Free Rad. Res. Comms., Vol. 2, No. 2, pp. 115-124
8755-0199/86/0202-0115 $25-00/0

Printed in Great Britain

IN VITRO SCREENING OF IRON CHELATORS USING MODELS OF FREE RADICAL DAMAGE

GEORGE J. KONTOGHIORGHES, MALCOLM J. JACKSON,† JOSEPH LUNEC††

Department of Haematology, Royal Free Hospital School of Medicine, Pond Street, Hampstead NW3 2QG.
† *University Department of Medicine, Royal Liverpool Hospital, P.O. Box 147, Liverpool L69.*
†† *Biochemistry Department, Selly Oak Hospital, Raddlebarn Road, Birmingham. B29 6JD*

(Received January 31, 1986, in final form March 11, 1986)

The effect of chelators on free radical damage to deoxyribose induced by iron, on IgG by UV irradiation, and on mouse muscle by homogenisation has been studied using the Thiobarbituric acid method and the increase in fluorescence in IgG mixtures.

Although there were some variations on the effects of the chelators in the three models, it was shown that most of the chelators could inhibit the noxious effects of the free radicals and some which are orally effective in increasing iron excretion in animals could be potentially useful in preventing iron toxicity in related pathogenic diseases.

Key words: Chelators, iron, Deoxyribose, IgG, tissue homogenates.

INTRODUCTION

Iron is an essential metal for normal cell growth and development. Specific biomolecules have been evolved for its regulation, e.g. the siderophores in fungi and bacteria[1], and the proteins of iron transport and storage in animals namely transferrin and ferritin. If the regulatory processes controlling iron break down and "free", non protein bound iron is present or if iron is released during cellular damage, an increase in the production of oxygen activated products and other radical species may occur which would potentially damage all known biomolecules, cells and tissues. Other biomolecules such as superoxide dismutase, catalase and Vit. E. have been evolved to prevent or elimate toxic oxygen species such as O_2^-, H_2O_2 and $OH^\cdot$ which could be generated by iron catalysis. Mechanisms of iron toxicity involving production of toxic oxygen radicals have been proposed in human diseases such as iron overload in thalassaemia[2,3] and in rheumatoid arthritis[4]. Iron chelators could, in principle, be used to

Author for correspondence: G.J. Kontoghiorghes.

minimise this damage by chelating effectively "free" iron and preventing its noxious catalytic properties. Desferrioxamine which is a fungal siderophore is the only established chelator which is used routinely in the treatment of tranfusional iron overload by daily 8–10h subcutaneous injections. However, this chelator was ineffective in rheumatoid arthritis patients and because of its high cost and oral inactivity is of limited use[5,6,33].

The most important property of an effective iron chelator *in vivo* is likely to be the ability to remove iron from the body, but an additional useful property would be the ability to inhibit iron catalysed production of oxygen radical species. We have therefore tested iron chelators for their ability to inhibit iron induced free radical damage on the sugar and DNA component deoxyribose on the protein IgG and on tissue homogenates. The chelators examined include the N-alkyl α-ketohydroxy pyridines which are orally active in increasing iron excretion in animals to comparable levels to that caused by injected desferrioxamine[7,8]; the naturally occurring α-ketochydroxy pyrones maltol and kojic acid whose iron binding properties are well established[9,10]; the catechol type chelators, 2,3 dihydroxybenzoic acid (2,3-DHB), pyrogallol, caffeic acid and catechol; purpurogallin which contains both an α-ketohydroxy and catechol binding sites, and 8-hydroxyquinoline a known β-hydroxyquinoline. With the exception of desferrioxamine (DF) which is a hexadentate chelator and binds iron at 1:1 molar ratio all other chelators are bidentate forming 3 chelator to 1 iron complexes at physiological pH.

