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FREE RADICAL AND CYTOTOXIC EFFECTS OF CHELATORS AND THEIR IRON COMPLEXES IN THE HEPATOCYTE

L.J. MOSTERT, J.A.L.M. VAN DORST, J.F. KOSTER†, H.G. VAN EIJK

Department of Chemical Pathology and Biochemistry I†, Erasmus University Rotterdam, Rotterdam. The Netherlands

and

G.J. KONTOGHIORGHES

Department of Haematology, The Royal Free Hospital, London, United Kingdom

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In a comparative screening study of chelators intended for clinical use eleven iron chelators have been tested for their ability to mobilize (^{59}Fe) iron from ^{59}Fe-labelled ferritin and from hepatocytes of rats labelled with ^{59}Fe-transferrin. The toxic effects of the chelators were also studied using microsomal lipid peroxidation induced by Fe^{3+}/ADP and NADPH. From these tests it was shown that 1,2-dimethyl 3-hydroxypyrid-4-one (L1) and mimosine were the most effective iron chelators in iron mobilization and did not catalyse lipid peroxidation. In conclusion it can be stated that besides to investigate the iron binding capacity of new chelators also their ability to catalyse lipid peroxidation has to be ruled out.

KEY WORDS: Iron chelators, Ferritin, Hepatocytes, Lipid peroxidation, Iron mobilization.

INTRODUCTION

Most of the intracellular iron is stored in ferritin. This storage is generally considered as a protective mechanism in contrast to the toxicity exerted by "free" iron which catalyses the formation of hydroxyl or hydroxyl like radicals.[1-3] Desferrioxamine is the only clinically used iron chelator for the treatment of transfusional iron overload. However it is highly expensive and not orally active. Several experimental iron chelators were shown previously to be orally active in animals[4-7] but none yet reached the state for clinical use.

Iron bound to a chelator can either increase, e.g. Fe^{3+}-EDTA or decrease e.g. ferrioxamine, the process of lipid peroxidation and other forms of free radical damage.[8-10] These processes can lead to severe tissue damages. Therefore it is of importance to rule out a possible lipid peroxidation catalysis by the iron-chelator complexes before the chelator is used *in vivo*.

In this paper a chelator screening system involving:

a. the removal of iron from ferritin
b. the intracellular mobilization of ^{59}Fe from ^{59}Fe-labelled hepatocytes
c. the capacity of the iron-chelator complex to catalyse the process of lipid peroxidation

has been used to examine eleven iron chelators including those containing a hydroxypyridone iron binding site, which were previously shown to be effective in the mobilization of iron from transferrin[11] and ferritin[12] and also *in vivo*.[6]

MATERIALS AND METHODS

All reagents were of analytical reagent grade. Desferrioxamine, DTPA and desferrithiocin were obtained from Ciba-Geigy (Switzerland). 1,2-Dimethyl 3-hydroxy pyrid-4-one (L1) was prepared according to the method of Kontoghiorghes[13] similar to the preparation of other pyridone derivatives[14] and 2-hydroxy 4-methoxy pyridine-N-oxide (L6) was prepared according to the method of Mizukami *et al.*[15] 2-Hydroxy pyridine-N-oxide (L4), 2-methyl 3-hydroxypyr-4-one (maltol) and 2-hydroxy 5-hydroxymethylpyr-4-one (kojic acid) were obtained from Aldrich (Gillingham, U.K.).

1-Aminopropionic acid 3-hydroxypyrid-4-one (mimosine), 3,4-dihydroxybenzoic acid, 8-hydroxyquinoline and 2-mercaptopyridine-N-oxide (omadine) were obtained from Sigma (U.K.). The chemical formula and the structural formula of some chelators are given in Table I. Apoferritin from horse spleen was obtained from Boehringer (W.-Germany). ^{59}Fe-citrate was obtained from Amersham (U.K.). Collagenase from *Clostridium histolyticum* was obtained from Sigma (U.S.A.), Sepharose-CNBr and Sephadex G-50 were obtained from Pharmacia (Sweden), DEAE-Trisacryl M was obtained from LKB (Sweden).

