm$ 1.7	4.8 $\pm$ 1.4	8.3 $\pm$ 6.2	14.0 $\pm$ 2.2

maintained at 10  $\mu\text{M}$  (Table 2), weakly enhanced scavenging of iron was seen when DFO was combined with deferiprone and CP358, but not with CP502.

The rate of iron removal from 80% saturated transferrin in serum was also investigated. Combinations of HPO and DFO were incubated with serum samples (Figure 6), and the amount of iron removed by 100  $\mu\text{M}$  deferiprone alone in the presence of serum was found to be comparable with that removed from purified transferrin (Table 2). Addition of 10  $\mu\text{M}$  DFO did not result in an increase in the amount of iron removed from transferrin when compared with 100  $\mu\text{M}$  HPO alone. However, in the presence of equimolar concentrations of deferiprone and DFO, a significant increase in iron removal from serum transferrin was observed when compared with deferiprone alone. The addition of 200  $\mu\text{M}$  deferiprone did not result in any further increase in iron removal compared with 100  $\mu\text{M}$  deferiprone. The combination of CP502/DFO did not result in enhanced scavenging activity, even at high concentrations of chelator; however, the combination of 100  $\mu\text{M}$  CP358 and 100  $\mu\text{M}$  DFO did result in marginal enhancement of iron removal (Figure 6).

Iron removal from iron-saturated transferrin was also monitored using the FO assay (Figure 7). The addition of 10  $\mu\text{M}$  DFO



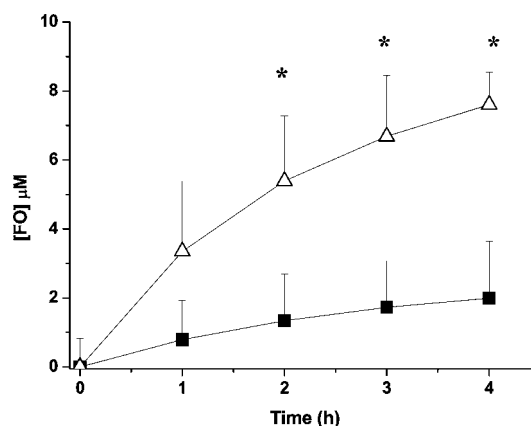
**Figure 6** Iron removal from serum transferrin

Serum samples were incubated with equimolar concentrations of HPO and DFO (either 100  $\mu\text{M}$  each or 200  $\mu\text{M}$  each) at 37 °C as follows: **(A)** deferiprone and DFO, **(B)** CP358 and DFO, and **(C)** CP502 and DFO. Dark grey bars denote HPO alone, light grey bars denote HPO in the presence of an equimolar concentration of DFO. Iron removal from transferrin was calculated using densitometry scanning. Results are means  $\pm$  S.D. ( $n=6$ ). Asterisk (\*) denotes a significant difference between HPO alone and the combination of HPO and DFO, determined using the Student's  $t$  test at the 5% significance level.

alone resulted in marginal (non-significant) iron removal from transferrin. The addition of 30  $\mu\text{M}$  deferiprone resulted in a large increase in the rate of iron removal. Addition of 100  $\mu\text{M}$  deferiprone to 10  $\mu\text{M}$  DFO resulted in slightly greater FO formation compared with that observed in the presence of 30  $\mu\text{M}$  deferiprone and 10  $\mu\text{M}$  DFO (results not shown). It is clear that the FO assay is more sensitive than the urea gel method for determining iron removal from transferrin at lower chelator concentrations.

## DISCUSSION

Although current chelation therapy, namely the use of either DFO or deferiprone, is effective at increasing the life expectancy of patients with transfusion-dependent anaemias, there are some disadvantages associated with their use. It has been hypothesized

