Multidentate pyridinones inhibit the metabolism of nontransferrin-bound iron by hepatocytes and hepatoma cells

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The therapeutic effect of iron (Fe) chelators on the potentially toxic plasma pool of nontransferrin-bound iron (NTBI), often present in Fe overload diseases and in some cancer patients during chemotherapy, is of considerable interest. In the present investigation, several multidentate pyridinones were synthesized and compared with their bidentate analogue, deferiprone (DFP; L1, orally active) and desferrioxamine (DFO; hexadentate; orally inactive) for their effect on the metabolism of NTBI in the rat hepatocyte and a hepatoma cell line (McArdle 7777, Q7). Hepatoma cells took up much less NTBI than the hepatocytes (< 10%). All the chelators inhibited NTBI uptake (80–98%) much more than they increased mobilization of Fe from cells prelabelled with NTBI (5–20%). The hexadentate pyridinone, N,N,N-tris(3-hydroxy-1-methyl-2(1H)-pyridinone-4-carboxaminoethyl)amine showed comparable activity to DFO and DFP. There was no apparent correlation between Fe status, Fe uptake and chelator activity in hepatocytes, suggesting that NTBI transport is not regulated by cellular Fe levels. The intracellular distribution of iron taken up as NTBI changed in the presence of chelators suggesting that the chelators may act intracellularly as well as at the cell membrane. In conclusion (a) rat hepatocytes have a much greater capacity to take up NTBI than the rat hepatoma cell line (Q7), (b) all chelators bind NTBI much more effectively during the uptake phase than in the mobilization of Fe which has been stored from NTBI and (c) while DFP is the most active chelator, other multidentate pyridinones have potential in the treatment of Fe overload, particularly at lower, more readily clinically available concentrations, and during cancer chemotherapy, by removing plasma NTBI.

Keywords: non-transferrin bound iron; liver cells; iron chelation therapy; chemotherapy.

Iron (Fe) is transported in blood plasma bound tightly in a nontoxic form to the plasma iron-binding protein, transferrin (Tf). Under normal conditions, Tf is 20–50% saturated with Fe. However, in some cases, particularly when the concentration of Fe in the plasma exceeds the Fe-binding capacity of Tf, there is additional Fe circulating in non-Tf bound forms (NTBI). This is of particular concern in diseases of Fe overload such as the genetic disorder hemochromatosis [1–3], in which there is an abnormally high absorption of Fe leading to saturation of the plasma Tf. Patients with the hereditary anemia thalassemia [4,5] also have increased plasma Fe, primarily due to the obligatory treatment of the anemia with blood transfusions. The contribution of plasma NTBI to the toxicity associated with Fe overload in these disorders is uncertain, as is the form of NTBI. Significant levels of NTBI in plasma also occur in cancer as a result of some chemotherapeutic regimes [6–8]. The source of this Fe, its toxicity, and whether it can be cleared by the liver or taken up by cancer cells and used in Fe-dependent reactions essential for growth and proliferation, is uncertain. Hence, it is of interest to investigate the uptake and metabolism of NTBI in normal and cancer cells, and the effect of Fe chelators on these processes.

In the present study we have characterized these processes in the rat hepatocyte and its neoplastic counterpart, the rat hepatoma cell line (Q7). The form of NTBI used was ferric citrate, as several studies indicate that citrate (normal plasma concentration, 70–150 µM) may be a major NTBI transport molecule in the plasma under Fe overload conditions, and is also implicated in intracellular Fe metabolism [3,9,10]. An important aspect of this work was the assessment of the effect of novel Fe chelators on the uptake and fate of NTBI and to investigate the potential of these chelators for therapeutic use in Fe overload diseases and cancer chemotherapy. Desferrioxamine (DFO), the only chelator in widespread clinical use, is expensive and not active when given orally [11,12]. Deferiprone (DFP, L1; 1,2-dimethyl,3-OH pyridin-4-one; CP 20), the most promising alternative, is in extensive clinical trials and is orally active. However, there is some evidence of toxicity [13,14] which may be related to its bidentate nature, due to the formation of transient intermediate Fe complexes with chelator/Fe ratios of 1 : 1 and 2 : 1 before formation of the stable hexadentate 3 : 1 complex. The present study
documents the effects of DFP and novel tetradentate and hexadentate analogues on NTBI uptake and mobilization from rat hepatocytes and hepatoma cells. DFO was included as a reference chelator.