MATERIALS AND METHODS

Chelators

The structure of the chelators is shown in Fig 1. 1,2-Dimethyl-3-hydroxyprid-4-one (L_1) was prepared as previously described[8] using a method similar to that of Harris[11] for the preparation of other pyridone derivatives. Two other methods of preparation of L_1 have also been reported[12,13]. 3-Hydroxy-1-methyl-pyrid-2-one (L_2) was prepared as previously described[8] similar to the method of Mohrle and Weber[14]; 1-hydroxy-6-methoxypyrid-2-one (L_6) was prepared by the method of Mizukami et al[15] and 1,2-dihydroxypyrid-2-one (L_3) from 2,4-dimethoxypyridine-N-oxide[16] by acid hydrolysis (20% HCl, 150°C, 13h)[8]. Maltol (L_5), kojic acid and purpurogallin were obtained from Aldrich Gillingham UK; catechol, 2,3-dihydroxy benzoic acid, caffeic acid, pyrogallol and 8-hydroxyquinoline from Sigma London, UK and desferrioxiamine from Ciba Geigy, Horsham UK.

1. *The effect of chelators on deoxyribose*

The thiobarbituric acid method was used to study the formation of malonaldehyde-like breakdown products of deoxyribose in the presence of iron, as previously described[17]. To deoxyribose (100 μl, 40 mM) H_2O_2 (20 μl, 200 μM) and ascorbic acid (20 μl, 200 μM) were added, followed by a fresh solution of ammonium ferrous sulphate (100 μl, 100 μM) and variable amounts of a chelator to a final concentration of 0–400 μM. The solution was made to a final volume of 1 ml using phosphate buffer (0.05 M, pH 7.4) and incubated at 26°C for 30 min. Trichloroacetic acid (1 ml, 2.8%) and thiobarbituric acid (0.5 ml, 1% in 0.5 M NaOH) was added and the

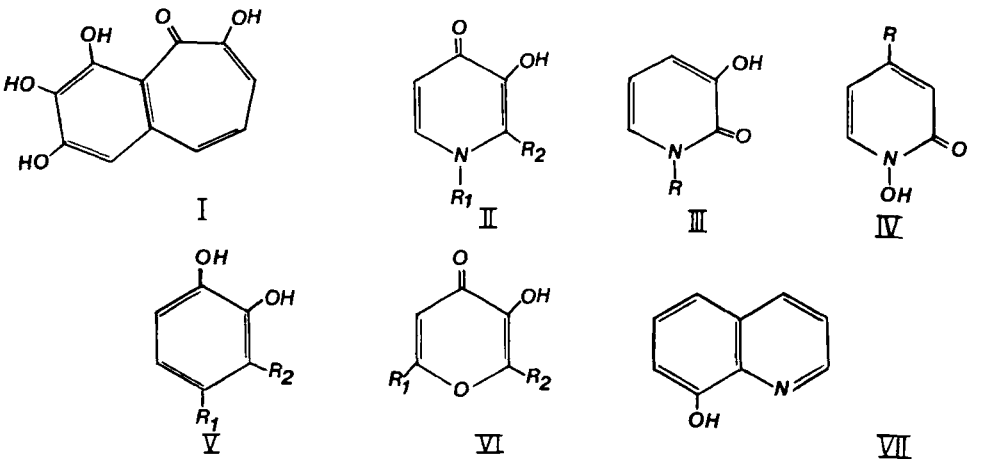

FIGURE 1 *The chemical structure of the chelators:*
I — purpurogallin; II — ($R_1 = R_2 = CH_3$) 1,2-dimethyl-3-hydroxypyrid-4-one (L_1); III — ($R = CH_3$) 3-hydroxy-1-methyl-pyrid-2-one (L_2); IV — ($R = OH$) 1,4-dihydroxypyrid-2-one (L_3); IV — ($R = OMe$) 1-hydroxy-4-methoxypyrid-2-one (L_6); V — ($R_1 = R_2 = H$) Catechol; V — ($R_1 = H$, $R_2 = COOH$) 2,3-dihydroxybenzoic acid (2,3 DHB); V — ($R_1 = H$, $R_2 = OH$) Pyrogallol; V — ($R_1 = H$, $R_2 = CH = CHOOH$) Caffeic acid; VI — ($R_1 = H$, $R_2 = CH_3$) Maltol (L_5); VI — ($R_1 = CH_2OH$, $R_2 = H$) Kojic acid; VII — 8-hydroxyquinoline.

mixture heated in boiling water for 20 minutes. The absorbance at 532 nm was measured when the solution reached room temperature.

