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Mobilization of iron from cells by hydroxyquinoline-based chelators

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DFO, desferrioxamine
Fe, iron
HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
8-HQ, 8-hydroxyquinoline
L1, deferiprone
MEM, minimal essential medium
MO-8HQ, 7-morpholinomethyl-8-hydroxyquinoline
MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PIH, pyridoxal isonicotinoyl hydrazone

ABSTRACT

With the aim of identifying an iron (Fe) chelator which is effective at mobilizing intracellular Fe, two novel ligands were synthesized and tested. Hydroxyquinoline is known to possess a high affinity for Fe and was thus chosen as the Fe binding motif for the hexadentate chelators, C1 (2,2′-[ethane-1,2-diylbis(iminomethylene)]diquinolin-8-ol) and C2 (2,2′-[cyclohexane-1,2-diylbis(iminomethylene)]diquinolin-8-ol). Both chelators are lipophilic, with Fe^{3+} complexes slightly more hydrophilic than the free ligands. C1 and C2 were equally toxic to K562 cells, and partial protection was afforded by supplementing the culture medium with human holotransferrin, suggesting that some of the toxicity of the ligands is due to cellular Fe depletion. Micromolar concentrations of both ligands effectively mobilized ^{59}Fe from reticulocytes and K562 cells. In reticulocytes, 50 μM C1 caused the release of 60% of the cells' initial ^{59}Fe uptake after a 4 h incubation. Under the same conditions, C2 revealed a release of 50% of the ^{59}Fe. Overall, both ligands merit in vivo study for oral activity. Their effectiveness at low concentrations makes them candidates for therapeutic use.

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PMS, phenazine methosulfate
RPMI-1640, Rosewall Park Memorial Institute medium
Tf, transferrin

Patients suffering from iron-loading anemias such as thalassemia, aplastic anemia, and sideroblastic anemia require regular blood transfusions in order to maintain adequate tissue oxygen levels. However, in the absence of a significant physiological iron (Fe) excretory pathway, patients must receive chelation therapy to allow excretion of the excess iron. The only chelator in routine clinical use is desferrioxamine B (DFO) [1]. Although effective, DFO must be administered by subcutaneous or intravenous infusion for up to 8–10 h daily [2]. This is a cumbersome process, and is often met with patient non-compliance. Furthermore, its synthesis and mode of administration are expensive, making the drug unavailable for widespread use. In view of these considerations, there is an urgent need for the development of alternative, orally effective, inexpensive iron-chelating drugs.

Within recent years more than a thousand candidates for therapeutically valuable iron chelators have been screened in animal models [3]. The search for the ideal chelator has been a difficult task. Recently, this search has led to the discovery of deferiprone (L1), a bidentate ligand which has been extensively studied as an alternative to DFO. L1 has high affinity for Fe and can easily permeate cell membranes. While initially showing much promise, clinical trials of L1 yielded controversial results. It has not yet been resolved whether hepatic iron levels can be sufficiently decreased by oral L1 therapy [4], or whether the incidence of adverse effects is acceptable [5,6]. With the aim of successfully identifying a compound which is both effective and efficient, novel chelators were modeled upon the successes and failures of previously tested ligands. Chelators containing the 8-hydroxyquinoline moiety such as *O*-Trensox and 7-morpholinomethyl-8-hydroxyquinoline (MO-8HQ), have recently proven to be effective iron chelators in vitro [7–11]. The hydroxyquinoline moiety is known to possess a high affinity for iron [12] and was thus chosen as the iron binding motif. With the objective of synthesizing orally effective chelators, the ligands described in this study have been designed as hexadentate structures, which are expected to coordinately saturate the Fe^{3+} ion, thereby minimizing the potential of the complexes to redox cycle. C1 (2,2′-[ethane-1,2-diylbis(iminomethylene)]diquinolin-8-ol) was originally described as an analytical reagent. C2 (2,2′-[cyclohexane-1,2-diylbis(iminomethylene)]diquinolin-8-ol) has not to our knowledge, been reported.

1. Materials and methods

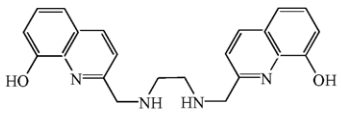
1.1. Materials

Succinylacetone and human holo- and apo-transferrin (Tf) were purchased from Sigma Chemical Co. (St. Louis, MO). Modified minimum essential medium (MEM), Rosewall Park Memorial Institute medium (RPMI-1640), fetal bovine serum (FBS), penicillin-streptomycin and L-glutamine were obtained from Gibco Laboratories, Canada. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium) was purchased from Promega (Madison, WI). Phenazine methosulfate (PMS) was obtained from Aldrich (Oakville, Canada) and phenylhydrazine hydrochloride was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). Iron-59 (as ferric chloride in 0.1 M HCl) was purchased from Amersham (Baie d'Urfe, Canada). Sodium citrate, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and phosphate-buffered saline (PBS), were purchased from Bioshop (Burlington, Canada). Ferric citrate was purchased from Fisher Scientific (Franklin Lakes, NJ), and CD1 mice were obtained from Charles River (Lasalle, Canada).