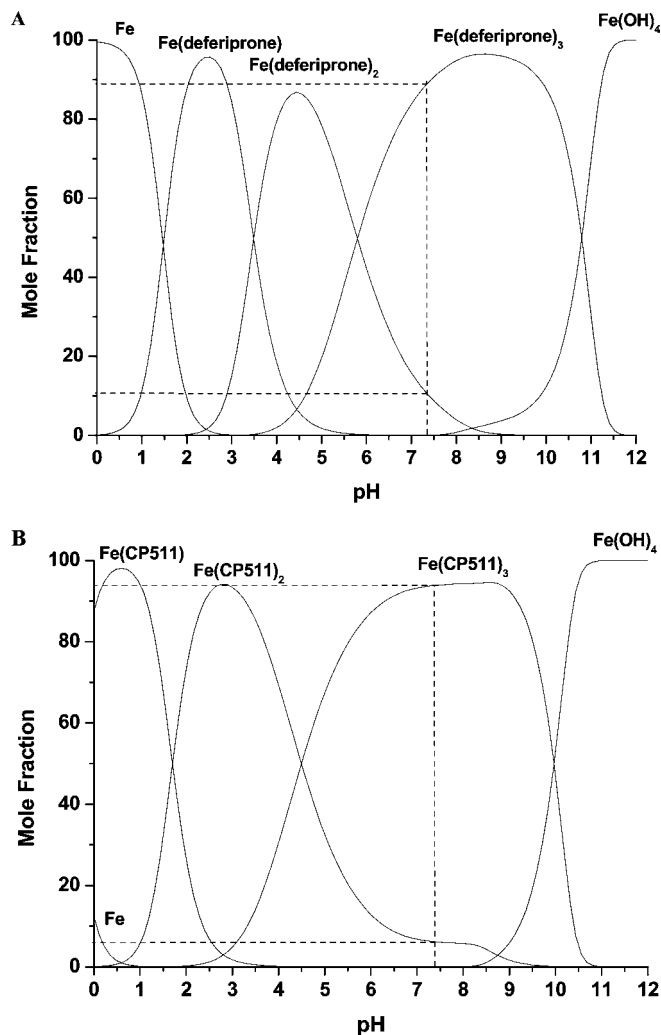


**Figure 7** Iron removal from transferrin measured by FO formation following incubation with deferiprone and DFO

Transferrin (32.5  $\mu\text{M}$ ) was incubated with 30  $\mu\text{M}$  deferiprone and 10  $\mu\text{M}$  DFO ( $\Delta$ ) and 10  $\mu\text{M}$  DFO alone ( $\blacksquare$ ). Samples were prepared in 20 mM Mops buffer (pH 7.0) and incubated at 37 °C. Results are means  $\pm$  S.D. ( $n=3$ ). Asterisk (\*) denotes a significant difference between DFO alone and the combination of deferiprone and DFO, determined using the Student's  $t$  test at the 5% significance level.

that the combined administration of these two chelators may increase the total amount of iron removed from the body, without an increase in chelator dose [7]. Studies have been carried out which indicate that combined chelation therapy may not only prove to be effective at decreasing body iron burden, but may also decrease the cost and increase compliance with these pharmaceuticals [26].

It has been suggested that enhanced scavenging activity arising from combined chelation therapy is based on the transfer of iron from the smaller bidentate chelator to the hexadentate chelator (Scheme 1) and is therefore dependent on the rate of iron transfer between the two chelators [7]. In this present study, the rate of iron transfer between a range of bidentate HPO chelators and DFO was determined using UV-visible spectroscopy (Figure 1). The lower-affinity chelators deferiprone and CP95 ( $p\text{Fe}^{3+}$  values of 19.4 and 19.9 respectively) were the most effective at iron transfer to DFO. The rates of iron transfer from CP357 and CP358 to DFO were approx. 2-fold lower than the rate of iron transfer from CP95. Both of these compounds possess long side-chains at the 2' position on the pyridinone ring. The presence of this side-chain could possibly interfere with the formation of the postulated DFO ternary complex (Scheme 1). The high-affinity chelators are the slowest iron donors to DFO, and this is probably the result of a decrease in the rate of dissociation of the  $\text{Fe}(\text{HPO})_3$  complex to the intermediary  $\text{Fe}(\text{HPO})_2$  complex [27], which is suggested to be necessary for DFO chelation (Scheme 1). From these results, it appears that there is a limited relationship between the  $p\text{Fe}^{3+}$  value and the rate of transfer of iron to DFO, with an increase in  $p\text{Fe}^{3+}$  resulting in a decrease in the rate of transfer. However, other structural factors must also be involved in controlling the rate of transfer of iron from the bidentate chelator to DFO, since both CP95 and CP40, which have similar  $p\text{Fe}^{3+}$  values to deferiprone, have much slower rates of transfer. In the presence of citrate, the rate of transfer of iron from HPO to DFO was decreased by approx. 1.5-fold in most cases (results not shown). The high concentration of citrate could possibly encourage transfer of iron to citrate. Once formed, dimeric and oligomeric iron-citrate species [28], which are not readily accessible to DFO, would be predicted to retard the rate of iron transfer to DFO.