Materials and methods

Animals

Hepatocytes were obtained from 7- to 10-week-old male Wistar rats. The procedure was approved by the Animal Experimentation Ethics Committee of the University of Western Australia and is in accordance with the Australian Code of Practice for the care and use of animals for scientific purposes as well as the guidelines published by the National Institutes of Health, USA. Animals were fed on the normal chow diet (control) or diet supplemented with carbonyl Fe and had access to water ad libitum.

Reagents

$^{59}$Fe as FeCl$_3$ was obtained from Dupont (North Ryde, Australia). Collagenase H and pronase were from Boehringer Mannheim (Mannheim, Germany). Eagle’s Minimum Essential Medium (MEM) was purchased from Flow Laboratories (Irvine, Scotland). Foetal bovine serum and insulin were both supplied by Commonwealth Serum Laboratories (Melbourne, Australia). Fungizone was from Trace Bioscience (Sydney, Australia), penicillin and glutamine from Gibco BRL (Auckland, New Zealand) and streptomycin sulphate from Calbiochem (La Jolla, USA). Hepes and bovine serum albumin (BSA) were from Sigma (St Louis, USA). Tris (hydroxymethyl) methylamine was from BDH Chemicals, Australia. All other chemicals were of analytical reagent quality and purchased from Sigma or Ajax (Sydney, Australia).

Chelators

DFO was purchased from Sigma (St Louis, USA). The multi-dentate pyridonines were provided by the research group of K. N. Raymond (University of California, Berkeley, USA) and prepared as described previously in US patents no. 5,624,901, April 29, 1997 ‘3-Hydroxy-2-(1H)-Pyridinone Chelating Agents’ and no. 5,892,029, April 6, 1999 ‘3-Hydroxy-2(1H)-Pyridinone Chelating Agents’.

The structures of the chelators used are presented in Fig. 1, and include the hexadentate N,N,N-tris(3-hydroxy-1-methyl-2(1H)-pyridinone-4-carboxaminoethyl)amine (Tren-N-Me-3,2-HOPO), Tren-Bis-3,2-HOPO-Bis-acetic acid and DFO, tridentate pyridoxal isonicotinyl hydrazone (PIH), tetradentate 4LI-Me-3,2-HOPO (4LI) and 5LIOMe-3,2-HOPO (5LIO) and the bidentate deferiprone (DFP).

Protein purification

Ferritin was isolated using the method of Huebers and colleagues [15] and used to raise an antiserum in rabbits [16].

Radiolabelling of ferric citrate

Ferric citrate solution was prepared by adding $^{59}$FeSO$_4$/$^{59}$FeCl$_3$ (both in 0.1 M HCl; pH 1.3) at a molar ratio of 10 : 1. Trisodium citrate (pH 8.0) was then added to the mixture to yield a Fe/citrate ratio of 1 : 100 (pH 5.5). The mixture was incubated for 10 min at 37 °C and then added to an isotonic solution of MEM containing 20 μM Hepes/Tris and 10 mg BSA mL$^{-1}$ to give a final concentration of 1 μM Fe and 100 μM citrate at pH 7.4. It has been shown that all the Fe is converted to the ferric form over the 10 min incubation [10].

Isolation and culture of rat hepatocytes

Adult rat hepatocytes were isolated and cultured after liver perfusion with collagenase (0.05%) as described previously [10,16].

Culture of rat hepatoma cells

The rat hepatoma cell line, McA-RH 7777 (McArdle Laboratory for Cancer Research, Wisconsin) was grown in MEM containing 100 μg mL$^{-1}$ streptomycin, 3.75 μg mL$^{-1}$ Fungizone, 100 U mL$^{-1}$ penicillin and 10% fetal bovine serum, and were seeded on tissue culture plates. The plates were used when cells had reached 90–100% confluency.

