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Structure/Iron Binding Activity of 1-Hydroxypyrid-2-one Chelators Intended for Clinical Use

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Abstract

Several bidentate 1-hydroxypyrid-2-ones which resemble aromatic hydroxamates have been synthesised and tested for their iron binding properties at physiological pH. In contrast to the increase in the pK_a and iron binding properties observed with the 4-hydroxy and 4-methoxy 1-hydroxypyrid-2-one derivatives, the introduction of one or two nitro substituents in the heteroaromatic ring had the reverse effect. Coloured, water soluble, neutral, 3 chelator:1 iron complexes with iron binding constants comparable to those of transferrin were formed at pH 7.4 using the 4-hydroxy, 4-methoxy and 4-(2'-methoxyethoxy) derivatives. When these three chelators were incubated with transferrin and ferritin, large amounts of iron were released which were equivalent to those caused by 1,2-dimethyl-3-hydroxypyrid-4-one and higher than those caused by desferrioxamine. The methodology used for increasing the affinity of the chelators by introducing the right substituents in the heteroaromatic ring could help in the design of other chelators intended for clinical use. The high iron binding and other properties of some 1-hydroxypyrid-2-ones increase the prospect for their use in iron overload and other diseases of metal imbalance.

Introduction

Many chelators have been tested for replacing desferrioxamine in the treatment of iron overload because this linear hydroxamate which is produced by fungi is highly expensive and orally inactive [1]. Few chelators have been shown to be effective in iron removal *in vivo* such as the pyridoxal isonicotinoyl hydrazone [2], phenolic ethylene diamines [3], deferriferrithiocin [4], spermidine catecholates [5] and α-ketohydroxypyridines [6, 7]. In many cases the inactivity of chelators *in vivo* is related to the high affinity for other metals namely Mg^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+} (e.g. DTPA, EDTA), susceptibility towards oxidation (e.g. catechols) rapid elimination or biotransformation, toxicity and ability to donate iron to micro-organisms. Most synthetic chelators are designed to mimic the structure of microbial siderophores which have high affinity for iron [8], but other factors should also be considered e.g. desferrioxamine which is positively charged and hydrophilic at physiological pH cannot readily permeate membranes [9] and is orally inactive, but heteroaromatic neutral chelators e.g. the α-ketohydroxy pyridines have oral activity. Several heteroaromatic 1-hydroxypyrid-2-one naturally occuring chelators which resemble aromatic hydroxamates have been shown to possess antibiotic and metal binding properties e.g. the aspergillic acids [10] and 1-hydroxy-5-methoxy-6-methylpyrid-2-one [11, 12]. Similarly the synthetic parent compound 1-hydroxypyrid-2-one was also shown to have metal binding and antimicrobial activity [13].

In this work, several synthetic 1-hydroxypyrid-2-one derivatives have been designed and tested *in vitro* for their iron binding properties at physiological pH using a new structure activity correlation protocol and using other known chelators for comparison. The 1-hydroxypyrid-2-ones are known to exist as a mixture of tautomers, the 2-hydroxy-*N*-oxide being the other tautomeric form.

Experimental

Materials

Human transferrin, horse spleen ferritin and Tris were obtained from Sigma, desferrioxamine from Ciba-Geigy, Phosphate buffer saline (PBS) from Oxoid and $^{59}FeCl_3$ from Amersham U.K. The chelators 1-hydroxypyrid-2-one, 2,3-dihydroxypyridine, 1-hydroxypyridinethi-2-one, 3-hydroxypyridinethi-2-one, maltol and all other chemicals were obtained from Aldrich U.K.

Synthesis of Chelators

The following compounds were prepared as previously described; 1,2-dimethyl-3-hydroxypyrid-4-one [14]; 1-methyl-3-hydroxypyrid-2-one [15]; 5-nitro-

