Relative Oral Efficacy and Acute Toxicity of Hydroxypyridin-4-One Iron Chelators in Mice


The relationship between the oral efficacy and the acute toxicity of hydroxypyridin-4-one iron chelators has been investigated to clarify structure-function relationships of these compounds in vivo and to identify compounds with the maximum therapeutic safety margin. By comparing \(^{55}\text{Fe}\) excretion following oral or intraperitoneal administration of increasing doses of each chelator to iron-overloaded mice, the most effective compounds have been identified. These have partition coefficients (Kpart) above 0.3 in the iron-free form with a trend of increasing oral efficacy with increasing Kpart values (\(r = .8\)). However, this is achieved at a cost of increasing acute toxicity, as shown by a linear correlation between \(^{55}\text{Fe}\) excretion increase per unit dose and 1/LD\(_{50}\) (\(r = .83\)). A sharp increase in the LD\(_{50}\) values is observed for compounds with Kpart values above 1.0, suggesting that such compounds are unlikely to possess a sufficient therapeutic safety margin. Below a Kpart of 1.0, acute toxicity is relatively independent of lipid solubility. All the compounds are less toxic by the oral route than by the intraperitoneal route, although iron excretion is not significantly different by these two routes. At least five compounds (CP51, CP94, CP93, CP96, and CP21) are more effective orally than the same dose of intraperitoneal desferrioxamine (DFO) (\(P < .02\)) or orally administered L1(CP20) (\(P < .02\)).

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In the search for oral iron chelators, many compounds have been rejected because they lack sufficient oral activity to produce negative iron balance in chronically transfused patients, or because of unacceptable toxicity. Even desferrioxamine (DFO), which has been used effectively for over 2 decades, has significant toxicity, particularly in patients with little iron overload.\(^{12}\) Although the toxicity of iron chelators can be reduced by the use of compounds with a high selectivity for iron (III), unfortunately many of the physico-chemical properties which are advantageous for oral absorption also tend to lead to increased toxicity. This toxicity is due to the fact that the properties of compounds which favor absorption from the gastrointestinal tract, namely low molecular weight, neutral charge, and lipid solubility, may also favor penetration of the central nervous system (CNS)\(^{14}\) and access to the intracellular iron pools necessary for normal cellular function. A fine balance exists, therefore, between effective oral activity and unacceptable toxicity. This balance may be influenced by the state of iron overload.

When selecting chelators for clinical use it will be important to distinguish between the toxic effects that are related to the chelation of metals, and those which are independent of this mechanism. A failure to distinguish between these effects may lead to the unnecessary exclusion of compounds because their acute toxicity is a function of rapid and effective chelation of iron (III). Therefore, the initial screening of iron chelators should involve both iron-overloaded and non-overloaded animals.

The bidentate 3-hydroxypyridin-4-one series of iron chelators have properties well suited to the chelation of iron in vivo by the oral route. These properties include a high specificity and selectivity for iron (III) and the possession of a neutral charge in the iron-free and iron-complexed form, allowing both forms of the chelator to cross biological membranes. A number of these hydroxypyridin-4-ones have been shown to be effective by mouth in animal models\(^{57}\) with a trend of increasing activity in the more lipophilic compounds.\(^6\) One of these compounds, CP20 (L1), is effective at increasing urinary iron excretion in humans.\(^{8}\) The wide range of hydroxypyridin-4-ones that has been synthesized offers the opportunity to study in detail the relationship between the physico-chemical properties of iron chelators, their toxicity, and their effectiveness. Such studies may give an insight into the general physico-chemical properties of iron chelators best suited for safe and effective iron chelation as well as identifying which hydroxypyridin-4-ones are best suited for further development as oral iron chelators. In this study we have sought to identify these properties and to determine how iron overload may influence this relationship.