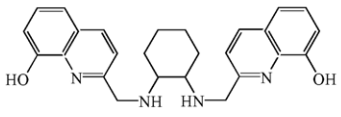
1.2. Chelators

C1 and C2, the structures of which are shown in Fig. 1, were synthesized and purified as described for C1 [7]. C2 was

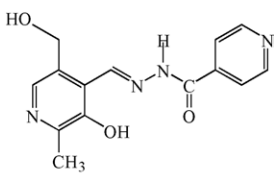
C1: *bis*(8-hydroxyquinoline-2-carbaldehyde)-*N,N'*-ethylenediamine



C2: *bis*(8-hydroxyquinoline-2-carbaldehyde)-*N,N'*-cyclohexane-1,2-diamine



PIH: pyridoxal isonicotinoyl hydrazone



8-HQ: 8-Hydroxyquinoline

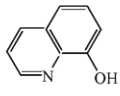


Fig. 1 – Structures of chelators used.

synthesized from a racemic mixture of 1,2-diaminocyclohexane. The identities and purities of the products were determined by elemental analysis (Robertson Microlit Laboratories Madison, NJ) and ^{1}H NMR. Stock solutions of the ligands were prepared in 1 M NaOH, and then immediately diluted with medium or buffer and neutralized with HCl. PIH was synthesized as described [13].

1.3. Spectrophotometry

Absorbance readings were made using a Varian Cary 1 spectrophotometer. Fe citrate was prepared by dissolving $FeCl_3$ in 500 mM sodium citrate, giving a final concentration of 5 mM Fe. Complex formation was analyzed from spectra collected between 190 and 700 nm.

Affinity of C1 and C2 for Fe^{3+} was determined in PBS by competition with EDTA. Displacement of EDTA from its Fe^{3+} complex by C1 and C2 (i.e., formation of Fe–C1 or Fe–C2) was measured spectrophotometrically at wavelengths at which neither EDTA nor its Fe^{3+} complex absorbed significantly. Fe–EDTA and C1 or C2 (starting concentrations were 10 μM) were added to PBS, and spectra were collected every 10 min until equilibrium was reached. Data were collected in triplicate. Concentrations of Fe–C1 at equilibrium were determined from calibration curves, and used to calculate the stability constant for the complexes according to the following equilibrium:

$$\text{Fe}-\text{EDTA} + \text{C1} \leftrightarrows \text{Fe}-\text{C1} + \text{EDTA} \quad (1)$$

from which the following equation was derived:

$$\frac{K_{\text{C1}}}{K_{\text{EDTA}}} = \frac{[\text{Fe}-\text{C1}][\text{EDTA}]}{[\text{Fe}-\text{EDTA}][\text{C1}]} \quad (2)$$

The value for K_{EDTA} of 5×10^{23} at pH 7.4 was determined using data from Critical Stability constants [12]. The stability constant for Fe–C2 was calculated analogously.

Specificity of C1 and C2 for Fe^{3+} was determined by absorbance changes following the addition of 1 mM Ca^{2+} or Mg^{2+} to solutions of 25 μM Fe complexes of the ligands. Stoichiometries of Fe–ligand complexes were determined using the slope ratio method.

Ethyl acetate/PBS partition coefficients were determined from the absorbance at local spectrophotometric maxima in ethyl acetate-saturated PBS before and after the addition of measured quantities of ethyl acetate. Volumes of ethyl acetate were chosen such that no more than 90% and no less than 10% of the species of interest entered the organic phase. Experiments were performed in triplicate. Ethyl acetate was chosen over octanol, the usual solvent for such measurements, as the former allowed greater sensitivity in distinguishing small differences in partition coefficients among the compounds under investigation.

1.4. ^{59}Fe mobilization from reticulocytes

To induce reticulocytosis, CD1 mice received intraperitoneal injections of phenylhydrazine hydrochloride (50 mg/kg body weight), for 3 consecutive days. On the sixth day, the mice were sacrificed and their blood collected via cardiac puncture. A consistent percentage of reticulocytes (33–55%) was identified in all experiments by staining with new methylene blue and counting the percentage of reticulocytes. Cells were washed with PBS and incubated for 30 min with 1 mM succinylacetone to inhibit heme synthesis [14]. A final concentration of 10 μM $^{59}Fe_2$-transferrin was then added to the cells, incubated for 1 h, and washed. This procedure yields reticulocytes with a radioactively labeled non-heme iron pool, referred to as '^{59}Fe-labeled reticulocytes' [14,15]. Aliquots of cells (30–35 μl) were incubated in MEM media with the chelators as described for each experiment. The supernatant containing ^{59}Fe–ligand complexes was then collected and counted. Protein precipitation was achieved after cell lysis with 250 μl of cold ddH_2O followed by the addition of 1 ml ice-cold 95% ethanol. Samples were centrifuged at $588 \times g$ for 20 min to yield an ethanol-soluble fraction containing intracellular chelator-bound ^{59}Fe, and an ethanol-insoluble fraction containing protein-bound ^{59}Fe [16]. Both fractions were collected and counted. ^{59}Fe in each fraction was expressed as a percentage of the initial ^{59}Fe taken up by the cells. Mass balance was preserved in all experiments (radioactivity from supernatant, ethanol-soluble, and ethanol-insoluble fractions equaled 100%, defined as the radioactivity found in intact, ^{59}Fe-labeled cells).