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1-hydroxypyrid-2-one [13], 3,5-dinitro-1-hydroxypyrid-2-one [16], 4-methoxy-1-hydroxypyrid-2-one [17]. 4-Nitro-1-hydroxypyrid-2-one was prepared from the acid hydrolysis [17] of 4-nitro-2-methoxy-1-hydroxypyridine [18]; 4-(2′-methoxyethoxy)-1-hydroxypyrid-2-one was prepared using a similar method to that of the preparation of 4-methoxy-1-hydroxypyridine-*N*-oxide [17], by stirring 4-nitro-2-chloro-1-hydroxypyridine (1.75 g) with sodium methoxyethoxide solution (Na 0.239 g, methoxyethanol 30 ml) for 28 h at 20 °C, removing the excess methoxyethanol by distillation, washing with diethyl ether (25 ml), dissolving in water (25 ml), extracting into chloroform, drying over anhydrous sodium sulphate and evaporating *in vacuo* giving a yellow solid on cooling, 2-chloro-4-(2′-methoxyethoxy)-1-hydroxypyridine. To the solid, NaOH (15 ml, 10%) was added, left in a steam bath for 3 h, acidified to pH 2 using conc. HCl, reduced in volume by evaporating *in vacuo*, and left to crystallise. Recrystallisation from ethanol gave a white solid (4-(2′-methoxyethoxy)-1-hydroxypyrid-2-one (^{1}H NMR, Table I)).

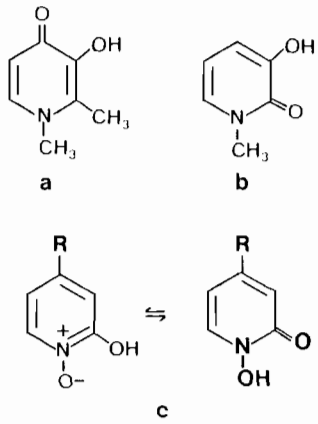
1,4-Dihydroxypyrid-2-one was prepared from the 2,4-dimethoxy-1-hydroxypyridine [19] by acid hydrolysis using HCl (20%, at 105 °C, for 13 h) giving an orange-white solid following reduction in volume by evaporation *in vacuo* and recrystallisation in water (^{1}H NMR, Table I). The use of milder conditions (HCl, 10%, in a steam bath, 5 h) was not effective and NMR tracing of the reaction indicated initially the removal of one $-CH_3$ group. It could be envisaged that 1,4-dihydroxypyrid-2-one could also be prepared from 4-methoxy-1-hydroxypyrid-2-one under the same conditions.

Methods

The reactions of the chelators with iron(III), protons and other metals were studied spectrophotometrically. All the solutions were freshly prepared and the pH adjustments were carried out using HCl (16 M) and NaOH (10 M). The pH titrations of the chelator iron complexes were carried out with the chelator in 3 or 4 fold molar excess in the presence of $NaClO_4$ (0.1 M) for maintaining constant ionic strength. The stoichiometry of the chelator iron complex (Job's plot [20]) and the conditional stability constant K' or K_{sol} [21, 22] were determined at an acidic and neutral pH using the method of continuous variation [23] and a computing method [24]. The log β_3 and log K_{eff} of some chelators were estimated from the conditional stability constants K' using the following formulae as previously described [14] $\log \beta_3 = \log K' - 21 + pK_{sp} + n\log \alpha_{L(H)}$; $\log K_{eff} = \log \beta_3 - \log \alpha_{Fe(OH)} - n\log \alpha_{L(H)}$, where n is the number of chelators binding iron(III), $K_{sp} = 10^{-39}$ is the solubility product of $Fe(OH)_3$, and where $\log \alpha_{Fe(OH)} = 8.4$ at pH 7.4 [22].

The pK_a determination of the chelators were carried out using HCl (16 M) and NaOH (10 M) in the presence of NaCl or $NaClO_4$ (0.1 M). The absorption spectra changes of the chelators in the UV region were recorded after each pH modification and plots of the change in absorbance at a selected wavelength against pH, gave a sigmoidal curve from which the pK_a was determined.

The protein studies were carried out as follows; ^{59}Fe transferrin (1.1×10^{-5} M, in Tris 0.1 M, pH 7.4) which was prepared as previously described [25] was mixed with the chelators (4×10^{-3} M, in Tris 0.1 M, pH 7.4) for 5 h at 26 °C, the reactive mixture transferred in a dialysis tubing and dialysed for 16 h in 2 litres buffer, and the ^{59}Fe activity remaining in the dialysis tubing compared to ^{59}Fe transferrin solutions with no chelators added (control). The reactions of the chelators with ferritin were studied spectrophotometrically as previously described [26], by estimating the amount of iron released in the ferritin ([iron] = 1.42×10^{-3} M) chelator (1×10^{-3} M) mixture at 26 °C in Tris, $NaClO_4$ (0.1 M, pH 7.4) (method A) or in the diffusate of ferritin ([iron] = 1.7×10^{-3} M) in a dialysis tubing, dialysed against a chelator solution (3.8×10^{-3} M) at 37 °C in PBS, pH 7.3 (method B) using the absorption coefficients of the chelator iron complexes ([ϵ M^{-1} cm^{-1}]) as follows: ferritin $\epsilon_{420} = 560$; 1,2-dimethyl-3-hydroxypyrid-4-one $\epsilon_{460} = 3600$; 1,4-dihydroxypyrid-2-one $\epsilon_{400} = 4050$; 1-hydroxypyrid-2-one $\epsilon_{400} = 2410$; 4-methoxy-1-hydroxypyrid-2-one $\epsilon_{420} = 3350$ and 4-(2′-methoxyethoxy)-1-hydroxypyrid-2-one $\epsilon_{450} = 2510$.