MATERIALS AND METHODS

Reagents. The N-alkyl-3-hydroxypyridin-4-one iron chelators were synthesized and purified as previously described\(^{11,12}\) and their purity confirmed by reverse phase high performance liquid chromatography (HPLC), \(^1\)H nuclear magnetic resonance, and elemental analysis. Their general structure is shown in Fig 1, and Table 1 lists the compounds investigated. CP20 and CP21 have also previously been referred to as L1 and LINEt.\(^{11}\) DFO was purchased from Ciba-Geigy, UK, and Imferon from Fisons, UK. All other chemicals were of analytical grade.

Determination of partition coefficients. The partition coefficient (Kpart) was measured as the ratio of the concentration of the compounds between n-octanol and Tris HCl (20 mmol/L, pH 7.4) at 20°C; the concentration of free ligands was 10\(^{-4}\) mol/L, and that of the neutral 3:1 (ligand:iron) complexes was 10\(^{-4}\) mol/L, as previously described.\(^{14}\)

Acute toxicity studies. Modified LD\(_{50}\) studies were performed as follows. Groups of age-matched, albino, male mice (Balb c) aged 6 to 8 weeks were purchased from Harlan-Olac, UK, and fed an ad libidium diet (rat and mouse special diet services [S.D.S.] expanded diet No. 1). Each group was given intraperitoneal injections of iron chelator or phosphate-buffered saline (PBS) at the lowest test dose.
Iron excretion studies. Iron excretion studies were performed essentially as previously described. Briefly, male albino mice aged 8 to 10 weeks were iron overloaded by weekly intraperitoneal injections of iron dextran (Imferon) for 4 weeks (8 mg of iron in total), followed by a 2-week equilibration period. Human apolactoferrin (<10% saturated) (Sigma, UK) was saturated to 90% with $^{59}$Fe, and separated from free iron by gel chromatography. One milligram of lactoferrin (specific activity 3 pCi/mg of iron) was administered by the oral route (PO) were determined from the dose response curves in Fig 4, where the correlation coefficient and standard error of the slope and intercept values of each were used to calculate $^{59}$Fe excretion for a given dose. The standard error was calculated from the standard error of the slope and the relative effectiveness of each chelator compared by linear regression analysis.

RESULTS

General properties of iron excretion model. Basal daily $^{59}$Fe excretion represented approximately 0.6% to 0.9% of total body $^{59}$Fe. With all compounds studied, the majority of radio-iron excretion at lower doses was fecal, in agreement with previous findings. As the dose of chelator administered increased, the percentage excreted in the urine increased (Fig 4). The proportion of urinary iron was maximal within 24 hours of oral administration of chelator and decreased steadily thereafter (Fig 4).

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dose, the duration of increased iron excretion was examined at increasing doses of various chelators. Figure 2 shows the effect of increasing doses of compounds CP93, CP22, and CP51 on the duration of iron excretion (urine + feces). It can be seen that at doses of less than approximately 800 mg/kg, the increased 59Fe excretion had ceased by 48 hours. At higher doses, the increased excretion continued for at least 72 hours. Therefore, for comparative dose response studies, 48-hour collections were used for doses below 800 mg/kg (Fig 3).

Comparison of 59Fe excretion following escalating oral doses of chelators. In Fig 3, oral dose response curves for nine hydroxypyridin-4-one iron chelators are shown. Increments are expressed as total 59Fe excretion (urine + feces) as a percentage of baseline excretion, over a 48-hour period after the intragastric administration of each compound. The correlation coefficient was determined over the linear range of the dose response curve for each compound, and from the slope of the graph, the increase in excretion of 59Fe for each milligram per kilogram dose administered was estimated (Table 1). From the slope of the graph and the y-axis intercept, the dose (in milligrams per kilogram) required for a nominal 200% increase in iron excretion was calculated (Table 1). The dose response curves were linear for compounds CP20, CP21, CP22, CP51, CP93, and CP96 (r ≥ .82) over the range of 0 to 750 mg/kg. CP94 showed a linear dose response to 400 mg/kg and plateaued above this dose (Fig 3). CP55 was only marginally effective over the dose range studied. From Table 1 it can be seen that the most effective compounds orally are CP22, CP51, and CP94. CP22 (P < .001), CP94 (P < .001), CP96 (P < .001), CP51 (P < .001), and CP21 (P < .02) are all significantly more effective than CP20 (L1) by the oral route (Table 1).