Experimental procedures

Uptake studies. Hepatocytes and hepatoma cells were washed with Hank’s balanced salt solution and the medium replaced with the incubation medium (MEM/Hepes/Tris/BSA, pH 7.4) containing the radiolabelled ferric citrate with or without chelators (0.1 and 1 mM). The cells were incubated for 0–3 h at 37 °C, after which the amount of radioactivity internalized by the cells was estimated by DNA estimation, by the method of Hinegardner [18,19]. All data were calculated as mmol Fe $^{-1}$ DNA to correct for variation in cell density. The efficacy of chelators was calculated from changes in internalized Fe levels expressed as a percentage of the control in each experiment. Some uptake data were also expressed as molecules per cell using our measured values of 21 ± 2 pg DNA per hepatocyte (mean ± SEM; n = 5) and 31 ± 1 pg DNA per hepatoma cell (mean ± SEM; n = 5).

Mobilization studies. Cells were preincubated with the radiolabelled ferric citrate in MEM containing Hepes/Tris/BSA (pH 7.4) at 37 °C for 2 h. The cell monolayer was then washed before reincubation with the control medium (no chelator) or the test media (with chelators) for 2 h. The efflux medium was then collected and the cells treated as in the uptake experiments.

Subcellular fractionation. In several experiments the incorporation of radioactive Fe into stroma-mitochondrial...
membrane, ferritin and ferritin-free cytosol was also measured as previously described [16].

**Fe-loading in vivo.** Hepatocytes were Fe-loaded in vivo by feeding 3-week-old male Wistar rats 2% (20 g kg⁻¹ diet) carbonyl Fe (pure form of elemental Fe) for 8 weeks prior to cell isolation [20,21]. The nonheme Fe levels in the livers of the Fe-loaded animals were 10-fold greater than the controls. In the isolated Fe-loaded hepatocytes, nonheme Fe was sixfold greater, 0.89 ± 0.08 and 5.56 ± 1.3 nmol·µg⁻¹ DNA for control and Fe-loaded rats, respectively (n = 6). Uptake and mobilization studies were performed on these Fe-loaded hepatocytes for comparison with normal hepatocytes, using a wider range of chelators.

**Toxicity studies.** Aspartate aminotransferase (AST) release from cells was measured using the optimized AST kit purchased from Sigma. This was carried out at every step of the experiments to assess chelator toxicity. The morphology of the cells was also monitored. Results are expressed as mean ± SD unless stated otherwise. Student’s unpaired t-test was used to determine any significant difference at the 95% confidence level.

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**Fig. 1. Structures of chelators.** DFP (bidentate); PIH (tridentate); 4L1, 5L1, 5L1O (tetradentate); DFO, Tren-Me-3,2-HOPO, Tren-bis-3,2-HOPO (hexadentate).
Results

Kinetics of NTBI uptake

Hepatocytes took up and rapidly internalized NTBI over time, up to at least 3 h incubation (Fig. 2A). Fe uptake by hepatoma cells was linear over the same 3 h time period but the rate of binding and internalization was very much slower (Fig. 2B). The membrane-bound Fe uptake was approximately 10% of the total Fe uptake at 3 h in both the hepatocytes and hepatoma cells. There was a marked difference in NTBI uptake between the two cell types, with hepatocytes internalizing 19624 ± 4068 (n = 11) nmol Fe per g DNA per 2 h, over 10-fold greater than hepatoma cells, which took up 1457 ± 124 (n = 9) nmol Fe per g DNA over a 2-h incubation. When uptake was expressed as atoms Fe per cell, the values for Fe internalization were (239 ± 50) × 10^6 and (26 ± 2) × 10^6 for hepatocytes and hepatoma cells, respectively.