Preparation of the hepatocyte suspension

^{59}Fe-transferrin (60 μCi, 1 mg transferrin) was injected in a male Wistar rat (300–380 g) and after three days a perfusion was peformed as described by Dekker *et al.*[16] the isolated hepatocytes were then suspended in an incubation medium containing 50 mM Hepes, 110 mM NaCl, 5 mM KCl, 1.6 mM $CaCl_2$, 0.74 mM $MgCl_2$ and 0.25% BSA, to a final cell concentration of 1–3* 10^7 hepatocytes/ml.

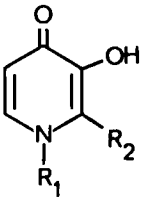
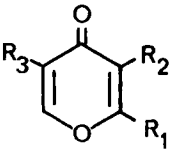
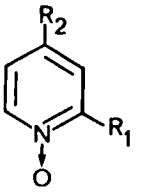
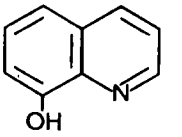
Preparation of the 80,000 g cytosol

After incubating the hepatocytes for 80 minutes with a chelator solution (4 mM) at 37°C in a shaking waterbath, the hepatocytes were separated from the incubation medium by centrifugation at 1700 g for 10 min. Subsequently the cell pellet was lysed in 4 ml bidistillated water with a Branson B-10 sonifier for 2 min. while cooling on ice. Finally the homogenate was centrifuged in a Beckman LB-70 ultracentrifuge at 80,000 g for 45 min. and the supernatant (= 80,000 g cytosol) was separated from the pellet.

Hepatocyte iron mobilization experiments

Three chelators resp. desferrioxamine, L1 and mimosine were added to a hepatocyte suspension (preparation described earlier) to a final concentration of 1 mM. Their effect on iron mobilization was compared to that of a blank which consisted of the hepatocyte suspension without any addition. At t = 0, 20 and 80 min. 1 ml samples were taken. All samples were centrifuged at 1700 g and the cell pellet and incubation

TABLE I

Chelator	chemical formula	structural formula	substituents
L1	1,2-dimethyl 3-hydroxy pyrid-4-one		R_1=-CH_3 R_2=-CH_3
Mimosine	1-aminopropionic acic 3-hydroxy pyrid-4-one		R_1=-CH_2-CH(NH$_2$)-COOH R_2=-H
Kojic acid	5-hydroxy 2-hydroxymethyl pyr-4-one		R_1=-CH_2-OH R_2=-H R_3=-OH
Maltol	2-methyl 3-hydroxy pyr-4-one		R_1=-CH_3 R_2=-OH R_3=-H
L4	2-hydroxy pyridine-N-oxide		R_1=-OH R_2=-H
L6	2-hydroxy 4-methoxy pyridine-N-oxide		R_1=-OH R_2=-OCH_3
Omadine	2-mercapto pyridine-N-oxide		R_1=-SH R_2=-H
8-hydroxyquinoline			

medium separated. To correct for leakage of cellular labelled ferritin-iron in the incubation medium, this was treated with anti-rat ferritin coupled with Sepharose beads in order to precipitate the labelled ferritin. After incubation for 1 hour at 25°C with anti-rat ferritin all samples were centrifuged at 1700 g. The ferritin containing pellet and the incubation medium were separated. Next all samples were counted in a gamma counter (Packard 500 C auto gamma spectrophotometer). From radioactiv-

ity present in cell pellet and incubation medium the percentage of iron mobilization can be calculated. The rate of iron mobilization was calculated from the slope of the mobilization curve (not shown) between $t = 20$ and $t = 80$ min.