Generally, within each experiment, variability among identically prepared samples is extremely low. Variability among experiments is somewhat greater, probably due to non-uniform levels of non-heme ^{59}Fe in various reticulocyte preparations. As an internal control for variability among experiments, PIH was used as a control in each experiment to establish reproducibility [16]. Since the effect of PIH was similar in all experiments in this study, quantitative comparisons among the experiments can be safely made.

1.5. Cell culture

K562 cells were grown in RPMI-1640 media, supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. For the toxicity experiments, 14,000 cells were added to each well of 96-well plates with fresh media. Varying concentrations of ligand were added as described for each experiment. Cells were incubated for 72 h at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, 14 μl of an MTS/PMS solution was added to each well, and the cells re-incubated for 1 h under the same conditions. MTS is reduced by dehydrogenases in metabolically active cells into a formazan [17], which was detected at 492 nm using a Titertek Multiscan plate reader (MTX Lab Systems, VA). Samples were prepared in quadruplicate.

1.6. ^{59}Fe mobilization from K562 cells

Log phase cells were collected and samples were prepared at a density of 10^6 cells/ml. $^{59}Fe_2$-transferrin was added to yield a final concentration of 10 μM, and cells were incubated for 2 h. Succinylacetone was not added since it has been shown that K562 cells use less than 10% of their iron for heme synthesis [18]. After incubation, cells were washed three times with PBS, and twice with RPMI-1640. Cells were centrifuged at $315 \times g$ for 15 min, and their supernatants removed and counted for gamma radioactivity. Sample processing and analysis were as described for reticulocytes, except that K562 cells were

suspended in 250 μl buffer (10 mM HEPES/150 mM NaCl, pH 8) prior to lysis with ethanol.

2. Results

2.1. Chemical characterization of C1 and C2

C1 and C2 are very similar in structure (Fig. 1), differing only by the linkers joining the hydroxyquinoline moieties. Both compounds were designed as hexadentate ligands, and were therefore expected to bind one iron ion per molecule of ligand. Fe^{3+}–ligand complexes were formed by incubating Fe^{3+} citrate with the ligands at room temperature for 1 h, and complex formation was observed spectrophotometrically. Data were analyzed using two-parameter linear least squares fits, and the stoichiometries of the complexes were calculated using the slope ratio method:

$$\text{stoichiometry} = \frac{m_{Fe}}{m_L} \quad (3)$$

in which m_{Fe} and m_L are the slopes of the lines generated as the concentrations of Fe citrate and ligand increased, respectively. Since the calculated stoichiometries for Fe–C1 and Fe–C2 were both close to 1, both ligands-bound Fe^{3+} in a 1:1 ratio.

The log conditional stability constants at pH 7.4 for the Fe^{3+} complexes of C1 and C2 in PBS, measured by competition with EDTA, were 23.4 ± 0.3 and 22.8 ± 0.3 (averages of triplicate determinations ± standard deviations), respectively, indicating high affinity for Fe^{3+}, similar to that of EDTA. Selectivity of C1 and C2 for Fe^{3+} in the presence of divalent metal ions of biological interest was evaluated. Neither Ca^{2+} nor Mg^{2+}, at concentrations of 1 mM, competed with 25 μM Fe^{3+} complexes of the ligands, indicating that physiological levels of these ions are unlikely to significantly displace iron from its complexes with either ligand.

The lipophilicity of a molecule is an important property with regards to its ability to permeate biological membranes [19,20] in the absence of a specific transport system. The rate of passive diffusion of a chelator into cells, as well as its efflux as an iron complex, is largely a function of its lipophilicity. Lipophilicity was measured by the partition coefficient, P, of a species between ethyl acetate and PBS, as described in Section 1. A high log *P* value indicates a highly lipophilic species, which is likely to easily penetrate cell membranes. The following equation was used to calculate the log *P* values:

$$\log P = \log \frac{y(1 - (A_x/A_0))}{x(A_x/A_0)} \quad (4)$$

in which *A* represents the absorbance of the drug in the PBS phase of volume *y* after the addition of a volume *x* of the ethyl acetate.

The log of the partition coefficients (log *P*) of C1 and C2 were 1.05 ± 0.07 and 1.07 ± 0.05 (means ± standard deviations of triplicate measurements), respectively. These values are similar to that obtained for pyridoxal benzoyl hydrazone [20], an iron chelator which readily accesses intracellular compartments [21]. The lipophilic character of the Fe–ligand complexes however, differed. The log *P* of the Fe–C1 complex was 0.00 ± 0.03, whereas that of C2 was 0.43 ± 0.04. Thus, C1 became much more hydrophilic upon forming a complex with Fe than did the structurally similar C2.

2.2. ^{59}Fe mobilization from reticulocytes by C1 and C2

Reticulocytes in which heme synthesis was inhibited by succinylacetone were labeled with ^{59}Fe by incubation for 60 min with $^{59}Fe_2$-transferrin, followed by incubation with chelators as described in Section 1. This in vitro model in which cells have a relatively large pool of non-heme Fe has been successfully used to study the effects of PIH and its analogs [16,21,22]. When ^{59}Fe-reticulocytes were washed and incubated for 2 h in MEM in the absence of chelators, approximately 1% of the ^{59}Fe was released into the medium, probably due to release of a small amount of $^{59}Fe_2$-transferrin from which ^{59}Fe was not dissociated during the labeling period.