Mobilization of NTBI

The release of Fe from membrane-bound and intracellular pools was measured during reincubation after a 2-h preincubation with ^59Fe-citrate in hepatocytes and hepatoma cells. There was little release of Fe from either Fe pool in both cell types over 2 h reincubation in the absence of added chelators (not shown). Internalized Fe in hepatoma cells decreased slightly (≈ 5%). The membrane bound Fe also fell slightly. The hepatocytes exhibited similar characteristics.

Effect of chelators on uptake of NTBI

The effects of the chelators on NTBI uptake by hepatocytes and Q7 hepatoma cells were marked and similar. All chelators decreased uptake to 20% or less of the controls. DFP and DFO almost completely abolished NTBI uptake at both 0.1 and 1 mM chelator concentrations (Fig. 3). The other pyridinones tested in this series were also effective (80–90% inhibition). On the whole, the chelators were slightly more effective at reducing NTBI uptake in hepatocytes than in hepatoma cells. The hexadentate molecule Tren-N-Me-3,2-HOPO decreased Fe uptake by ≈ 90% in hepatocytes but only ≈ 80% in hepatoma cells at the same concentration. The tetradentate molecule, 5L1O, was also active, decreasing uptake to about 10% of the control in both cell types.

Kinetics of NTBI uptake in the presence of chelators (Fig. 4) showed that DFP and DFO were the most active chelators, almost blocking membrane and internalized Fe uptake at all time points in hepatocytes (Fig. 4), and in hepatoma cells (not shown). The tridentate chelator, PIH, was almost as effective. Tren-N-Me-3,2-HOPO, a hexadentate chelator like DFO, with a similar Fe-binding affinity.
but orally active, was not as effective as DFO in inhibiting NTBI uptake but still reduced uptake to less than 10% of the control.

Effect of chelators on Fe mobilization after preincubation with NTBI

All the chelators studied were less effective in mobilizing cellular $^{59}$Fe than blocking NTB-$^{59}$Fe uptake (Fig. 5) and were similar in both hepatocytes and hepatoma cells (Fig. 5). DFP was the most active chelator in both cell types, releasing $\approx 20\%$ of intracellular $^{59}$Fe taken up over 2 h in hepatoma cells and in hepatocytes (Fig. 5). Tren-N-Me-3,2-HOPO, DFO and 5L1O were less effective, releasing approximately 10% at 1 mM. Both DFP and Tren-N-Me-3,2-HOPO at 0.1 mM reduced the amount of internalized Fe in hepatoma cells. Interestingly, an increase in chelator concentration by 10-fold had no significant effect on cellular Fe, suggesting Fe mobilization is limited by the size of an intracellular chelatable Fe pool or permeability of the Fe-chelator complex.

In view of the similar efficacy of the multidentate pyridinones to DFO, further experiments were conducted using a wider range of pyridinones on normal and Fe-loaded hepatocytes.

Comparison of normal and Fe-loaded hepatocytes

Effect of hepatocyte Fe loading on NTBI uptake. There was no apparent difference in the uptake and internalization of NTBI by normal hepatocytes and Fe-loaded hepatocytes at any time point (Fig. 6A). The mean rates of Fe internalization were $25349 \pm 4022$ and $24061 \pm 635$ nmol Fe per g DNA per 2 h for normal and Fe-loaded hepatocytes, respectively. The proportion of NTBI incorporated into ferritin increased with time to $\approx 60\%$ after 2 h (Fig. 6B). The difference between normal and Fe-loaded cells was not statistically significant.

Effect of hepatocyte Fe-loading on chelator activity in Fe uptake studies. The activities of DFO and five pyridinone chelators were compared in normal and Fe-loaded hepatocytes (Table 1). The bidentate DFP and the hexadentate DFO almost totally inhibited NTBI uptake in both cell types. The hexadentate Tren-Bis-3,2-HOPO-Bis-acetic acid was as effective as DFP and DFO, reducing Fe uptake to approximately 1% of the control, while the other chelators 5L1O, 4L1 and Tren-N-Me-3,2-HOPO were less active, although inhibition was still $\approx 90-95\%$. Apart from Tren-N-Me-3,2-HOPO, Fe-loading in vivo did not affect the efficacy of the chelators at the concentration used.