Gelfiltration of the 80,000 g cytosol on Sephadex G-50

One ml of the 80,000 g cytosol was applied on a Sephadex G-50 column (55 × 3 cm) and eluted with a buffer containing 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.2. The flow rate was 33 ml/hr. After fractionation all samples were counted in a gammacounter (Packard 500 C auto gamma spectrometr). The percentage ^{59}Fe in each fraction was calculated and compared to the total hepatocyte radio-iron.

Preparation of homogeneous ^{59}Fe-labelled horse spleen ferritin

Iron was incorporated into apoferritin by adding a mixture of ^{59}Fe ($^{59}FeCl_3$) and ^{56}Fe (ferrous ammonium sulfate) to give a ferritin iron content of 2000 +/− 100 Fe atoms/molecule as described by Hoy *et al.*[20]

Equilibrium dialysis of chelator against ^{59}Fe-labelled ferritin

One ml of the labelled ferritin solution was diluted with a 0.1 M phosphate buffer pH 7.4 to a final volume of 8 ml. The protein concentration was 150 μg/ml and ferritin contained 2200 ± 100 Fe atoms/molecule. The chelator was dissolved in 8 ml 0.1 M phosphate buffer pH 7.4 to a concentration of 2.5 mM.

The ferritin and the chelator solution were dialysed in a counter current dialysator for at least 24 hr. The solutions were separated by using a semi-permeable membrane (Cuprophane, Technicon), the flowrate was 4 ml/min. At different time intervals 1 ml sample was taken from the chelator solution in a gammacounter and put back into the chelator solution. The iron mobilization was calculated from the radioactivity present in the chelator solution at the indicated time interval and the total radioactivity present in the ferritin solution at $t = 0$.

As a control total iron in the chelator and the ferritin solution was measured spectrophotometrically using ferrozine at 562 nm after a Kjeldahl destruction. There was an excellent correlation between ^{59}Fe and total iron mobilization measured by these two methods (correlation 0.993).

Inhibition of microsomal lipid peroxidation induced by Fe^{3+}/ADP and NADPH

Microsomes were isolated as previously described[18] and dissolved in Tris-HCl 1 M, pH 7.5, to give a protein concentration of 1 mg/ml. A control mixture was prepared by adding a solution of Fe^{3+} (final conc. 0.1 mM), ADP (final conc. 0.5 mM) and NADPH (final conc. 0.4 mM) to the solution containing the microsomes. Samples were taken on $t = 0$, 5, 10, 15 and 20 min. In all samples the malondialdehyde formed was estimated spectrophotometrically.[19] The effect of the chelators was examined by adding to the incubating mixture a 4-fold excess chelator to iron. The absorbance at 532 nm was plotted against time. At $t = 20$ min. the absorbance at 532 nm of the control solution was taken 100% lipid peroxidation and from the absorbance at 532 nm of the chelator solution at $t = 20$ min. the percentage catalysis of lipid peroxidation was calculated using the equation:

$$\frac{(A\,(532\,nm)\,chelator)\,t\,=\,20^{*}\,100\%}{(A\,(532\,nm)\,control)\,t\,=\,20}$$

RESULTS

Iron mobilization from ferritin

The release of iron from ferritin as a function of time using L1, mimosine and 8-hydroxyquinoline is shown in Fig. 1 and the mobilization of (^{59}Fe) iron in 24 hr by the chelators is shown in Table II.