Scheme 1. The structure of the hydroxypyridone derivatives: (a) 1,2-dimethyl-3-hydroxypyrid-4-one; (b) 1-methyl-3-hydroxypyrid-2-one; (c) the tautomeric structures of 2-hydroxypyridine-*N*-oxide (left) and 1-hydroxypyrid-2-one (right) derivatives: R = H; 1-hydroxypyrid-2-one; R = OH, 1,4-dihydroxypyrid-2-one; R = $O-CH_3$, 4-methoxy-1-hydroxypyrid-2-one; R = $-O-CH_2-CH_2-O-CH_3$, 4-(2′-methoxyethoxy)-1-hydroxypyrid-2-one.

TABLE I. The ^{1}H NMR Spectra of the Synthetic 1-Hydroxypyrid-2-ones

Compound	Type of proton (ppm)				Solvent
1,4-Dihydroxypyrid-2-one	H(3) 6.08s	H(5) 6.12q	H(6) 7.88d		d-DMSO + D_2O (trace)
4-Methoxy-1-hydroxypyrid-2-one	H(3) 5.88s	H(5) 5.95q	H(6) 7.52d	CH_2 3.67s	D_2O
4-(2'-Methoxyethoxy)-1-hydroxypyrid-2-one	H(3) 6.05d	H(5) 6.05q	H(6) 7.62t	CH_3 3.42s	$CDCl_3$
	CH_2(2') 4.08t	CH_2(1') 3.7t			

TABLE II. The pK_a of the Chelators [a] and the Iron Binding Constants and Solubility of their Iron Complexes

Chelator	pK_a	Log β	Log K_{eff}	Log K_{sol}	Solubility at pH 7.0
1-Hydroxypyrid-2-ones					
4-Hydroxy-	6.7 (8.4)[b]	29.9	21.5	9.9	Soluble
4-Methoxy-	6.0	29.3	20.9	11.3	Soluble
4-(2'-methoxyethoxy)					Soluble
4-Hydrogen	5.9				Precipitation
4-Nitro	3.6				No complex
5-Nitro	3.7				No complex
3,5-Dinitro	2.7				No complex
1,2-Dimethyl-3-hydroxypyrid-4-one	9.7 (3.3)[b]	34.5	19.2	9.6	Soluble
1-Methyl-3-hydroxypyrid-2-one	8.8 (3.0)[b]	33.5	20.0	10.1	Soluble
Maltol	8.6	29.5	17.7	8.0	Soluble
Salicylic acid		36[c]	19.0	1.0	
Desferrioxamine		31[c]	24.0	6.0	Soluble
Transferrin		36[c]	24.0 20.0[d]	6.0	Soluble

[a]The chelators 2,3-dihydroxypyridine, 1-hydroxypyridinethi-2-one and 3-hydroxypyridinethi-2-one form precipitates with iron(III) at pH 7.0. These three chelators and all the others above form coloured, water soluble iron(III) complexes at pH 2 with a smaller stoichiometry of 3 chelator:1 iron. [b]pK_a of other ionisable groups. [c]Obtained from ref. 14. [d]According to P. Aisen *et al.* [28].

Results

The structure of the chelators and the ^{1}H NMR spectroscopic data of some of those prepared are shown in Scheme 1 and Table I respectively. The purity of the chelators as judged from their ^{1}H NMR spectra was greater than 96%. The pK_a of some of the chelators and the solubility of their iron complexes are shown in Table II. The pK_a of the hydroxyl group at the binding site of the 1-hydroxypyrid-2-ones seems to be effected by the presence of substituents on the pyridine ring. When compared to the parent compound 1-hydroxypyrid-2-one, a substantial decrease in the pK_a of the nitro derivatives in contrast to a small increase in the hydroxy and methoxy derivatives was observed. Although all the chelators formed soluble iron complexes at pH 2, only few formed soluble iron complexes at pH 7, while the nitro derivatives did not appear to bind iron and other chelators formed iron(III) precipitates at the same pH (Table II).