**Figure 8** Speciation plots of iron–deferiprone and iron–CP511 complexes

Speciation plots showing all complexes of iron and HPO and the predominant iron hydroxide species. **(A)** Iron–deferiprone speciation plot, **(B)** iron–CP511 speciation plot.  $[\text{Fe}^{3+}]_{\text{total}} = 1 \times 10^{-5}$  M,  $[\text{HPO}] = 3 \times 10^{-5}$  M. Broken lines show amounts of  $\text{Fe}(\text{HPO})_2$  and  $\text{Fe}(\text{HPO})_3$  present at pH 7.4. The plots were generated using HySS [20].

The marked retardation in the rate of exchange in the presence of excess chelator (Figure 3) is almost certainly associated with a shift in equilibrium of the dissociation process (Scheme 1). In the presence of excess chelator, the equilibrium will adjust, favouring the formation of the  $\text{Fe}(\text{HPO})_3$  complex, which will not donate iron to DFO, thereby reducing the rate of transfer. The lower-affinity chelators, such as deferiprone, CP40 and CP95, are not as greatly affected as the higher-affinity chelators and the bulky chelators. Thus even in the presence of excess chelator, the lower-affinity chelators are more likely to dissociate from iron than the higher-affinity chelators, and therefore the rate of iron transfer from these chelators is likely to be greater. The relative proportions of the partially co-ordinated  $\text{Fe}(\text{HPO})_2$  complex under any given condition can be calculated from speciation plots. Comparing the speciation plots obtained for deferiprone and CP511 at pH 7.4 provides an insight into the reasons for the variation in rates of transfer of iron from these two chelators to DFO (Figure 8). The presence of a higher proportion of the  $\text{Fe}(\text{deferiprone})_2$  complex compared with the  $\text{Fe}(\text{CP511})_2$  complex demonstrates that the higher  $\text{pFe}^{3+}$  of CP511 results in a decrease in the dissociation of

the  $\text{Fe}(\text{HPO})_3$  complex compared with deferiprone, and thereby decreases the rate of iron transfer to DFO. Even in the presence of a 3-fold molar excess of HPO compared with iron, a proportion of partially co-ordinated  $\text{Fe}(\text{HPO})_2$  will exist in solution, the exact concentration of which depends on the  $\text{pFe}^{3+}$  of the chelator.

To determine the efficacy of DFO as a sink molecule, the rate of transfer of iron from the Fe–DFO complex to deferiprone was investigated. The slow rates of transfer demonstrate that once iron is chelated by DFO, it is highly improbable that the iron could be mobilized under clinically achievable HPO concentrations. Thus although greater iron removal was observed at pH 5.5, the concentration of deferiprone required to mobilize iron was much higher than would be present under clinical conditions. Clearly DFO acts as an effective sink molecule and so is a good candidate for combined chelation therapy with HPOs, such as deferiprone.

After the demonstration that transfer of iron from bidentate chelators to DFO occurs under clinically relevant concentrations of chelator, the interaction of chelators with transferrin was investigated. Transferrin-bound iron is not readily accessible to DFO, and so it was of interest to determine whether this iron could be chelated in the presence of both a bidentate HPO and DFO. At high deferiprone concentrations, the combination of deferiprone and DFO resulted in marginally greater iron removal from transferrin compared with deferiprone alone.

Having demonstrated that enhanced scavenging in the presence of a combination of chelators is observed for iron removal from transferrin at high deferiprone concentrations, chelator concentrations were reduced to simulate conditions achievable *in vivo* (Table 2). In the absence of DFO, CP502 was found to be the most effective at removing iron from transferrin. CP502 has the highest affinity for iron out of the chelators tested and is also relatively small, thereby enabling access to the iron-binding sites on transferrin. CP358 would be restricted in its access to the iron-binding sites on transferrin, because of the larger size of the molecule. In the presence of DFO, there is also a variation in the enhancement in iron removal observed with each HPO chelator, with a greater increase observed with the combination of deferiprone and DFO (approx. 2.4-fold increase in iron removal compared with deferiprone alone) and lower increases observed with the combination of CP502/DFO and of CP358/DFO (approx. 1.3-fold and 1.7-fold increases in iron removal respectively when compared with either HPO chelator alone). The reason for this discrepancy in scavenging activity between the HPO chelators is probably the result of their relative abilities to transfer iron to DFO. The rate of iron transfer from CP502 and CP358 to DFO is slower when compared with deferiprone (Figure 2).