Effect of hepatocyte Fe-loading on chelator activity in Fe mobilization studies. All chelators decreased internalized Fe levels slightly compared to the controls (Table 1). There was, however, little difference in the activity of the chelators in Fe-loaded hepatocytes compared to normal hepatocytes. DFP, 5L1O and Tren-N-Me-3,2-HOPO reduced internalized Fe significantly to 75-80% control ($P < 0.05$), in the normal hepatocytes. The decrease in internalized Fe caused by chelators in the Fe-loaded hepatocytes was similar.
Effect of chelators and Fe status on subcellular distribution of NTBI in hepatocytes.

The effect of the chelators on the intracellular distribution of Fe in normal and Fe-loaded hepatocytes in the Fe uptake and mobilization studies are shown in Tables 2 and 3, respectively. In the Fe uptake studies in normal hepatocytes, all chelators caused a major shift of Fe from the ferritin fraction to the cytosolic compartment, and a slight shift to the membrane-bound fraction, particularly by 4L1 (Table 2). This change in intracellular distribution with all chelators suggests they act intracellularly as well as extracellularly. In contrast, only DFP, DFO and Tren-N-Me-3,2-HOPO caused a major shift in Fe distribution to the cytosolic compartment in Fe-loaded hepatocytes. There was little effect on intracellular distribution of Fe by 5L1O, while 4L1 increased Fe accumulation in the membrane-bound compartment, as seen in normal hepatocytes. In the Fe mobilization studies, there appeared to be a shift from the membrane-bound fraction to the cytosolic compartments, while the proportion of Fe incorporated into ferritin remained within the range obtained for the control (Table 3). The changes in the cellular distribution of Fe in the Fe uptake studies were much more marked compared to those observed in the Fe mobilization studies.

Toxicity studies

Chelators showed no apparent cytotoxicity. Table 4 shows AST release values observed for hepatocytes and a typical hepatoma cell line (Q7). There was no obvious morphological change detected by phase contrast microscopy and only 1–6% of cellular AST was released under all conditions by all chelators.

Discussion

The main aims of this study were: (a) To examine NTBI uptake and metabolism in normal and Fe-loaded hepatocytes, and neoplastic liver cells, using rat hepatocytes and the rat hepatoma cell line (Q7); (b) to determine the effect of chelators on uptake, intracellular distribution and mobilization of NTBI; (c) to assess and compare the activity and toxicity of novel tetradeutate and hexadentate pyridinones with bidentate DFP, whose toxicity may be due to the transient formation of reactive ligand-Fe species before forming the stable Fe(L)3 species (see Richardson, 2001 [41]).

Both hepatocytes and hepatoma cells took up NTBI in the form of Fe-citrate, however, the hepatocytes accumulated NTBI at a far greater rate than the rat hepatoma cell line in confluent culture (over 10-fold faster). In addition, hepatocyte uptake of NTBI was not regulated by intracellular Fe levels, as judged by the lack of effect of a sixfold increase in hepatocyte nonheme Fe. This strongly suggests a role for the liver in binding, storing and detoxifying excess body Fe in the form of plasma NTBI. While these hepatoma cells took up much less NTBI than hepatocytes, they did take up a significant amount. Indeed, another study on other hepatoma cell lines has shown a much higher uptake of NTBI [22] but the uptake times and concentration employed were different to that used in the current study. This is of concern as several studies have shown the presence of NTBI in the plasma of patients undergoing chemotherapy for cancer [6]. This may be Fe derived from reticuloendothelial cells, accumulating in the plasma while the marrow is ablated. Thus it appears possible that cancer cells could take up NTBI and utilize it in cell proliferation.

