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Uptake and intracellular distribution of iron from transferrin and chelators in erythroid cells

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Summary. Iron chelators of different physicochemical properties were studied for their ability to donate iron in vitro to uninduced K562 cells, human bone marrow cells and purified human erythroblasts. To a large extent uptake was found to be related to lipophilicity and those chelators able to deliver iron to the cells in significant amounts were also able to deliver iron to ferritin and haem. Some differences in the distribution of iron delivered was observed but no chelator showed exclusive delivery to or rejection of a particular cellular iron compartment. Several chelators could probably substitute for transferrin and be used to probe metabolic events subsequent to iron removal from transferrin. Two chelators which were excellent iron donors were also found to cause considerable inhibition of iron incorporation into haem from transferrin. The implications of this for in vivo toxicity are briefly discussed.

Key words: Erythroid cells - Iron uptake - Oral chelators

Introduction

Recent years have seen some exciting new developments in the design and use of oral iron chelators for the treatment of iron overload (Hoy et al. 1979; Kontoghiorghes 1982). One of these, namely 1,2-dimethyl-3-hydroxypyrid-4-one (L1), has been shown to be effective in removing iron from man (Kontoghiorghes et al. 1987a, b). L1 is now undergoing multicentre clinical evaluation: 130 patients have so far taken L1, some for a maximum period of 15 months. Although a long-term toxicology study in animals is required before any chelator could become commercially available, their use in studies in vitro may be valuable in determining their possible mode of action and toxicity in vivo. Furthermore, the chelators can be useful for the in vitro investigation of the normal pathways of iron metabolism (Barnekow and Winkelman 1978; Landschulz et al. 1984; Bottomley et al. 1985; Ponka and Schulman 1985).

Chelators vary in their physicochemical properties, iron-binding affinities, toxicity, protein and cellular interactions and ability to mobilise iron. When a chelator is administered in vivo or is introduced in vitro it partly forms a complex with iron. Chelators and their iron complexes could affect extracellular and intracellular iron metabolic pathways by removing, donating and distributing iron to different iron compartments (Kontoghiorghes 1986, 1987a, b; Lawson et al. 1967; Yamamoto et al. 1971).

Developing erythroid cells with their large uptake of iron from transferrin and their ability to put considerable amounts of iron into both haem and ferritin provide a good model system for studying chelators. In this work the donation of iron and its intracellular distribution by chelators and by transferrin in the presence of chelators was studied in human erythroid cells. The chelators used were 1-methyl (L1), 1-ethyl (L1NEt) and

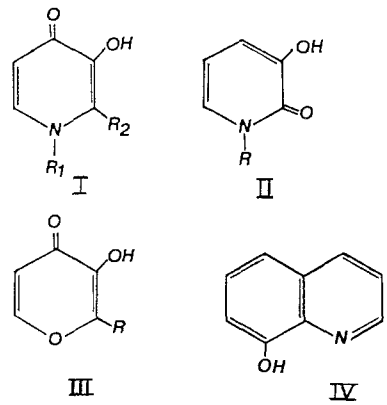

Fig. 1. Structure of the chelators. (I) $R_2=CH_3$ $R_1=CH_3$ (L1), $R_1=CH_2CH_3$ (L1NEt), $R_1=CH_2CH_2CH_3$ (L1NPr). (II) $R=CH_3$ (L2), $R=CH_2CH_3$ (L2NEt), $R=CH_2CH_2CH_3$ (L2NPr). (III) $R=CH_3$ (maltol). (IV) 8-Hydroxyquinoline

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1-propyl (L1NPr) 3-hydroxy-2-methylpyrid-4-ones; the 1-methyl (L2), 1-ethyl (L2NEt) and 1-propyl (L2NPr) 3-hydroxypyrid-2-ones, mimosine, maltol, 8-hydroxyquinoline, tropolone, tetracycline and desferrioxamine (Fig. 1). All the chelators with the exception of mimosine, desferrioxamine and tetracycline are neutral. From the neutral chelators L1, L1NEt and L2 form relatively more hydrophilic iron complexes with lower lipid/water partition coefficients K_{par} ($K_{par} < 0.5$) than the other more lipophilic chelators ($K_{par} > 0.5$).

The aim of this study was to identify the mode of interaction of these chelators or their iron complexes with erythroid cells and in particular whether any of these were able to donate iron to haem, ferritin and other cell compartments in the presence and absence of transferrin.