To assess the ligands' efficiency at mobilizing intracellular iron, ^{59}Fe-labeled reticulocytes were incubated for 1 h with chelator concentrations ranging from 10 to 200 μM. C1 displayed a dose dependent release of ^{59}Fe up to a concentration of 40 μM, releasing up to 36% of the initial ^{59}Fe taken up by the cell (Fig. 2). However, intracellular chelator-bound ^{59}Fe also increased with greater concentrations, suggesting that the free ligand quickly penetrates the cell membrane and forms an intracellular ^{59}Fe complex, but that once formed, the complex may exit the cell relatively slowly. This may be the limiting factor in the mobilization efficiency of C1. C2 displayed similar ^{59}Fe binding with a dose dependence curve saturating at a concentration near 50 μM, and causing the release of approximately half the ^{59}Fe taken up by the cells. In contrast with C1, intracellular chelator-bound ^{59}Fe did not change significantly with increasing dose, remaining in the range of 2–10% over the concentration range of the experiment (Fig. 2).

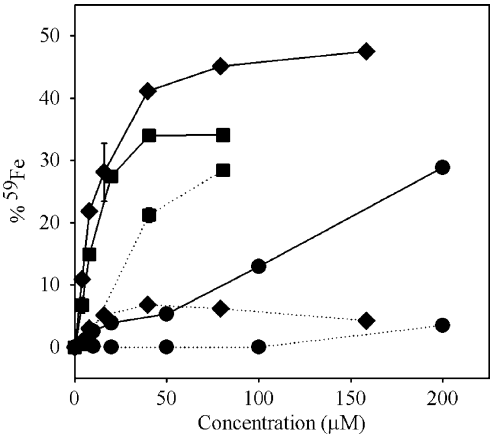


Fig. 2 – Concentration dependence of ^{59}Fe mobilization from reticulocytes by C1, C2, and PIH. ^{59}Fe-labeled reticulocytes were re-incubated for 1 h in the presence of PIH (●), C1 (■), or C2 (◆). ^{59}Fe released from cells was collected and counted (solid line). Cells were then lysed and resuspended in 95% ethanol to yield the ethanol-soluble fraction containing intracellular chelator-bound ^{59}Fe (dotted line). Data are means and standard deviations of triplicate determinations from which control values were subtracted.

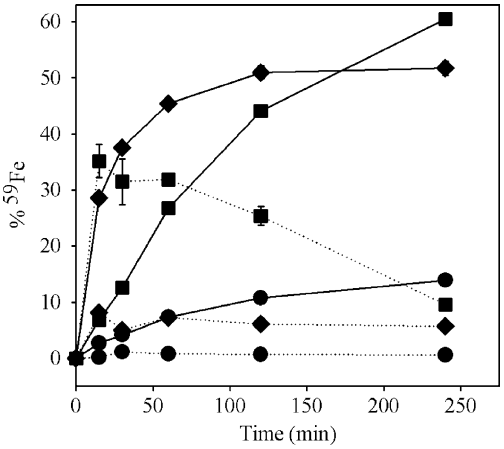


Fig. 3 – Kinetics of ^{59}Fe mobilization from reticulocytes by PIH (●), C1 (■), and C2 (◆). ^{59}Fe-labeled reticulocytes were incubated with 50 μM ligand for 15–240 min. ^{59}Fe released from cells was counted (bold line). Cells were then lysed and resuspended in 95% ethanol to yield an ethanol-soluble fraction containing intracellular chelator-bound ^{59}Fe (dotted line). Data are means and standard deviations of triplicate determinations from which control values were subtracted.

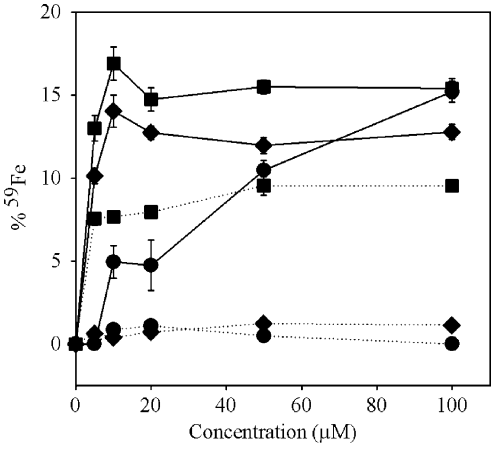


Fig. 4 – Concentration dependence of ^{59}Fe mobilization from K562 cells by PIH (●), C1 (■), and C2 (◆). ^{59}Fe-labeled K562 cells were incubated for 2 h in the presence of 5–100 μM chelators. ^{59}Fe released from cells was counted (bold line). Cells were then lysed and resuspended in 95% ethanol to yield an ethanol-soluble fraction containing intracellular chelator-bound ^{59}Fe (dotted line). Data are means and standard deviations of triplicate determinations from which control values were subtracted.