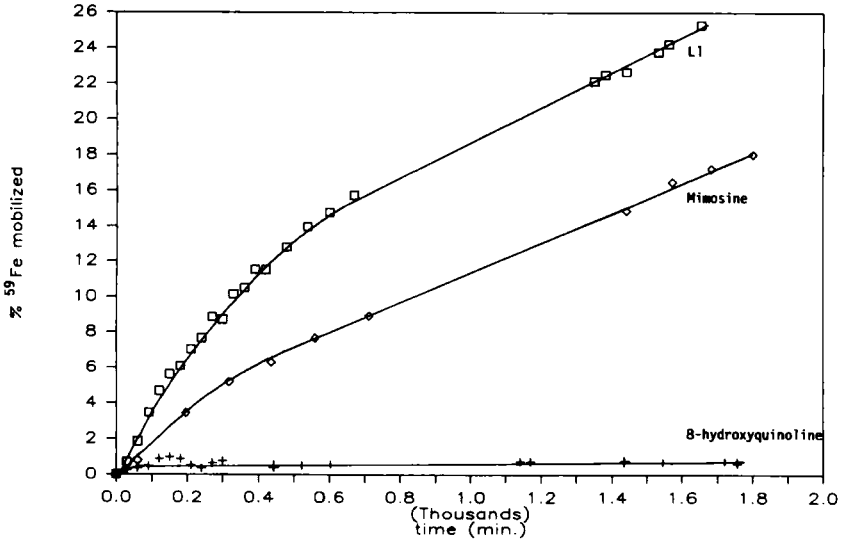


FIGURE 1 Equilibrium dialysis of ^{59}Fe-labelled horse spleen ferritin against chelator dissolved in 0.1 M phosphate buffer pH 7.4. Ferritin 150 μg/ml, chelator 1.25 mM, ferritin contained 2200 Fe atoms/molecule.

TABLE II
Iron mobilization from ^{59}Fe labelled horse spleen ferritin. Ferritin 150 μg/ml, chelator 1.3 mM, ferritin 2200 Fe atoms/molecule

chelator	%^{59}Fe mobilization in 24 hr.
desferrioxamine	12
L1	21
L4	23
Maltol	9
L6	16
Mimosine	14
8-hydroxyquinoline	1
2-mercapto pyridine-N-oxide	2
3,4-dihydroxybenzoic acid	3
DTPA	5
Desferrithiocin	6
Kojic acid	6

Intracellular accumulation

A typical curve for the elution of the hepatocyte 80,000 g cytosol following incubation of intact ^{59}Fe-labelled hepatocytes with L1 over Sephadex G-50 is shown in Fig. 2. Besides the ^{59}Fe located in ferritin also a considerable amount (7.2%) of the total hepatocyte ^{59}Fe was present inside the cel and appeared as a low molecular weight complex. The intracellular formation of a low molecular iron pool induced by the various chelators is shown in Table III. The chelators L1, maltol, mimosine, desferrioxamine and DTPA caused higher increases of intracellular ^{59}Fe release than the other chelators. At the end of each incubation experiment cell viability was determined with Trypan Blue and LDH determinations. While 8-hydroxyquinoline resulted in a high percentage of non-viable cells (96%), the remaining chelators always had a cell viability of 70–80% at $t = 80$ min, the same as the control had.

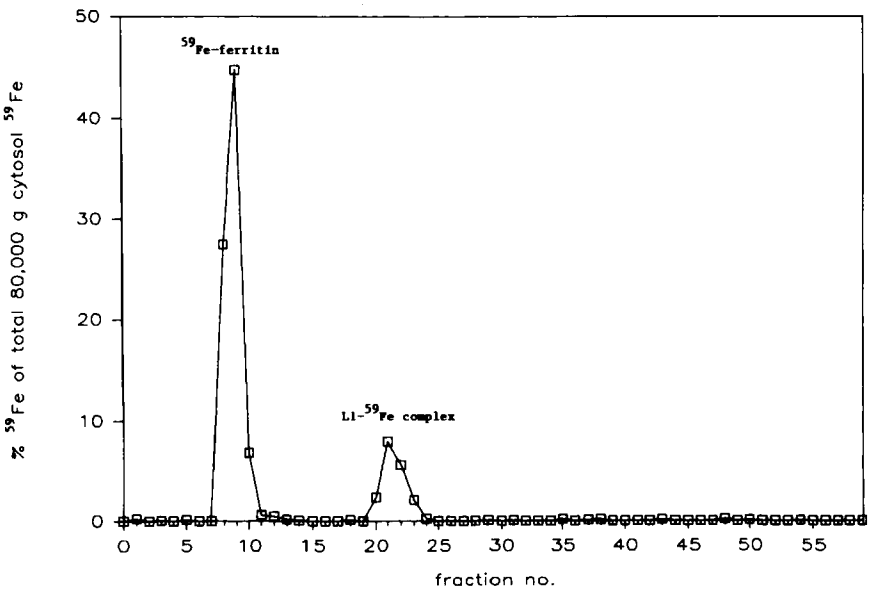


FIGURE 2 Gelfiltration of the 80,000 g cytosol of L1 over Sephadex G-50.