Materials and Methods

Materials. L1, L1NEt, L1NPr, L2, L2NEt and L2NPr were prepared as previously described (Kontoghiorghes 1982; Kontoghiorghes and Sheppard 1987). Mimosine, 8-hydroxyquinoline and tropolone were obtained from Sigma London; maltol from Aldrich UK, desferrioxamine from Ciba-Geigy UK and tetracycline from Lederle, UK. Transferrin used was apotransferrin (Behringwerke). ^{59}Fe saturation of transferrin (8 mg/ml) to about 40% was achieved using [^{59}Fe]iron citrate (Amersham International; approx. 10 μCi/μg; 10 μg/ml) in the presence of excess HCO_3^- and cleared of unbound iron citrate using an anion-exchange resin (Cavill 1971). The chelators were used as 2 mM aqueous solutions, stored at 4° C. For chelator iron uptake experiments, $^{59}FeCl_3$ (Amersham International, approx. 10 μCi/μg; 10 μg/ml) was diluted fivefold with water and added to the chelator. This was then made isotonic with 2× phosphate-buffered saline. The final chelator/iron molar ratio was 139:1 for three experiments and 24:1 for another.

Cells. K562 cells were harvested following on overnight incubation after subculturing. Bone marrow cells were obtained from patients undergoing total hip replacement surgery (all samples taken in the manner approved by the S. G. Joint Ethical Committee). Pure erythroblast fractions (E) were prepared from fresh human bone marrow by antibody lysis of non-erythroid cells followed by Percoll density gradient fractionation (Ali et al. 1982). E_1 is the least dense (76% erythroid: 20% early erythroblasts, 20% intermediate, 60% late and a few red cells), E_2 is of intermediate density (92% erythroid: 7% early, 30% intermediate, 64% late and red cells equivalent in number to the nucleated cells) and E_3 the most dense fraction (99% erythroid: 0.3% early, 23% intermediate, 77% late and many red cells). Eagles minimum essential medium, buffered to pH 7.4 at 37° C with 20 mM Hepes and 1.3 mmol/l glycine, was used for washing and incubating the cells. Before incubation, the cells were washed several times at room temperature in incubation medium without transferrin. Further washings at 37° C (Hemmaplardh and Morgan 1974) were found not to increase transferrin binding (Hodgetts 1984) and so were not used.

Iron uptake experiments. Six experiments were carried out: four measuring ^{59}Fe delivery from the chelators (two using K562 cells, one using bone marow cells and one using erythroblasts) and two preliminary ones measuring the effect of four chelators on ^{59}Fe delivery from transferrin using bone marrow cells. The number of nucleated cells per tube was kept constant for each experiment but between experiments they varied over $(1\text{–}13) \times 10^6$. Iron uptake was carried out by adding equal amounts of ^{59}Fe/transferrin or ^{59}Fe/chelator (20–60 μl) to 0.4 ml cell suspension and incubating for 1 h at 37° C. For the experiments in which the iron-free chelators were tested for their effect on transferrin delivery of ^{59}Fe to human bone marrow cells, exactly the same procedure was followed except that the addition of $^{59}FeCl_3$ was omitted and the chelators were added to the cells just prior to the start of the incubation with ^{59}Fe/transferrin.

Uptake and incorporation of ^{59}Fe. ^{59}Fe uptake and ^{59}Fe incorporation into the cell stroma, haem and ferritin were estimated using previously described methods (May et al. 1982). The cell stroma is that fraction spun down at 45000 *g* from sonicated cells; the haem was extracted using acidified butanone and ferritin was immunoprecipitated. All determinations are single ones.

Results

The results show a great difference in the abilities of the chelators to donate iron to the erythroid cells (Ta-

Table 1. Uptake and distribution of ^{59}Fe from chelators and transferrin by uninduced K562 cells

Chelator	^{59}Fe uptake into cells (cpm)	^{59}Fe in stroma (%)	^{59}Fe in ferritin (%)	^{59}Fe in haem (%)	^{59}Fe in haem (cpm)
L1	28	*	*	*	*
L1NPr	414	41	27	1.4	5.8
L2	459	62	15	0.6	2.6
L2NPr	1812	40	30	0.8	13.8
Tropolone	2003	35 (28)	33 (41)	1.2 (0.4)	24.5
8-Hydroxyquinoline	4477	38 (27)	34 (37)	0.8 (0.6)	35.8
Maltol	582	58	15	0.6	3.7
Mimosine	5	*	*	*	*
Tetracycline	583	64 (69)	12 (15)	0.3 (*)	1.8
Transferrin	996	35 (34)	32 (40)	1.3 (0.5)	12.6
Desferrioxamine	—	— (*)	— (*)	— (*)	—