Although purified transferrin has been shown to be an effective model for iron removal studies, experiments were also carried out using iron-loaded serum samples (Figure 6). The total iron removed from transferrin in serum by 100  $\mu\text{M}$  HPO alone was comparable with that achieved from purified transferrin. The addition of equimolar concentrations of deferiprone and DFO resulted in appreciable enhancement of iron removal. In contrast, the combination of CP358/DFO and of CP502/DFO failed to demonstrate such an effect. The most probable reason for this marked difference in behaviour is that both CP358 and CP502 do not donate iron(III) to DFO as readily as deferiprone (Figures 2 and 3).

It was not possible to use urea gel analysis to monitor iron removal from transferrin in the presence of low concentrations of bidentate chelator (for example, 30  $\mu\text{M}$ ). Therefore an alternative procedure based on the quantification of FO was adopted to monitor iron removal from transferrin at lower concentrations of bidentate chelator. This method provided a time course for the removal of iron. Any increase in FO formation in the presence

of deferiprone must have occurred as a result of the shuttling of iron from transferrin to DFO by the bidentate chelator, as DFO is unable to directly remove iron from transferrin under these conditions. Iron removal using a combination of 30  $\mu\text{M}$  deferiprone and 10  $\mu\text{M}$  DFO resulted in faster iron removal compared with DFO alone (Figure 7). The complete conversion of DFO to FO takes approx. 5 h. A similar effect was observed with 100  $\mu\text{M}$  deferiprone. This observation confirms the findings reported previously which relate to the iron removal from transferrin by deferiprone [9].

The experiments described in this present study demonstrate that the combination of the bidentate chelator deferiprone and the hexadentate chelator DFO results in enhanced scavenging activity of iron from transferrin at clinically achievable levels of both chelators. In the presence of DFO, this mobilized iron is donated to the hexadentate ligand (Figures 2 and 3). Thus it is possible to predict that simultaneous administration of the two ligands will lead to more efficient removal of iron from iron-overloaded patients. Furthermore, it is suggested that the combination of a chelator, such as deferiprone, with a sink molecule, such as DFO, could further improve the efficacy of chelation of tissue iron by ligand exchange of iron. It should be noted, however, that enhanced scavenging activity of iron may also lead to increased toxicity owing to enhanced iron removal from essential iron-containing enzymes. HPO chelators have been shown to inhibit lipoxygenase activity *in vitro* [29], an effect which may be accentuated in the presence of a sink molecule, such as DFO. Inhibition of ribonucleotide reductase [30] and tyrosine and tryptophan hydroxylases [31] have also been shown to occur in the presence of HPO chelators. Limited information exists regarding the toxic effects of combined chelation therapy.

Combined chelation therapy with deferiprone and DFO has been demonstrated to enhance iron scavenging activity *in vivo* in a number of clinical cases and has been shown to be especially beneficial in decreasing cardiac iron burden [32]. This phenomenon has been attributed to the shuttling of iron from the bidentate chelator to the hexadentate chelator [7,33]. It has been established previously that deferiprone removes iron from transferrin. However, the present study is the first to conclusively demonstrate that in the presence of both deferiprone and DFO *in vitro*, the chelated iron is shuttled on to DFO to form the FO complex, which is not formed in the absence of deferiprone. This phenomenon demonstrates the possibility of shuttling iron from a bidentate chelator to a hexadentate chelator and the subsequent removal of iron from a source unavailable to DFO alone.

In summary, the results presented here demonstrate that in the presence of a bidentate and a hexadentate chelator, enhanced iron scavenging activity occurs at clinically relevant chelator concentrations and, among the HPO chelators, deferiprone would appear to be particularly well suited for such applications.