Thus, the limiting factor in ^{59}Fe mobilization is likely either membrane permeability of C2, or its complex formation with intracellular Fe.

From the data in Fig. 2, the minimal concentration required to achieve maximal ^{59}Fe release from ^{59}Fe-labeled reticulocytes was determined to be approximately 50 μM for both ligands. ^{59}Fe-labeled reticulocytes were thus incubated with 50 μM of C1 and C2 for periods of 15–240 min. Incubation with C1 revealed a nearly linear increase in ^{59}Fe mobilization with time (Fig. 3). After an incubation of 15 min with the chelator, ~44% of cellular ^{59}Fe was bound to the chelator, most of which remained inside the cell, presumably as a ^{59}Fe–ligand complex. As the incubation time increased, the percentage of intracellular chelator-bound ^{59}Fe decreased and the fraction of chelator-bound ^{59}Fe released from the cell increased to a remarkable 62% (Fig. 3). It is evident that the free ligand can quickly penetrate the cell membrane and form an intracellular complex with ^{59}Fe. Furthermore, these data confirm that once formed, the ^{59}Fe–C1 complex is relatively slow to exit the cell. Binding of ^{59}Fe by the ligand may result in structural changes in the molecule, which may in turn alter its lipophilicity, and the ease with which it exits the cell. Moreover, the complex may be large, thus limiting its efflux rate.

C2 also bound ~40% of the cell's initial ^{59}Fe uptake after an incubation of 15 min with the ligand. However, in contrast with C1, the majority of C2-bound ^{59}Fe had already been released from the cell. ^{59}Fe mobilization by C2 was non-linear, and reached a maximal release of ~50% after 120 min. Levels of intracellular chelator-bound ^{59}Fe remained steady at ~8% throughout the incubations (Fig. 3). These data suggest that free C2 can easily penetrate the cell membrane and rapidly form an intracellular ^{59}Fe–ligand complex, much like C1, but in contrast, it exits the cell as a complex with considerably higher efficacy.

2.3. *^{59}Fe mobilization from K562 cells by C1 and C2*

The second model of ^{59}Fe mobilization used consisted of K562 cells, a human leukemia cell line. K562 cells have high ferritin levels which store excess Fe, but following a short-term incubation with $^{59}Fe_2$-transferrin, the majority of ^{59}Fe is found in a cytoplasmic pool of chelatable iron [18]. Thus, K562 cells are an appropriate model for cell types for which the major destination of Fe is ferritin, in contrast with reticulocytes, in which iron is targeted to heme synthesis.

^{59}Fe-labeled K562 cells were incubated for 2 h with the ligands at concentrations of 5–100 μM (Fig. 4). ^{59}Fe release by C1 reached ~36% at concentrations as low as 30 μM. Intracellular levels of ligand-bound ^{59}Fe remained near constant at 10%, slightly increasing at higher ligand concentrations. The structurally similar C2 required similar concentrations of to release ~33% of its ^{59}Fe. Its intracellular ligand-bound ^{59}Fe remained low at ~4% at all concentrations (Fig. 4). ^{59}Fe mobilization of these chelators in this cell line was similar to that seen with reticulocytes, although the percentages are lower. This may be due to the fact that as the incubation time approached 2 h, a significant amount of the iron was being stored in ferritin, which is probably inaccessible to chelators [18,23].

^{59}Fe-labeled K562 cells were incubated with 50 μM C1 and C2 for periods of 15–240 min (Fig. 5). Release of ^{59}Fe by C1 increased with time for the first 120 min, and then remained unchanged for the remaining incubation. The maximal release attained was ~36% of the cell's initial ^{59}Fe uptake, as observed in Fig. 4. Intracellular levels of chelator-bound ^{59}Fe remained nearly constant. ^{59}Fe mobilization by C2 gradually increased over time. It too released 36% of the ^{59}Fe taken up by the cells, although it required the full 4 h to do so. Its intracellular chelator-bound ^{59}Fe slightly

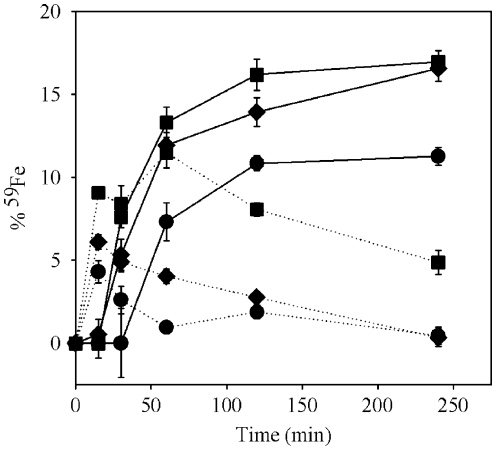


Fig. 5 – Kinetics of ^{59}Fe mobilization from K562 cells by PIH (●), C1 (■), and C2 (◆). ^{59}Fe-labeled K562 cells were re-incubated with 50 μM ligand for 15–240 min. ^{59}Fe released from cells was counted (bold line). Cells were then lysed and resuspended in 95% ethanol to yield an ethanol-soluble fraction containing intracellular chelator-bound ^{59}Fe (dotted line). Data are means and standard deviations of triplicate determinations from which control values were subtracted.