In contrast to NTBI uptake by Q7 cells, the uptake of Tf-bound Fe (TBI) by receptor-mediated endocytosis is much lower in hepatocytes in comparison with hepatoma cells [23] and regulated by intracellular nonheme Fe levels [24]. However, the intracellular distribution of Fe from these two sources are similar (Table 2 cf. 16, 25) and competition studies indicate that NTBI and TBI have at least one common step in uptake by hepatocytes [25–27].
Chelator uptake studies revealed that DFO and DFP were the most potent compounds. DFO, the only widely used chelator in clinical therapy for Fe overload diseases, almost completely abolished NTBI uptake in both the hepatocytes and hepatoma cells. DFO could possibly chelate Fe directly from citrate as it has a much higher affinity for Fe. The ferrioxamine complex (Fe-chelator complex) does not donate Fe to cells [16]. DFP, like DFO, almost completely blocked NTBI uptake. Its mechanism of inhibition may be similar to that of DFO. DFO is a relatively slow-permeating chelator compared to DFP, as it is a much more hydrophilic molecule. Hence its ability to rapidly (within 2 min) and almost totally block Fe uptake from citrate, with the same kinetics as DFP suggests that

Table 1. Effect of chelators on internalization of NTBI by normal and iron-loaded hepatocytes, and on Fe internalized from NTBI by normal and iron-loaded hepatocytes. For uptake assays, cells were incubated for 2 h at 37 °C in the presence or absence of 1 mM chelator, after which the cells were treated with pronase as described in Materials and methods. For mobilization results, cells were incubated for 2 h with radiolabelled ferric citrate at 37 °C and then reincubated with medium with or without 1 mM chelator for 2 h at 37 °C. The cells were then treated with pronase as described in Materials and methods. Results are the mean and standard deviation of iron internalized by normal and iron-loaded hepatocytes from three separate experiments, and are expressed as a percentage of the control.

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Uptake (% control cells)</th>
<th>Mobilization (% control cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Fe-loaded</td>
</tr>
<tr>
<td>DFP</td>
<td>0.18 ± 0.09</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>4L1</td>
<td>5.16 ± 0.94</td>
<td>4.80 ± 0.59</td>
</tr>
<tr>
<td>5L1O</td>
<td>9.31 ± 0.78</td>
<td>10.39 ± 1.42</td>
</tr>
<tr>
<td>Tren-N-Me-3,2-HOPO</td>
<td>9.41 ± 0.45</td>
<td>4.25 ± 0.31</td>
</tr>
<tr>
<td>Tren-Bis-3,2-HOPO</td>
<td>1.36 ± 0.48</td>
<td>0.72 ± 0.02</td>
</tr>
<tr>
<td>DFO</td>
<td>0.11 ± 0.05</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. The effect of chelators on the cellular distribution of iron taken up from NTBI by normal and Fe-loaded hepatocytes. Cells were incubated with radiolabelled ferric citrate for 2 h in the presence or absence of 1 mM chelator at 37 °C. The cells were then fractionated into the subcellular compartments; membrane-bound, cytosol and ferritin, as described in Materials and methods. Results are presented as mean ± SD, from three separate experiments, and are expressed as a percentage of total iron taken up from NTBI.

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Normal hepatocytes</th>
<th>Fe-loaded hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Membrane</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Control</td>
<td>15.8 ± 4.5</td>
<td>7.2 ± 1.7</td>
</tr>
<tr>
<td>DFP</td>
<td>19.2 ± 0.5</td>
<td>34.7 ± 10.5</td>
</tr>
<tr>
<td>DFO</td>
<td>20.2 ± 16.0</td>
<td>46.0 ± 3.0</td>
</tr>
<tr>
<td>Tren-N-Me-3,2-HOPO</td>
<td>23.6 ± 5.4</td>
<td>40.1 ± 22.5</td>
</tr>
<tr>
<td>Tren-Bis-3,2-HOPO</td>
<td>24.8 ± 5.0</td>
<td>19.3 ± 10.3</td>
</tr>
<tr>
<td>5L1O</td>
<td>27.8 ± 10.8</td>
<td>26.3 ± 19.4</td>
</tr>
<tr>
<td>4L1</td>
<td>31.8 ± 0.9</td>
<td>20.6 ± 14.2</td>
</tr>
</tbody>
</table>