Comparison of oral efficacy with that achieved by the intraperitoneal route. Iron excretion following oral and intraperitoneal administration was compared using increasing doses of CP21, CP93, CP51, and CP20 using a 48-hour collection of urine and feces. Figure 5 compares the relative 59Fe excretions at an arbitrary dose of 200 mg/kg, calculated from the regression line for increasing doses of each compound by the respective routes as in Fig 3. The results show that iron excretion after intragastric or intraperitoneal administration does not differ significantly (P > .05). DFO, which is inactive by the oral route, produces less increase in 59Fe excretion per unit dose intraperitoneally (0.81% increase per mg/kg ± 0.085) than do CP94, CP51, CP22, CP96, CP21, and CP93 intragastrically (Table 1).

LD50 and its relationship to lipid solubility. Table 1 shows the estimated LD50 for the compounds shown via the intraperitoneal route. This route was chosen because there was less variability in acute toxicity than by the oral route, making comparisons easier, and because toxic doses following oral administration were so high as to make such dosing impractical. The LD50 for each compound via the oral route has therefore not been determined formally; however, in the escalating dosage studies on 59Fe excretion (Fig 3) less than half the mice died at doses of 990 mg/kg with compounds CP20, CP21, CP22, CP52, CP55, CP93, CP94, CP96.

Figure 6A shows the relationship of the Kpart of the free ligands to 1/LD50. The reciprocal of the LD50 has been plotted so that this value increases as toxicity increases. It can be seen that there is a trend of increased acute toxicity (1/LD50) with increased lipid solubility. At lethal doses of each compound, death occurred by convulsions. The time interval between intraperitoneal administration of compound and death was shorter with the lipophilic compounds (within
In addition to the LD<sub>50</sub> studies, which were performed on non-overloaded mice, acute toxicity was also compared in overloaded mice and non-overloaded mice for selected compounds. During the increasing dose study by the intraperitoneal route (performed on overloaded mice), non-overloaded, age-matched mice were administered equal doses of compounds CP51, CP21, and CP20. In each case the estimated dose at which half the animals died was greater in the overloaded than in the non-overloaded mice (data not shown), suggesting a protective effect of iron loading against acute toxicity. Although only small numbers of animals were used (n = 5 to 7), the number of mice alive following a 20-mg (=800 mg/kg) dose for CP51 was significantly greater for the overloaded than in the non-overloaded mice (P < .05, χ² analysis) and for CP21 at 40 mg (=1,600 mg/kg) (P < .001, χ² analysis). The differences for CP20 did not reach statistical significance.

Relationship between lipid solubility and efficacy of chelators. Figure 6C shows the relationship between the lipid solubility (K<sub>part</sub>) of the iron-free form of each chelator and the iron excretion per unit dose (mg/kg). It can be seen that there is a weak linear correlation between these parameters (r = .6). CP52 is clearly less effective than would be predicted from the K<sub>part</sub> value alone. If this compound is eliminated, the other compounds lie close to the predicted relationship between K<sub>part</sub> and relative efficacy (r = .84). By contrast, there is no relationship apparent between efficacy and the lipid solubility (K<sub>part</sub>) of the iron-chelator complex of each chelator (Table 1).

Relationship between oral effectiveness and acute toxicity. Figure 6B shows the relationship between the relative oral efficacy of each compound (expressed as the increase in <sup>59</sup>Fe excretion per unit increase in dose administered in milligrams per kilogram) and the relative acute toxicity (expressed as 1/LD<sub>50</sub>). There is a linear correlation of increasing efficacy with increasing toxicity (r = .84). CP51
Efficacy and toxicity of iron chelators

Fig 4. The percentage of \(^{55}\)Fe iron in the urine as a function of total iron excretion (urine + feces) for each 24-hour period following the oral administration of chelators, CP93, CP22, and CP51. Values shown are the mean of four observations for each dose.

and CP94 lie to the left of this line, indicating that these compounds have a more favorable efficacy to toxicity ratio than other compounds which lie closer to or to the right of the line.