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## REFERENCES

- Cappellini, M. D., Cohen, A., Piga, A., Bejaoui, M., Perrotta, S., Agaoglu, L., Aydinok, Y., Kattamis, A., Kilinc, Y., Porter, J. et al. (2006) A phase 3 study of deferasirox (ICL670), a once-daily oral iron chelator, in patients with  $\beta$ -thalassaemia. *Blood* **107**, 3455–3462
- Hider, R. C., Liu, Z. D. and Piyamongkol, S. (2000) The design and properties of 3-hydroxypyridin-4-one iron chelators with high  $\text{pFe}^{3+}$  values. *Transfus. Sci.* **23**, 201–209
- Peng, C.-T., Chow, K.-C., Chen, J.-H., Chiang, Y.-P., Lin, T.-Y. and Tsai, C.-H. (2003) Safety monitoring of cardiac and hepatic systems in  $\beta$ -thalassaemia patients with chelating treatment in Taiwan. *Eur. J. Haematol.* **70**, 392–397
- Kalinowski, D. S. and Richardson, D. R. (2007) Future of toxicology-iron chelators and differing modes of action and toxicity: The changing face of iron chelation therapy. *Chem. Res. Toxicol.* **20**, 715–720
- Gaeta, A. and Hider, R. C. (2005) The crucial role of metal ions in neurodegeneration: the basis for a promising therapeutic strategy. *Br. J. Pharmacol.* **146**, 1041–1059
- Boddaert, N., Le Quan Sang, K. H., Rotig, A., Leroy-Willig, A., Gallet, S., Brunelle, F., Sidi, D., Thalabard, J.-C., Munnich, A. and Cabantchik, Z. I. (2007) Selective iron chelation in Friedreich ataxia: biological and clinical implications. *Blood* **110**, 401–408
- Grady, R. W., Bardoukas, V. A. and Giardina, P. J. (1998) Iron chelation: combined therapy could be a better approach. *Blood* **92** (suppl 1), 16b
- Schubert, J. and Derr, S. K. (1978) Mixed ligand therapy for plutonium and cadmium poisoning. *Nature* **275**, 311–313
- Evans, R. W., Sharma, M., Ogowang, W., Patel, K. J., Bartlett, A. N. and Kontoghiorghes, G. J. (1992) The effect of alpha-ketohydroxypyridine chelators on transferrin saturation *in vitro* and *in vivo*. *Drugs Today* **28** (suppl A), 19–23
- Pollack, S., Aisen, P., Lasky, F. D. and Vanderhoff, G. (1976) Chelate mediated transfer of iron from transferrin to desferrioxamine. *Br. J. Haematol.* **34**, 231–235
- Pollack, S., Vanderhoff, G. and Lasky, F. (1977) Iron removal from transferrin. An experimental study. *Biochim. Biophys. Acta* **497**, 481–487
- Dobbin, P. S., Hider, R. C., Hall, A. D., Taylor, P. D., Sarpong, P., Porter, J. B., Xiao, G. and van der Helm, D. (1993) Synthesis, physicochemical properties, and biological evaluation of N-substituted 2-alkyl-3-hydroxy-4(1H)-pyridinones: orally active iron chelators with clinical potential. *J. Med. Chem.* **36**, 2448–2458
- Liu, Z. D., Kayyali, R., Hider, R. C., Porter, J. B. and Theobald, A. E. (2002) Design, synthesis and evaluation of novel 2-substituted 3-hydroxypyridin-4-ones: Structure-activity investigation of metalloenzymes inhibition by iron chelators. *J. Med. Chem.* **45**, 631–639
- Liu, Z. D. and Hider, R. C. (2002) Design of clinically useful iron(III) selective chelators. *Med. Res. Rev.* **22**, 26–64
- Piyamongkol, S. (2001) The design and synthesis of 3-hydroxide-4-one chelators, Ph.D. Thesis, University of London, London, U.K.
- Piyamongkol S., Liu Z. D. and Hider, R. C. (2001) Novel synthetic approach to 2-(1'-hydroxyalkyl)- and 2-amido-3-hydroxypyridin-4-ones. *Tetrahedron* **57**, 3479–3486
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M. (1986) Data for Biochemical Research, Oxford University, Oxford.
- Rose, J. (1964) Advanced Physico-chemical Experiments: A Textbook of Practical Physical Chemistry and Calculations, pp. 153–157, Sir Isaac Pitman & Sons, London.
- Evans, R. W. and Williams, J. (1980) The electrophoresis of transferrins in urea/polyacrylamide gels. *Biochem. J.* **189**, 541–546
- Alderighi, L., Gans, P., Ienco, A., Peters, D., Sabatini, A. and Vacca, A. (1999) Hyperquad simulation and speciation (HySS): a utility program for the investigation of equilibria involving soluble and partially soluble species. *Coord. Chem. Rev.* **184**, 311–318
- Baer, C. F. and Mesmer, R. E. (1976) The Hydrolysis of Cations, John Wiley & Sons, New York.
- Motekaitis, R. J. and Martell, A. E. (1991) Stabilities of the iron(III) chelates of 1,2-dimethyl-3-hydroxy-4-pyridinone and related ligands. *Inorg. Chim. Acta.* **183**, 71–80
- Liu, Z. D., Khodr, H. H., Liu, D. Y., Lu, S. L. and Hider, R. C. (1999) Synthesis, physicochemical characterization, and biological evaluation of 2-(1'-hydroxyalkyl)-3-hydroxypyridin-4-ones: novel iron chelators with enhanced  $\text{pFe}^{3+}$  values. *J. Med. Chem.* **42**, 4814–4823
- Kontoghiorghes, G. J., Goddard, J. G., Bartlett, A. N. and Sheppard, L. (1990) Pharmacokinetic studies in humans with the oral iron chelator 1,2-dimethyl-3-hydroxypyridin-4-one. *Clin. Pharmacol. Ther.* **48**, 255–261
- Porter, J. B. (2001) Deferoxamine Pharmacokinetics. *Semin. Hematol.* **38**, (1 Suppl 1), 63–68
- Gomber, S., Saxena, R. and Madan, N. (2004) Comparative efficacy of desferrioxamine, deferiprone and in combination on iron chelation in thalassaemic children. *Indian Pediatr.* **41**, 21–27
- Devanur, L. D., Neubert, H. and Hider, R. C. (2007) The Fenton activity of iron(III) in the presence of deferiprone, *J. Pharm. Sci.*, doi:10.1002/jps.21039
- Evans, R. W., Rafique, R., Zarea, A., Rapisarda, C., Cammack, R., Evans, P. J., Porter, J. B. and Hider, R. C. (2007) Nature of non-transferrin-bound iron: studies on iron citrate complexes and thalassaemic sera, *J. Biol. Inorg. Chem.*, doi: 10.1007/s00775-007-0297-8
- Abeyasinghe, R. D., Roberts, P. J., Cooper, C. E., Maclean, K. H., Hider, R. C. and Porter, J. B. (1996) The environment of the lipoxygenase iron-binding site explored with novel hydroxypyridinone iron chelators. *J. Biol. Chem.* **271**, 7965–7972