The pH titrations of some of the soluble chelator iron complexes are shown in Fig. 1. In the case of the 4-hydroxy, 4-methoxy and 4-(2'-methoxyethoxy) derivatives there is a plateau level in the absorbances of their iron complexes in the physiological pH

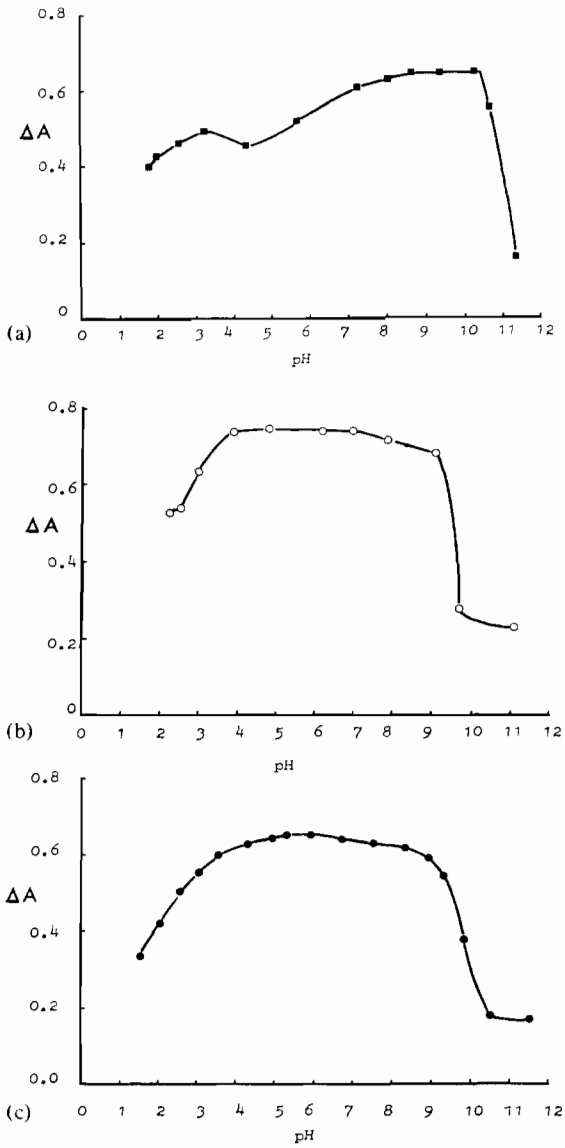


Fig. 1. The pH titration of the chelator iron complexes. The change in absorbance (ΔA) with pH in the presence of $NaClO_4$ (0.1 M) of (a) 1,4-dihydroxypyrid-2-one (7.5×10^{-4} M) iron (2.5×10^{-4} M), (b) 4-methoxy-1-hydroxypyrid-2-one (25×10^{-4} M) iron (7.5×10^{-4} M) and (c) 4-(2'-methoxyethoxy)-1-hydroxypyrid-2-one (8×10^{-4} M) iron (2×10^{-4} M) was studied at 400, 450 and 400 nm, respectively.