Another way of comparing the efficacy to the toxicity of each compound is shown in Table 1. The oral doses of different compounds required to produce a nominal 200% increase in total iron excretion were calculated from the slopes and intercepts of the regression lines for each dose response curve. The ratio of the LD\(_{50}\) (milligrams per kilogram) to the dose required for a nominal 200% increase in excretion (milligrams per kilogram) was then calculated (Table 1). It can be seen that CP51 and CP94 are the compounds with the highest ratios and CP55 the compound with the lowest.

DISCUSSION

The main purpose of this study was to clarify the relationships between the oral efficacy of hydroxypyridinone iron chelators, their lipid solubility, and their acute toxicity. In principle, such studies should facilitate the identification of oral iron chelators for longer term toxicity and efficacy evaluation. The close structural similarities between the hydroxypyridin-4-one chelators studied in this report, all possessing similar iron binding constants,\(^{14}\) permits a systematic evaluation of these structure-function relationships.

The compounds chosen for these studies were selected on the basis of previous results using hepatocyte cultures, which demonstrated that chelators with high lipid solubility (K\(_{\text{part}} > 1\)) were likely to be toxic\(^{3,6,14}\) and that highly hydrophilic compounds were likely to be inactive by the oral route. Thus, all compounds where iron excretion was measured had a K\(_{\text{part}}\) for the free ligand between 0.2 and 1.5. CP24 and CP25 (Table 1) were eliminated from further study of iron excretion due to their high acute toxicity in mice\(^6\) and in tissue culture.\(^{14}\) CP40 was eliminated because of its low efficacy in tissue culture.\(^{14}\) However, these compounds have been included in the LD\(_{50}\) studies (Table 1) to illustrate...
the relationship between lipid solubility and acute toxicity more clearly.

Excretion of $^{59}$Fe in this mouse model is likely to be derived primarily from hepatocytes. The majority of $^{59}$Fe is found in liver (80%) and most of this is in parenchymal cells rather than in reticuloendothelial cells. The majority of iron excretion was fecal for all compounds studied, including DFO. Urinary excretion of iron by DFO is mainly derived from the reticuloendothelial system (RES) and iron chelation is largely independent of the ability of compounds to permeate the RES. Thus, the increased fecal iron excretion seen with the more lipophilic compounds is likely to be hepatocyte derived. The finding that compounds which were most effective at mobilizing intracellular iron in the hepatocyte model (eg, CP22, CP24, and CP51) were also the most effective in this study supports this hypothesis.

The relative affinity of a drug for lipid and water is a major factor in determining the permeation of drugs into cells. The Kpart of a drug between octanol and water has been shown to be good approximation to the relative solubilities between aqueous and lipid (membrane) phases. Kpart values close to 1 signify approximate equal solubility in each. It is clear from the comparative LD$_{50}$ intraperitoneal values of the non-overloaded mice, that the more lipophilic compounds, CP22, CP24, and CP25, have a greater toxicity (Table 1, Fig 6A) than the more hydrophilic compounds. However, the hydroxyypyridin-4-ones with Kpart values less than 1 show relatively small differences in LD$_{50}$ values (Fig 6A). Furthermore, these differences are more closely related to the ability of the compounds to increase iron excretion (Fig 6B) than to their Kpart values. This demonstrates, therefore, that below a critical lipid solubility (Kpart approximately equal to 1) acute toxicity is relatively independent of the Kpart.

It is likely that the acute toxicity is related to penetration of the CNS for several reasons. First, it is known that CNS penetration by drugs is closely related to lipid solubility, provided that the molecular weight is below approximately 400. Second, the mode of death in these animals suggests a CNS related cause, death being immediately preceded by convulsions. Hydrophobic compounds produced death almost immediately after intraperitoneal administration (eg, CP22, CP24, and CP25), whereas death was delayed and often preceded by a period of hyperexcitability with the more hydrophilic compounds (eg, CP20). As the molecular weight of the chelator-iron 3:1 complex is above 400, CNS penetration of this complex is unlikely, rendering it likely that the free chelator rather than iron complex is responsible for the acute toxicity.