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- 30 Cooper, C. E., Lynagh, G. R., Hoyes, K. P., Hider, R. C., Cammack, R. and Porter, J. B. (1996) The relationship of intracellular iron chelation to the inhibition and regeneration of human ribonucleotide reductase. *J. Biol. Chem.* **271**, 20291–20299
- 31 Waldmeier, P. C., Buchle, A.-M. and Steulet, A.-F. (1993) Inhibition of catechol-*o*-methyltransferase (COMT) as well as tyrosine and tryptophan hydroxylase by the orally active iron chelator, 1,2-dimethyl-3-hydroxypyridin-4-one (L1,CP20), in rat brain *in vivo*. *Biochem. Pharmacol.* **45**, 2417–2424
- 32 Fabio, G., Minonzio, F., Delbini, P., Bianchi, A. and Cappellini, M. D. (2007) Reversal of cardiac complications by deferiprone and deferoxamine combination therapy in a patient affected by a severe type of juvenile hemochromatosis (JH). *Blood* **109**, 362–364
- 33 Link, G., Konijn, A. M., Breuer, W., Cabantchik, Z. I. and Hersko, C. (2001) Exploring the “iron shuttle” hypothesis in chelation therapy: Effects of combined deferoxamine and deferiprone treatment in hypertransfused rats with labeled iron stores and in iron-loaded rat heart cells in culture. *J. Lab. Clin. Med.* **138**, 130–138

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