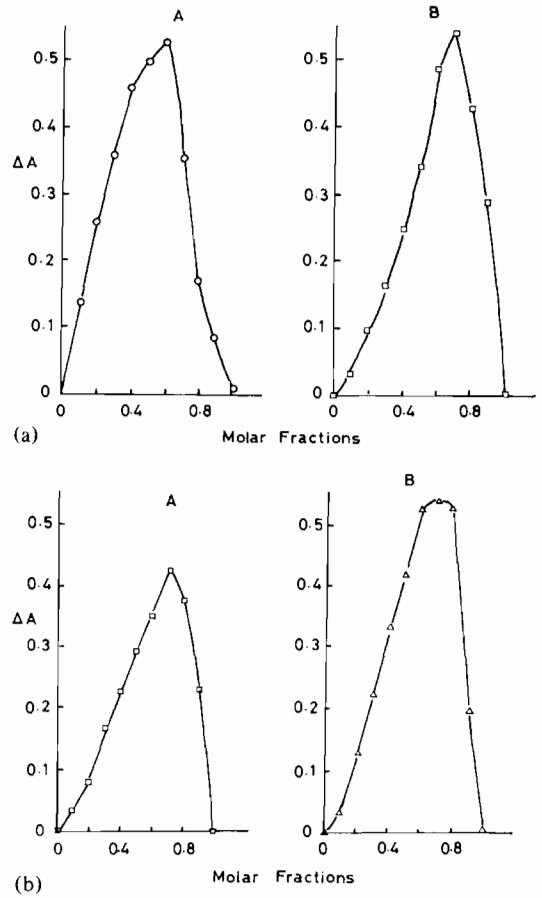


Fig. 2. Job's plots of 1-hydroxypyrid-2-ones. The Job's plots (change in absorbance (ΔA) *versus* the molar fraction of the chelator in the presence of 0.1 M $NaClO_4$) of (a) 1,4-dihydroxypyrid-2-one (1×10^{-3} M) with iron (1×10^{-3} M) was studied at pH 3.25, 530 nm (A) and at pH 7.4, 465 nm (B); and of (b) 4-methoxy-1-hydroxypyrid-2-one (4×10^{-4} M) iron (4×10^{-4} M) at pH 7.4, 460 nm (A) and pH 2.3, 420 nm (B).

region, which corresponds to the 3:chelator:1 iron complex of these chelators as judged from their Job's plots (Fig. 2). At lower pH values the stoichiometry of the chelator iron complexes are lower than 3:1 (Fig. 2).

The log β_3, log K_{eff} and log K_{sol} of the iron binding of the 1-hydroxypyrid-2-ones and other chelators is shown in Table II. Some of the 1-hydroxypyrid-2-ones in Table II which seemed in principle to be able on thermodynamic grounds to remove iron from transferrin and ferritin, were incubated with these two proteins at pH 7.4 and shown to be effective in mobilising substantial amounts of ^{59}Fe from ^{59}Fe transferrin within 5 h and also some iron from ferritin after 6 h of incubation (Table III).

The most effective chelators in the removal of iron from transferrin within 5 h were 1,2-dimethyl-3-hydroxypyrid-4-one and 1,4-dihydroxypyrid-2-one which caused the release of 90% and 80% of the iron respectively. The other 1-hydroxypyrid-2-ones i.e. the parent compound, the 4-methoxy and 4-(2'-methoxyethoxy) derivatives were slightly less effective releasing 65–70% and the remaining chelators including desferrioxamine could only manage to release 5–10%. The percentage iron release from ferritin at 6 h incubation was much lower than that from transferrin and higher amounts were released

in the second experiment (B), 1,4-Dihydroxypyrid-2-one was the most effective of all the chelators in both ferritin experiments releasing up to 22% in the second experiment (B) while the other chelators tested 7–14% iron under the same conditions (Table III).

TABLE III. Iron Mobilisation from Transferrin and Ferritin[a]

Chelator	Transferrin % ^{59}Fe release	Ferritin % Iron release	
		A	B
1-Hydroxy-pyrid-2-one	68, 65		
1,4-Dihydroxy-pyrid-2-one	82, 79	5.8	21.9
4-Methoxy-1-hydroxypyrid-2-one	69, 69	4.2	12.9
4-(2'-Methoxyethoxy)-1-hydroxypyrid-2-one	70	2.0	
1,2-Dimethyl-3-hydroxypyrid-4-one	90, 89	4.4	13.9
1-Methyl-3-hydroxypyrid-2-one	11, 11		11.1
Maltol	5, 4		7.3
Desferrioxamine	7, 5		
Control	0, 0		

[a]The reaction of transferrin (1.1×10^{-5} M) with the chelators (4×10^{-3} M) was carried out for 5 h at 26 °C in Tris 0.1 M, pH 7.4 and of ferritin (A: [iron] = 1.42×10^{-3} M, [chelator] = 1×10^{-3} M, 26 °C, in Tris $NaClO_4$, 0.1 M, pH 7.4; B: [iron] = 1.74×10^{-3} M [chelator] = 3.8×10^{-3} M, at 37 °C in PBS, pH 7.3) for 6 h.