It is unclear why the most lipophilic compound, CP25, did not show additional toxicity over that of CP24. However, it has been noted that iron mobilization from hepatocytes in culture did not increase for compounds with Kpart values above approximately 1.0. It is possible, therefore, that the permeation of cells does not increase significantly above this value and may indeed decrease a little. This lack of cell permeation would also explain the lack of increased efficacy with compounds possessing high lipid solubilities.

There is a linear relationship between the Kpart of each chelator and iron excretion following oral administration (Fig 6C), unlike that between the Kpart and acute toxicity (Fig 6A). In contrast to the latter relationship, there is a trend of increasing efficacy with increasing lipid solubility.
without a sudden increase above a $K_{\text{part}}$ of 1. This difference has important implications on the selection of compounds. On the basis of the $LD_{50}$ values, there is relatively little to choose between compounds with $K_{\text{part}}$ values below 1.0. By contrast, compounds with $K_{\text{part}}$ values at the lower end of the range studied (eg, CP20, CP55) are significantly less effective at mobilizing iron than compounds with higher lipid solubilities (eg, CP94, CP96). This difference means that compounds with $K_{\text{part}}$ values close to but less than 1 (eg, CP20, CP55) will tend to be more effective than those of compounds with lower $K_{\text{part}}$ values (eg, CP20, CP55) while there is a relatively small difference in the $LD_{50}$ values.

Some compounds are clearly more effective (CP51) or less effective (CP52) than would be predicted from the $K_{\text{part}}$ values alone (Fig 6C), as in hepatocyte cultures, although this difference is more marked for CP52 in the mouse model. This fact suggests that other factors such as molecular shape, stability, and metabolism contribute to the overall efficacy of compounds in vivo. Indeed, when the efficacy of compounds is plotted against the $LD_{50}$ (Fig 6B) these two latter compounds fit closely to the predicted relationship between efficacy and toxicity ($r = 0.84$). This latter graph demonstrates that increased oral efficacy is gained at the cost of increased acute toxicity. The ratio of the $LD_{50}$ to the dose required for a 200% increase in excretion (Table 1), gives an indication as to which compounds possess the best balance between these parameters. These are the same compounds which lie to the left of the regression line in Fig 6B.

While some toxicities from iron chelators are dependent on the degree of iron loading, such as susceptibility to ophthalmologic, auditory, and cerebral toxicity with DFO, other toxic effects may be unrelated to the degree of iron overload, and, therefore, independent of the chelation mechanism, such as leukopenia with CP20(L1). The reduced acute toxicity in iron-loaded compared with nonloaded mice seen with CP51 and CP21 supports the idea that iron loading may protect against some toxicities of compounds, presumably by making less free chelate available to bind iron or other metals in the CNS.

Iron excretion following oral administration is approximately equal to that obtained following intraperitoneal administration of each compound (Fig 5). This observation contrasts with the results described previously. This difference may be due to the fact that 48-hour collections were analyzed, unlike previous studies where the shorter collection times could underestimate excretion increments (Fig 2). While the efficacy of compounds is not significantly different in this model by oral or intraperitoneal routes, the finding that less than half the animals died at doses above 950 mg/kg suggests that the safety therapeutic margin is likely to be even greater by the oral route.

The comparative iron excretion data show clearly that there are a number of compounds (CP94, CP96, CP93, and CP51) which are more effective on a weight for weight basis than CP20 (L1), which has already been given to humans. Because CP20 has a lower molecular weight than the other compounds, this difference is likely to be an underestimate rather than an overestimate. Several of these compounds also have a superior acute therapeutic safety margin by virtue of their superior iron excretion properties in comparison to their $LD_{50}$ values. Chronic efficacy and toxicity evaluation is indicated with these compounds, in overloaded and nonoverloaded animals, to determine whether the compounds with optimal properties in short-term studies also possess the widest therapeutic safety margins in the longer term.

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Relative oral efficacy and acute toxicity of hydroxypyridin-4-one iron chelators in mice [see comments]

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