Table 3. The effect of chelators on the cellular distribution of iron taken up from NTBI by normal and Fe-loaded hepatocytes following a 2 h reincubation with chelators. Cells were incubated with radiolabelled ferric citrate for 2 h at 37 °C, followed by reincubation with medium containing no chelator (control) or chelators at 1 mM for 2 h at 37 °C. Cells were then fractionated into the subcellular compartments; membrane-bound, cytosol and ferritin, as described in Materials and methods. Results are presented as mean ± SD, from three separate experiments, and are expressed as a percentage of total iron taken up from NTBI.

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Normal hepatocytes (% total)</th>
<th>Fe-loaded hepatocytes (% total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Membrane</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Control</td>
<td>15.1 ± 1.9</td>
<td>5.4 ± 2.0</td>
</tr>
<tr>
<td>DFP</td>
<td>9.9 ± 1.4</td>
<td>10.4 ± 1.3</td>
</tr>
<tr>
<td>DFO</td>
<td>7.9 ± 0.8</td>
<td>8.6 ± 2.1</td>
</tr>
<tr>
<td>Tren-N-Me-3,2-HOPO</td>
<td>8.9 ± 0.4</td>
<td>10.4 ± 0.6</td>
</tr>
<tr>
<td>Tren-Bis-3,2-HOPO</td>
<td>11.8 ± 1.5</td>
<td>8.6 ± 1.2</td>
</tr>
<tr>
<td>5L1O</td>
<td>13.2 ± 2.9</td>
<td>8.9 ± 2.5</td>
</tr>
<tr>
<td>4L1</td>
<td>12.9 ± 1.9</td>
<td>7.0 ± 0.8</td>
</tr>
</tbody>
</table>
Cells were incubated with ferric citrate for 2 h at 37°C in the presence or absence of chelators (uptake studies) or were incubated with ferric citrate for 2 h at 37°C (mobilization studies). 

AST release was measured at every step of the experiments as described in Materials and methods. Results are the mean and standard deviation from a typical experiment (n = 3), and are expressed as a percentage of total AST present in the cells.

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Incubation medium</th>
<th>Membrane fraction</th>
<th>Uptake (% total)</th>
<th>Mobilization (% total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.63 ± 0.21</td>
<td>0.82 ± 0.17</td>
<td>0.32 ± 0.26</td>
<td>0.89 ± 0.27</td>
</tr>
<tr>
<td>DFP 0.1 mM</td>
<td>3.63 ± 0.10</td>
<td>1.08 ± 0.16</td>
<td>0.43 ± 0.16</td>
<td>0.82 ± 0.27</td>
</tr>
<tr>
<td>DFP 1 mM</td>
<td>3.10 ± 0.08</td>
<td>0.46 ± 0.11</td>
<td>0.20 ± 0.02</td>
<td>0.43 ± 0.16</td>
</tr>
<tr>
<td>DFO 0.1 mM</td>
<td>2.54 ± 0.50</td>
<td>3.24 ± 0.50</td>
<td>0.36 ± 0.28</td>
<td>2.31 ± 0.50</td>
</tr>
<tr>
<td>DFO 1 mM</td>
<td>0.56 ± 0.09</td>
<td>0.36 ± 0.24</td>
<td>0.20 ± 0.02</td>
<td>0.30 ± 0.24</td>
</tr>
<tr>
<td>Tren-N-Me-3,2-HOPO 0.1 mM</td>
<td>4.76 ± 0.21</td>
<td>0.66 ± 0.08</td>
<td>0.36 ± 0.24</td>
<td>0.30 ± 0.24</td>
</tr>
<tr>
<td>Tren-N-Me-3,2-HOPO 1 mM</td>
<td>4.76 ± 0.21</td>
<td>0.66 ± 0.08</td>
<td>0.36 ± 0.24</td>
<td>0.30 ± 0.24</td>
</tr>
<tr>
<td>5L1O 1 mM</td>
<td>1.36 ± 0.21</td>
<td>0.73 ± 0.17</td>
<td>0.56 ± 0.3</td>
<td>0.30 ± 0.24</td>
</tr>
</tbody>
</table>