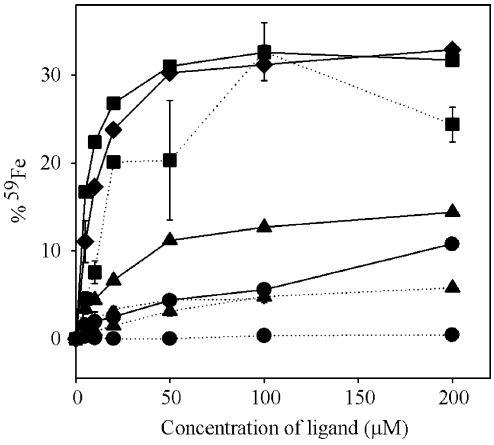


Fig. 6 – Mobilization of ^{59}Fe from reticulocytes by PIH (●), C1 (■), C2 (◆), and 8-hydroxyquinoline (8-HQ, ▲). ^{59}Fe-labeled reticulocytes were incubated for 1 h in the presence of ligand yielding final concentrations of 5–200 μM. ^{59}Fe released from cells was counted (bold line). Cells were then lysed and resuspended in 95% ethanol to yield an ethanol-soluble fraction containing intracellular chelator-bound ^{59}Fe (dotted line). Data are means and standard deviations of triplicate determinations from which control values were subtracted.

decreased over time, reaching a low of ~4% at the end of the incubation (Fig. 5).

C1 and C2 were designed as hexadentate ligands, which are expected to bind Fe more efficiently than simpler ligands. To determine whether these ligands, which possess two hydroxyquinoline moieties each, are more efficient than the equivalent concentration of hydroxyquinoline, the capacity of the novel ligands to mobilize ^{59}Fe from reticulocytes was compared to that of 8-hydroxyquinoline (8-HQ). 8-HQ caused a modest, concentration-dependent release of ^{59}Fe from reticulocytes after an incubation of 1 h. Concentrations of 200 μM only released 16% of the ^{59}Fe (Fig. 6), whereas C1 and C2 released 34 and 35%, respectively (Fig. 6). Since the amount of ^{59}Fe mobilized from cells by 8-HQ was much lower than that mobilized by C1 and C2, the high affinity of these ligands for Fe is greatly influenced by the linkers joining the two hydroxyquinoline groups, likely by influencing the preferred conformations of the ligands.

2.4. Toxicity of C1 and C2 toward K562 cells

K562 cells were incubated for 72 h in the presence of varying concentrations of chelator to determine the IC_{50}, the concentration at which cell growth is reduced to half that of control cells. Toxicity data were fitted to a weighted, three-parameter logistic equation;

$$a = \frac{a_{max}}{(c/IC_{50})^b + 1} \quad (5)$$

in which a is the number of cells, a_{max} the number of cells in the absence of the chelator, c is the concentration of chelator, and b is a Hill-type coefficient. The results revealed that the IC_{50} values of C1 and C2 were both low, 9.0 ± 0.3 and 8.5 ± 0.2 μM (means ± standard deviations of triplicate determinations), respectively (Table 1; Fig. 7).

In the presence of 10 μM human Fe_2-transferrin, which alone had no effect on cell viability or growth, the IC_{50} values of C1 and C2 increased to 13.4 ± 0.2 and 15.9 ± 0.3 μM, respectively (Table 1; Fig. 7). This protection against the toxicity of the chelators by additional transferrin suggests that the toxicity of these compounds toward K562 cells is in part due to the ligands' effectiveness at chelating cellular iron. It cannot be distinguished, from these experiments, whether increased Fe supply via transferrin diminishes the chelators' toxicity by preventing Fe depletion or by binding the chelators, thereby preventing some Fe-independent mode of action.

Toxicity of the Fe^{3+} complexes of C1 and C2 was also determined. Complexes were formed by incubation of equimolar amounts of Fe^{3+} citrate and ligand for 1 h at room temperature. That these conditions are sufficient to allow complete complex formation was determined spectrophotometrically. The IC_{50} values for the Fe^{3+} complexes of C1 and C2 were 143 ± 3 and 27 ± 0.5 μM, respectively, considerably higher than for the free ligands (Table 1; Fig. 7). Because the Fe–ligand complexes are likely to accumulate intracellularly, it must be considered that part of the toxicity of the free ligands may be due to effects of their Fe^{3+} complexes. It cannot be excluded, however, that these chelators have effects unrelated to Fe.