4 (or 2) × 10^6 cells were incubated for 1 h at 37° C in the presence of chelator/^{59}Fe (0.66 μmol/l ^{59}Fe; 92 μmol/l chelator) or transferrin/^{59}Fe (11 μmol/l ^{59}Fe; 15 μmol/l transferrin). An asterisk indicates that ^{59}Fe uptake too low to measure accurately. Results in parentheses are from a different experiment

Table 2. ^{59}Fe uptake into haem by bone marrow cells from chelator/^{59}Fe or transferrin/^{59}Fe

Chelator	^{59}Fe uptake into cells % total	^{59}Fe uptake into cells (cpm)	%^{59}Fe in haem
L1	0.1	49	*
LINEt	0.09	34	*
LINPr	49	20820	2.7
L2	6.2	2628	8.8
L2NEt	32	13620	1.3
L2NPr	56	23700	2.5
Tropolone	23	9552	9.0
8-Hydroxyquinoline	34	14460	5.6
Maltol	58	24420	1.9
Mimosine	0.02	11.4	*
Tetracycline	4	1638	9.0
Desferrioxamine	0.01	4.2	*
Transferrin	1.1	3122	30.3
Transferrin	1.2	2934	—

13×10^6 nucleated bone marrow cells (1×10^6 nucleated erythroid cells) were incubated for 1 h at 37° C in the presence of ^{59}Fe/chelator (0.63 μmol/l ^{59}Fe; 88 μmol/l chelator) or transferrin/^{59}Fe (11 μmol/l ^{59}Fe; 15 μmol/l transferrin). An asterisk indicates ^{59}Fe uptake which was too low to measure accurately

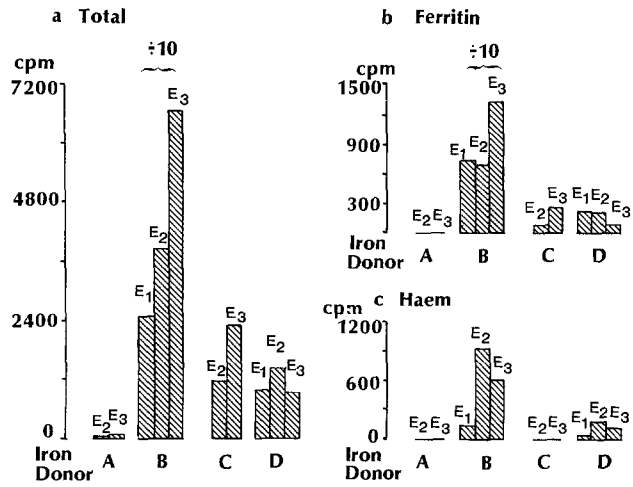

Fig. 2. Chelator iron uptake by erythroblasts. Iron uptake per 1×10^6 erythroblasts from (**A**) mimosine/^{59}Fe, (**B**) 8-hydroxyquinoline/^{59}Fe, (**C**) tetracycline/^{59}Fe, (chelator:iron 24:1; ^{59}Fe = 2.4 μmol/l) and (**D**) transferrin/^{59}Fe; (transferrin = 10.0 μmol/l; ^{59}Fe = 7.0 μmol/l)

bles 1 and 2). Three of the chelators form charged complexes with iron. Two of these (mimosine and desferrioxamine) were ineffective at delivering iron. In contrast, the tetracycline complex was a reasonable iron donor. Of the neutral iron-chelator complexes only L1 and L1NEt, the least lipophilic of the 3-hydroxypyrid-4- and -2-one derivatives, were ineffective at iron delivery. The iron delivery from the 3-hydroxypyrid-2-one derivatives (L2, L2NEt, L2NPr) was greater than the analogous less lipophilic 3-hydroxypyrid-4-one derivatives (L1, L1NEt, L1NPr). Maltol, 8-hydroxyquinoline and tropolone were also effective iron donors. Table 2 shows that, while cell uptake of transferrin iron was only a small percentage of the total, uptake from some of the chelators was significantly higher than transferrin.

In four experiments (one on bone marrow, two using K562 cells and one on pure erythroblast fractions from human bone marrow) the intracellular distribution into haem or into haem and ferritin of the radioactive iron subsequent to its uptake was measured (Tables 1 and 2, Fig. 2) and compared with that of transferrin iron. Except for those chelators which show an increased proportion of ^{59}Fe in the stroma (L2, tetracycline and maltol) and a consequently decreased proportion in ferritin and haem, the proportion of ^{59}Fe in ferritin was very similar irrespective of the source of ^{59}Fe.