The chelators markedly altered the intracellular distribution of Fe in the uptake studies (Table 2), with a much greater proportion of Fe in the ferritin-free cytosolic compartment. This suggests that the chelators may also be acting intracellularly, inhibiting Fe incorporation into ferritin. In comparison, the proportion of 59Fe in ferritin did not change significantly in the Fe mobilization studies, although there was a slight increase in 59Fe in the cytosol, derived from the membrane-bound fraction. The results indicate that the chelators may be acting on Fe present in a small transient or labile intracellular Fe pool, with limited access to Fe already incorporated into ferritin. DFP has been postulated to exert an effect on the intracellular labile Fe pool and ferritin in cancer cells (melanoma), by diffusing into cells and chelating Fe from these Fe pools, thus causing significant Fe mobilization. Our results do suggest the presence of an intracellular Fe pool but do not suggest DFP has the ability to mobilize Fe directly from ferritin in hepatocytes. It has been proposed that there are at least two distinct intracellular Fe subpools in rat hepatocytes, one of which is affiliated with Fe from endosomes, the other from lysosomal release of Fe.

Even though the nonheme Fe level in hepatocytes Fe-loaded in vivo was 6-fold greater than that of the control hepatocytes, there was no significant effect of Fe-loading on NTBI uptake, in agreement with our previous observations [10]. There was also no apparent effect of Fe-loading on intracellular distribution and chelator activity. DFP, 5L1O and Tren-N-Me-3,2-HOPO were the most effective chelators in reducing internalized Fe following reincubation after prelabelling both normal and Fe-loaded hepatocytes.
Fe-loading in vitro with ferric ammonium citrate, however, can lead to an up-regulation of NTBI uptake mechanisms [33–36], although this may be accompanied by a risk of cell membrane damage due to lipid peroxidation [37]. There was no cytotoxicity apparent in the hepatocytes or hepatoma cells in the present work in the presence or absence of any chelator (even at 1 mM). However, the exposure times were relatively short.

The use of DFP as a therapeutic drug in the treatment of Fe overload diseases is controversial. DFP administration has been found to cause toxic effects in some studies [13, 38–40] while not in others [41–44]. Despite this controversy, DFP is still considered to have potential as a chelator, particularly for the treatment of Fe overload [45]. Also, Olivieri et al. [13] only reported toxicity in patients administered with DFP for about 4.5 years. In 1998, Wonke and colleagues [46] administered DFP and DFO as a combined therapy and found no toxicity from either drug, with a promising drop in serum ferritin levels in patients accompanied by an increased urinary Fe excretion. While DFO and DFP are used to treat Fe overload, their potential as antineoplastic agents is also being assessed. DFP inhibits Fe uptake and cellular proliferation in liver cells [47,48]. However, further investigations are required to assess DFP’s potential as an anticancer drug. Our preliminary assessment suggests multidentate pyridinones such as Tren-N-Me-3,2-HOPO are also potential candidates for the assessment of Fe overload, particularly as they may be orally administered with DFP for about 4.5 years. In 1998, Wonke and colleagues [46] administered DFP and DFO as a combined therapy and found no toxicity from either drug, with a promising drop in serum ferritin levels in patients accompanied by an increased urinary Fe excretion. While DFO and DFP are used to treat Fe overload, their potential as antineoplastic agents is also being assessed. DFP inhibits Fe uptake and cellular proliferation in liver cells [47,48]. However, further investigations are required to assess DFP’s potential as an anticancer drug. Our preliminary assessment suggests multidentate pyridinones such as Tren-N-Me-3,2-HOPO are also potential candidates for the treatment of Fe overload, particularly as they may be orally active [29]. Further studies with appropriate detailed dose-response curves and varying exposure times to these chelators are warranted.

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