3. Discussion

3.1. Design of C1 and C2

Over the years, hundreds of ligands have been tested for their iron chelating properties. Of particular interest has been the

Table 1 – Lipophilicity and toxicity of C1 and C2

Chelator	Lipophilicity[a] (log *P*)	Toxicity (IC_{50}, μM)[b]	
		No added Fe_2Tf	10 μM Fe_2Tf[c]
PIH	−0.14 ± 0.01	89.0 ± 1.0	
C1	1.05 ± 0.07	9.0 ± 0.3	13.4 ± 0.2
C2	1.06 ± 0.05	8.5 ± 0.2	15.9 ± 0.3
Fe–C1	0.00 ± 0.03	143 ± 3[d]	
Fe–C2	0.43 ± 0.04	27 ± 0.5[d]	

[a] 80 μl ethyl acetate was added to 50 μM ligand (or Fe–ligand complex) with subsequent additions of 50 μl of ethyl acetate as necessary. The data are the averages of three determinations.
[b] Concentrations of ligand from 2 to 200 μM were incubated with K562 cells for 72 h ($n = 4$ for each concentration). MTS was added and absorbance measurements made at 492 nm.
[c] Concentrations of ligand from 2 to 200 μM were incubated with K562 cells for 72 h in the presence of 10 μM holotransferrin ($n = 4$ for each concentration). MTS was added and absorbance measurements made at 492 nm.
[d] Concentrations of Fe–ligand complexes from 2 to 200 μM were incubated with K562 cells for 72 h ($n = 4$ for each concentration). MTS was added and absorbance measurements made at 492 nm.

high binding affinity of the *O*-Trensox and MO-8HQ ligands for physiological metals. *O*-Trensox possesses an exceptionally high affinity for both ferric and ferrous ions [10]. Its structure contains three hydroxyquinoline moieties which together form the molecule's Fe binding domain. The MO-8HQ ligand, on the other hand, possesses one hydroxyquinoline moiety and was shown to chelate bivalent cations with high affinity [8]. The large stability constants of C1 and C2 are further evidence of the high affinity of hydroxyquinoline-based ligands for Fe^{3+}.

The number of atoms on the molecule which coordinately bind to an Fe ion largely influences the stability of the metal complex. Hexadentate ligands, which bind one Fe ion per molecule of ligand, generally make more stable complexes than do bidentate and tridentate ligands which require three and two molecules of ligand to bind one iron ion, respectively. Furthermore, hexadentate ligands have greater binding power at low concentrations and are therefore less likely to dissociate. Conversely, bidentate and tridentate ligands can form partially dissociated complexes at low concentrations, which can generate harmful hydroxyl radicals [1]. This may be one of the reasons for the toxicity of effective tridentate and bidentate chelators such as desferrithiocin [24] and L1 [25,26]. In the hopes of both improving the effectiveness of the ligands and reducing some of the toxicity seen with other ligands, the novel chelators have been modeled as hexadentate ligands. Stoichiometric analysis revealed that the ligands were in fact performing as such, revealing a 1:1 binding ratio with iron (Table 1).

In order for a chelator to be effective in a physiological setting, it must possess not only a high iron binding constant, but also a high degree of selectivity for Fe^{3+} in relation to other physiological ions. Chelators with selectivity for tripositive cations do not normally pose a large threat to the body since they generally do not selectively bind essential divalent cations such as calcium, magnesium, and zinc. In designing the new ligands, the high binding affinity of the hydroxyquinoline moiety was retained through its incorporation in the Fe binding motif of the ligands. Moreover, since the MO-8HQ ligand has been shown to chelate bivalent cations due to the hydroxyl group proximal to the nitrogen ring in the ligand, measures have been taken in the design of C1 and C2 to alter this property and make these novel ligands highly selective for Fe^{3+}. Experiments were performed in order to ensure that

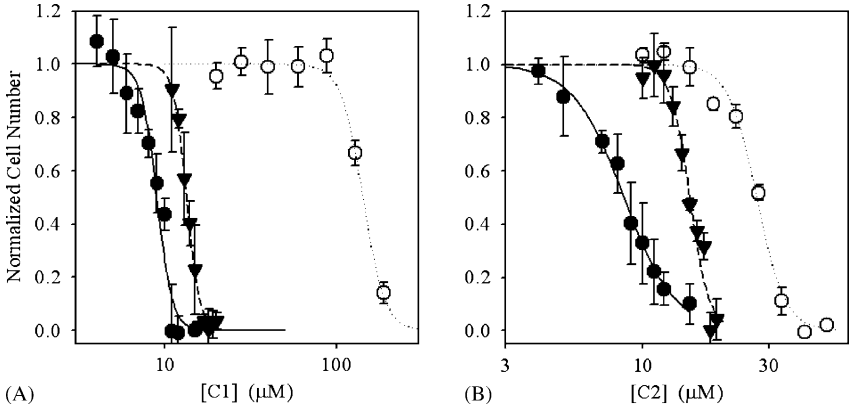


Fig. 7 – Toxicity toward K562 cells by C1 (A) and C2 (B) in the absence (●) and presence (▼) of 10 μM human Fe_2Tf, and of the Fe–ligand complexes (○). Cell viability was measured using the MTS assay as described in Section 1, and analyzed as described in Section 2. Fe_2Tf alone had no effect on cell viability. Data points and error bars are the averages and standard deviations, respectively, of six replicates. Curves are fits to Eq. (5). These data are individual determinations of the toxicity data in Table 1, which are the averages of three independent determinations.

these ligands in fact do not bind Ca^{2+} or Mg^{2+} under physiological conditions.