All the chelators which delivered iron to the cells in measurable amounts also managed to deliver iron to haem with variable efficiency (Tables 1 and 2). Efficiency here relating to the percentage iron delivered to the cell that was incorporated into haem. When compared with transferrin this proportion was in most cases low.

Since some of these chelators are so effective at delivering iron to cells a much larger amount of iron could be delivered to both ferritin and haem than by transferrin (Table 1, Fig. 2). Those chelators delivering most iron to haem in the bone marrow cells are the most lipophilic of the 1-alkyl-3-hydroxy-2-methylpyrid-4-one and 1-alkyl-3-hydroxypyrid-2-one series, the neutral maltol, 8-hydroxyquinoline and tropolone. These same chelators were found to deliver most iron to ferritin and haem in K562 cells. Out of the four chelators tested for their effect on iron uptake from transferrin by bone marrow cells (Table 3), tropolone was found to decrease the amount of iron incorporated into haem by over 40% and 8-hydroxyquinoline by over 80%. On the other hand, desferrioxamine and tetracycline made little difference (Table 3).

Table 3. The effect of chelators on the uptake of ^{59}Fe from transferrin by bone marrow cells

Chelator	^{59}Fe uptake into			
	cells (cpm)		haem (cpm)	
	A	B	A	B
Tetracycline	3240	1410	1199	984
8-Hydroxyquinoline	2676	1002	174	182
Tropolone	2436	870	709	590
Desferrioxamine	1956	1818	1051	1116
None (control)	2076	1614	1121	1062

(A) 3×10^6 or (B) 4×10^6 nucleated bone marrow cells incubated for 1 h at 37° C in the presence of 83 μmol/l (A) or 91 μmol/l (B), chelator and transferrin/^{59}Fe (6 μmol/l ^{59}Fe; 8.3 μmol/l transferrin)

Discussion

In this study we have shown that chelators differ in their ability to donate iron to erythroid cells, with substantial amounts of iron taken up from neutral/lipophilic chelator iron complexes in contrast to neutral/hydrophilic or charged complexes. In all the cases of ^{59}Fe uptake by cells the ^{59}Fe supplied in the form of chelator/^{59}Fe became incorporated into haem and ferritin, demonstrating that the ^{59}Fe was not simply confined to the cell membrane and extracellular or intracellular vesicles.

The purification of the erythroid cells and their separation into different density fractions allowed for the investigation of homogeneous populations. In the experiments described here, these cells had a very low capacity for ^{59}Fe incorporation into haem. Nevertheless, the results from their use confirmed the findings from K562 cells that much larger amounts of ^{59}Fe can be incorporated from chelator/^{59}Fe into erythroblast ferritin and haem than from partially saturated transferrin and even, in the case of ferritin at least, from that expected from fully saturated transferrin. Several of these chelators can therefore be used as iron donors independently or in conjunction with transferrin.

That two of the lipophilic chelators which were excellent iron donors could also inhibit iron incorporation into haem or cells from transferrin had previously been shown to be the case for pyridoxal isonicotinoyl hydrazone and other lipophilic chelators (Ponka et al. 1979; Forsbeck et al. 1987). Such chelators may perform a dual role of iron removal from certain pathways and indiscriminate iron loading. Such a diversion and redistribution of iron may lead to toxicity as previously shown in studies with leukaemic cells (Kontoghiorghes et al. 1986) and matured red cells (Kontoghiorghes 1988). In contrast, desferrioxamine and L1, which are hydrophilic and not good iron donors, have comparatively lower toxicity, i.e. higher median lethal doses in rats, than the more lipophilic L1NEt and L1NPr (Kontoghiorghes and Sheppard 1987; Kontoghiorghes 1987b).

The interactions of some chelators with erythroid cells observed in this study may have relevance to their effects in vivo. For example, increased absorption and body incorporation of iron was observed following the intragastric administration of neutral, lipophilic, soluble, iron complexes to mice. In all cases, the absorbed iron was delivered to the erythroid cells of the bone marrow and utilised primarily for the synthesis of haemoglobin (Kontoghiorghes 1990). In another study, where L1 was used in clinical trials with anaemic rheumatoid arthritis patients, there was an apparent increase in the production of haemoglobin (Vreugdenhil et al. 1989). This increase may be associated with the mobilisation of iron from the reticuloendothelial system by L1 its exchange with transferrin and its utilisation for the production of haemoglobin.

In conclusion, chelators can donate iron to all erythroid cells to a different extent depending on their lipophilicity. Those chelators delivering iron to erythroid cells in significant amounts can deliver iron to both haem and ferritin and should be useful in the investigation of intracellular iron metabolism although none showed exclusive delivery to, or rejection of, a particular pathway.

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