Discussion

In an attempt to find effective iron chelators for clinical use several known and new 1-hydroxypyrid-2-one chelators with different physicochemical properties have been synthesised and tested using an *in vitro* screening system at physiological pH. In examining the introduction of substituents on the activity of the chelators it was initially observed that the electron withdrawing effects of the nitro substituents caused a substantial decrease in the pK_a of the hydroxyl group and also the iron binding properties of the α-ketohydroxy chelating site at physiological pH. Furthermore, although the parent compound 1-hydroxypyrid-2-one formed a precipitate with iron(III) at this pH, the introduction of the 4-hydroxy, 4-methoxy and 4-(2' methoxyethoxy) substituents increased the pK_a, the iron binding properties of the chelators and the solubility of their iron complexes. The buffering capacity in the physiological pH region of these three water soluble iron-(III) complexes and the formation of a neutral 3:chelator:1 complex, which could facilitate the diffusion through membranes, adds two further properties of physiological importance to these chelators.

The estimation of the iron stability constant ($\log \beta_3$) alone at ideal conditions, which is generally reported could not provide sufficient information for the chelating properties of chelators at physiological pH. The estimation however of the $\log K_{eff}$ [22] and $\log K_{sol}$ [21] constants is more useful and relevant approach for *in vivo* conditions, and chelators with $\log K_{eff} > \log K_{eff}$ of transferrin should in principle be able to mobilise iron from this protein and those with $\log K_{sol} > 0$ should solubilise polynuclear iron e.g. ferritin iron. For example, while some chelators have high $\log \beta_3$ (e.g. salicilic acid, Table II) their $\log K_{eff}$ and $\log K_{sol}$ at pH 7.4 decreases substantially and are not effective *in vivo* [14]. Since some of the 1-hydroxypyrid-2-ones seemed to fulfill most of the criteria for effective chelation of iron at physiological pH, their ability for iron mobilisation from transferrin and ferritin was also examined in order to identify possible kinetic constrains of the protein molecular structures which could inhibit this process [26]. Large amounts of iron were removed following the incubation of the 1-hydroxypyrid-2-ones with both transferrin and ferritin. The levels of iron mobilisation by these chelators was equivalent to those caused by 1,2-dimethyl-3-hydroxypyrid-4-one and were higher than those caused by desferrioxamine. While high concentrations of the chelators were used in the removal of iron from transferrin, at low concentrations, the chelators donated their iron to apotransferrin (data not shown). It can be envisaged that these chelators could in principle be used at high concentrations for iron removal in iron overload, or for iron donation in iron deficiency if they are injected as their iron complexes at low concentrations. Iron complexes of these chelators which could be absorbed by the gastrointestinal tract could also be applied orally since they have been shown to hold on to iron(III) at acidic pH (Fig. 2). If however, their iron complexes are not absorbed in the gastrointestinal tract, the chelators could be used to decrease iron absorption in conditions of iron overload arising from increased dietary iron absorption e.g. idiopathic haemochromatosis and thalassaemia intermedia. Further work is required to examine these possibilities. It should be emphasised however, that in considering the possible clinical use of 1-hydroxypyrid-2-ones, other conditions may prevail in vivo which could minimise the prospects of their application such as toxicity, biotransformation, rapid excretion, chelation of other metals etc. In a preliminary study these chelators have been shown to have no affinity for

Mg^{2+} and Ca^{2+} but some affinity for Zn^{2+} and Cu^{2+}, which was however minimal in comparison to iron-(III). Furthermore, 1,4-dihydroxypyrid-2-one caused increased iron excretion when administered intraperitoneally and intragastrically to iron loaded mice at a 200 mg/kg dose [14] and also removal of Pu from transferrin and ferritin at pH 7.4 [27]. Many other analogs including oligomers and polymers with a 1-hydroxypyrid-2-one metal binding site could be synthesised and improvement of their iron binding properties at physiological pH could be envisaged if neutral, electron releasing substituents are introduced in the heteroaromatic ring. The cheap synthesis of these chelators and their high affinity for iron increases the prospects for their use in the treatment of iron overload and other diseases of metal imbalance.

In conclusion many factors have to be considered when designing iron chelators intended for clinical use, such as the structure of the backbone and the metal binding site, the effects of substituents, the water solubility and charge of the chelators and their iron complexes at physiological pH, their interaction with transferrin and ferritin [25, 26], their toxicity and other metabolic aspects. It was shown that some of the 1-hydroxypyrid-2-ones fulfill many of the criteria for chelators intended for clinical use and it is hoped that some would reach this stage.

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