3.2. C1

While C1, composed of two hydroxyquinoline moieties joined by an ethylenediamine linker (Fig. 1), has been tested for use as an analytical reagent [7], its value as a therapeutic agent has not been previously examined. ^{59}Fe mobilization experiments with reticulocytes (Figs. 2 and 3) and K562 cells (Figs. 4 and 5) revealed that the ligand entered the cell quickly as a free ligand and formed a complex with iron almost instantaneously. After incubation of reticulocytes for 15 min. with the ligand, approximately 44% of the intracellular ^{59}Fe was already in the form of a ^{59}Fe–ligand complex. However, only a small fraction of that had been released from the cell. As the incubation time was increased, the intracellular complexes were gradually released from the cell. After an incubation of 4 h with 50 μM of ligand, a remarkable 62% of cellular iron was released (Fig. 3). Furthermore, the amount of intracellular complexes rose as the concentration of chelator was increased (Fig. 2). It can thus be concluded that the rate-limiting step is the efflux of the ^{59}Fe–ligand complex from the cell. These findings have also been supported by lipophilicity experiments, which revealed that the free ligand is highly lipophilic with a log *P* value near 1, and is thus able to easily penetrate the cell membrane. However, upon forming a complex with iron, the ligand became more hydrophilic, with a log *P* value near 0 (Table 1).

Toxicity experiments using K562 cells (Table 1; Fig. 7A) demonstrated an IC_{50} of 9.0 μM for C1. In the presence of human Fe_2-transferrin, the IC_{50} nearly doubled, suggesting that the toxicity of C1 is partly due to Fe depletion by the chelator. Although the IC_{50} of Fe–C1 was more than an order of magnitude higher than that of the free ligand (Table 1), it is possible that the Fe complex has an important role in the toxicity of C1.

3.3. C2

C2 consists of two hydroxyquinoline moieties, linked not by a straight chain, but by a 1,2-cyclohexanediamine ring (Fig. 1). Mobilization experiments with reticulocytes revealed that the ligand enters the cell and binds intracellular ^{59}Fe as efficiently as C1. After a 15 min incubation with 40 μM ligand, ~40% of intracellular ^{59}Fe was bound to the chelator (Fig. 3). One important difference however is that after those 15 min had elapsed, 30% of the ^{59}Fe–ligand complexes formed with C2 had already been released from the cell, and only ~9% remained inside the cell, whereas with C1, only ~9% of the ^{59}Fe–ligand complexes had been released, and the additional 35% remained inside the cell (Fig. 3). These data demonstrate the importance of rapid efflux in efficient mobilization. C2 not only enters and binds intracellular iron rapidly, but also exits the cell with the same efficacy. This attribute is supported by its highly lipophilic character as a free ligand (log *P* = ~1), and the retention of its lipophilic character as a complex (log *P* = ~0.43, Table 1). A maximal release of 52% of intracellular ^{59}Fe was achieved after a 2 h incubation with a concentration of 50 μM of ligand. This value remained nearly constant with longer incubations (Fig. 3). In contrast, since the efflux of ^{59}Fe–C1 is impeded, longer incubations with the C1 resulted in release values of up to 62% (Fig. 3). Mobilization experiments with K562 cells revealed similar kinetics to those of reticulocytes, yet like C1, the effect was not as pronounced. After a 2 h incubation with ~20–30 μM ligand, a maximal release of ~13% above control was reached. With longer incubation times, this value slowly rose to 16% and kept rising. Intracellular values decreased with time, and then remained consistently low (Fig. 5).

The IC_{50} of C2 of 8.5 μM toward K562 cells doubled in the presence of human Fe_2-transferrin, suggesting a role for Fe depletion as a mechanism of toxicity (Table 1; Fig. 7B). Furthermore, the IC_{50} of the Fe–C2 complex was also low (Table 1), suggesting that the Fe–ligand complex which is formed intracellularly may contribute to the toxicity of C2. If the iron is incompletely bound to the ligand, it may participate in the formation of free radicals, thus damaging the cell. Furthermore, the ligand may be acting as an iron donor, as has been previously seen with other chelators, including PIH and its analog, salicylaldehyde isonicotinoyl hydrazone [27,28]. Thus, it is possible that C2 redistributes cellular Fe.

4. Conclusions

The search for an alternative treatment to secondary Fe overload has been the focus of many laboratories since the discovery of DFO in the early 1960s. While effective, its high cost of production and administration make it inaccessible to many individuals who require chelation therapy for survival. Furthermore, its mode of administration is a cumbersome process, resulting in patient non-compliance, which drives the search for an orally effective alternative.

The hydroxyquinoline-based ligands, C1 and C2, have high affinity for Fe^{3+} and lipophilicity consistent with membrane permeability, properties which underlie their mobilization of ^{59}Fe from labeled reticulocytes and K562 cells. Both compounds formed intracellular ^{59}Fe–ligand complexes, which likely diffused passively from the cell. Protection against the toxicity of these chelators by the addition of transferrin, the physiological Fe donor, indicated that their toxicity was at least partly due to Fe depletion in the K562 cell line, suggesting that the potential toxicity of these chelators in vivo may be limited by the state of Fe overload. Overall, both novel ligands have proven to be highly effective and efficient chelators, demonstrating the utility of the hydroxyquinoline moiety in the design of hexadentate ligands for therapeutic purposes. Their effectiveness at low concentrations makes them candidates for further study, including in vivo assessment of safety and oral efficacy.

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