TABLE III
Intracellular LMW ^{59}Fe pool induced by chelators in the hepatocyte

chelator	charge	polarity of iron complex	% LMW ^{59}Fe
desferrioxamine	charged	hydrophilic	4.6
L1	neutral	hydrophilic	7.2
L4	charged	lipophilic	0.9
Maltol	neutral	lipo/hydrophilic	3.7
L6	charged	lipophilic	0.4
Mimosine	charged	hydrophilic	4.3
8-hydroxyquinoline	neutral	lipophilic (cytotoxic)	0
2-mercapto pyridine-N-oxide	charged	lipophilic	0.7
3,4-dihydroxybenzoic acid	charged	lipo/hydrophilic	0.6
DTPA	charged	hydrophilic	6.7
Desferrithiocin	charged	hydrophilic	7.8
Kojic acid	neutral	hydrophilic	0.3

Lipid peroxidation

The effect of L1, L4 and maltol on microsomal lipid peroxidation compared to the extent of lipid peroxidation induced by Fe^{3+}/ADP is shown in Fig. 3. Table IV summarizes the results obtained for the various chelators to catalyse lipid peroxidation.

Iron mobilization from ^{59}Fe-labelled rat hepatocytes

Because L1 and mimosine released iron from ferritin most effectively and did not cause lipid peroxidation, their ability to release (^{59}Fe) iron from rat hepatocytes was investigated. Desferrioxamine was taken as a kind of reference to which L1 and mimosine were compared. From Table V can be seen that L1 did mobilize more iron from hepatocytes than desferrioxamine while mimosine did not.

DISCUSSION

In our screening procedure for iron chelators, described in the introduction, an "ideal" chelator for the treatment of iron overload should fullfill, amongst others, the following criteria.

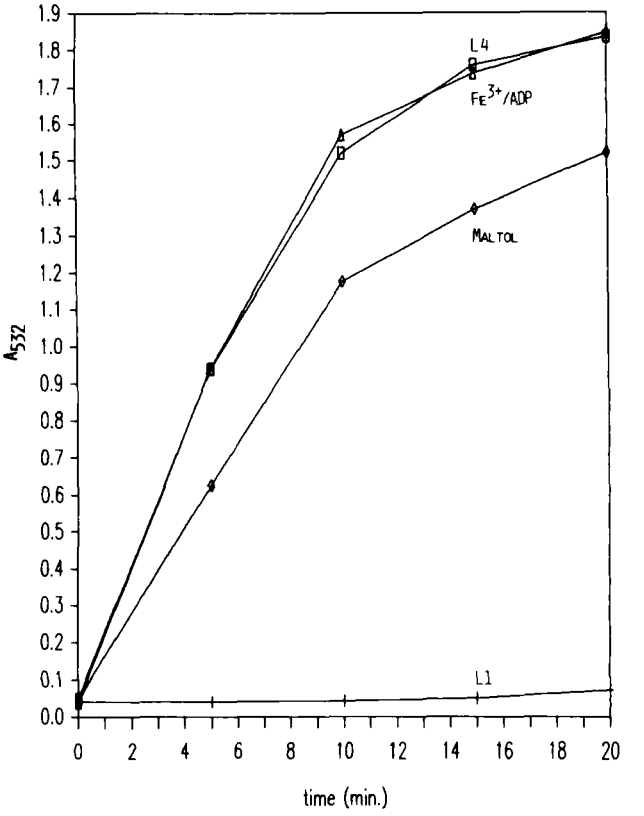


FIGURE 3 Microsomal lipid peroxidation induced by Fe^{3+}/ADP and NADPH. Chelator 0.4 mM, Fe^{3+} 0.1 mM, ADP 0.4 mM, NADPH 0.4 mM and rat liver microsomes 1 mg (protein)/ml.

TABLE IV
Microsomal lipid peroxidation. Microsomes 1 mg/ml, Fe^{3+} 0.1 mM, ADP 0.5 mM, NADPH 0.4 mM, chelator 0.4 mM. Incubation time 20 minutes

chelator	% catalysis lipid peroxidation
Fe^{3+}/ADP (control)	100
desferrioxamine	0
L1	0
L4	87
Maltol	100
L6	82
Mimosine	0
8-hydroxyquinoline	0
2-mercapto pyridine-N-oxide	0
3,4-dihydroxybenzoic acid	85
DTPA	0
Desferrithiocin	65
Kojic acid	100

TABLE V
Iron mobilization from ^{59}Fe-labelled rathepatocytes by the chelators L1, mimosine and desferrioxamine. The hepatocyte suspension contain $1\text{-}3 \cdot 10^7$ hepatocytes/ml, the incubation temperature was 37°C. The chelator concentration was 1 mM. The iron mobilization in 1 hr is calculated as described in Materials and Methods

% ^{59}Fe mobilization in 1 hr experiment	1	2	3	$\bar{x} \pm$ sd.
desferrioxamine	0.86	0.78	0.73	0.79 ± 0.05
L1	0.92	1.12	0.87	0.97 ± 0.11
Mimosine	0.25	n.d.	0.15	0.20 ± 0.05

First the chelator must be able to mobilize iron from ferritin since most of the iron in the body is stored as ferritin and/or haemosiderin. Most effective compared to desferrioxamine were L1, L4, L6 and mimosine (Table II).

Second the chelator should cause the removal of stored iron out of the cell into the circulation with subsequent urinary or faecal excretion. From Table III it can be seen that the chelators L1, maltol, mimosine, DTPA, and desferrioxamine caused expansion of the intracellular LMW ^{59}Fe pool (^{59}Fe-chelator complex). In relation to the chelator structure, the neutral and charged hydrophilic chelators accumulate to a greater extent in the hepatocyte cytosol than lipophilic chelators probably because the former have the same hydrophilic character as the cytosol. Additional data for desterrioxamine, L1 and mimosine presented in Table V, show an increased ^{59}Fe release from hepatocytes. It seems there is a good correlation between release of iron from ferritin (Figure 1, Table II) and hepatocytes (Table V) following the short term (80 min.) incubation with desferrioxamine, L1 and mimosine. This slow release of iron, which is minute in comparison to the total iron stored is indicative of the problems associated with polynuclear iron mobilization *in vivo*[12] and in iron overloaded patients.

A third important criterion for chelators intended for clinical use is that the chelators and the iron-chelator complexes should not be toxic. This toxicity can possibly be ascribed to lipid peroxidation by the iron-chelator complex.

For that reason it is particularly important in this respect to investigate whether iron complexes are able to catalyse lipid peroxidation. We propose that chelators

whose iron-chelator complexes catalyse lipid peroxidation (Table IV) are not useful for further in vivo screening. However the screening for other forms of toxicity e.g. cytotoxicity is also important because it may identify other compounds which do not cause lipid peroxidation and are yet highly toxic e.g. 8-hydroxyquinoline.

Combining the results from all experiments together (Table II, III and IV) only two chelators L1 and mimosine fullfill the three criteria mentioned above, and are worth being developed for clinical use. It must be noted that L1 has already been administered intragastrically in normal and iron overloaded mice[17] and was as effective as subcutaneous desferrioxamine. In iron overloaded rabbits orally administered L1 is roughly as effective as desferrioxamine (s.c).[6] It can be concluded that this test system could be useful for an initial screening of iron chelators intended for clinical use.

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