2. *The effect of chelators on IgG*

The effect of UV irradiation on IgG in the presence of the chelators was studied as previously described[18,34].

UV-irradiation A 50 mmol/l Tris HCl buffer in 0.1 mol/l NaCl, pH 7.65 was used. Human IgG was dissolved to a final concentration of 2.5 g/l. 4 × 3 ml volumes were irradiated in matched quartz cuvettes, 1 cm^2 in cross-section. Three of the cuvettes contained 5 μM, 20 μM and 100 μM (final concentration) of chelator under test. The contents of all the cuvettes were then made up to 3.5 ml, including the fourth cuvette which served as the control reaction (IgG only). Irradiation at 254 nm and 366 nm was carried out at a distance of 6 cm from the light source. Flourescence intensity measurements were made at 15 minute intervals, for up to 1h, in the region 454 nm when excited at 360 nm.

Fluorescence Measurements All fluorescence measurements were performed on a Perkin Elmer MPF-3L scanning spectrofluorimeter (Perkin Elmer, Beaconsfield, Bucks. UK). The excitation and emission slits were set at 12 and 14 nm respectively; sensitivity settings ranged from × 0.1 to × 10. Wavelength Calibration was performed with quinine sulphate (10 mmol/l in 100 mmol/l H_2SO_4). Fluorescence Intensity Calibration was made with a polymer block standard (Perkin Elmer block 5, Compound 610, approximate concentrations 5×10^{-5} mol/l) to read 100 units on a

TABLE 1
The effect of chelators on the production of thiobarbituric acid-reacting substances, formed from damage to deoxyribose by iron (II)

Chelator	Chelator Concentration μm					
	5	10	50	100	200	400
	% Inhibition[b] of Deoxyribose breakdown					
L_1	0	0	33	53	75	80
L_2	0	0	76	81	85	86
L_3	5	9	82	86	87	88
L_4	4	2[a]	77	82	85	86
L_6	9[a]	8[a]	73	81	83	85
Maltol (L_5)	7	2[a]	7	49	65	80
Kojic Acid	15[a]	—	18	18	54	—
DF	9	33	73	90	91	91
EDTA	9	2	7[a]	24[a]	12[a]	20[a]
2,3-DHB	5	8	29	53	80	88
Caffeic Acid	7	—	25	30	54	—
Purpurogallin	8	—	5[a]	13[a]	19[a]	—
8-Hydroxyquinoline	4	23	74	79	82	87

a — Activation
b — % Inhibition of deoxyribose damage caused by chelators at different concentrations is expressed in comparison to thiobarbituric acid-reacting substances formed in incubations in buffer with no chelator added.

sensitivity scale × 1 at an excitation wavelength, 400 nm; emission wavelength 475 nm. All measurements were made at ambient temperature.

3. *The effect of iron chelators on lipid peroxidation in tissue homogenates*

Lipid peroxidation of mouse skeletal muscle tissue was initiated by homogenisation in potassium phosphate buffer (100 mM, pH 7.4) using a Potter Elvehjeim homogeniser. The homogenate was rapidly filtered through gauze and aliquots were either immediately mixed with the colour reagent to provide a measure of the basal content of thiobarbituric acid reacting substances (expressed as malonaldehyde content) or bubbled with oxygen for 2 mins and then incubated exposed to air for 2 hours at 37°C, either alone or in the presence of the different chelators. The final concentration of tissue in the incubation mixtures was 20 mg wet wt./ml. of buffer and all chelators were added to a concentration of 500 μM. The thiobarbituric acid reacting substances were measured at the end of the incubation as previously described[19].

RESULTS

The effect of the chelators on the breakdown of deoxyribose in the presence of 10 μM iron (II) is shown in Table 1. At low chelator concentrations (10 μM) where iron is not fully chelated small inhibition or activation was generally observed. As the ratio of the chelators to iron was increased the inhibition of the deoxyribose breakdown increased except in the case of EDTA and purpurogallin where activation occurred (Table 1, Fig. 2). Although maximum inhibition of 80–90% was shown in most cases at higher

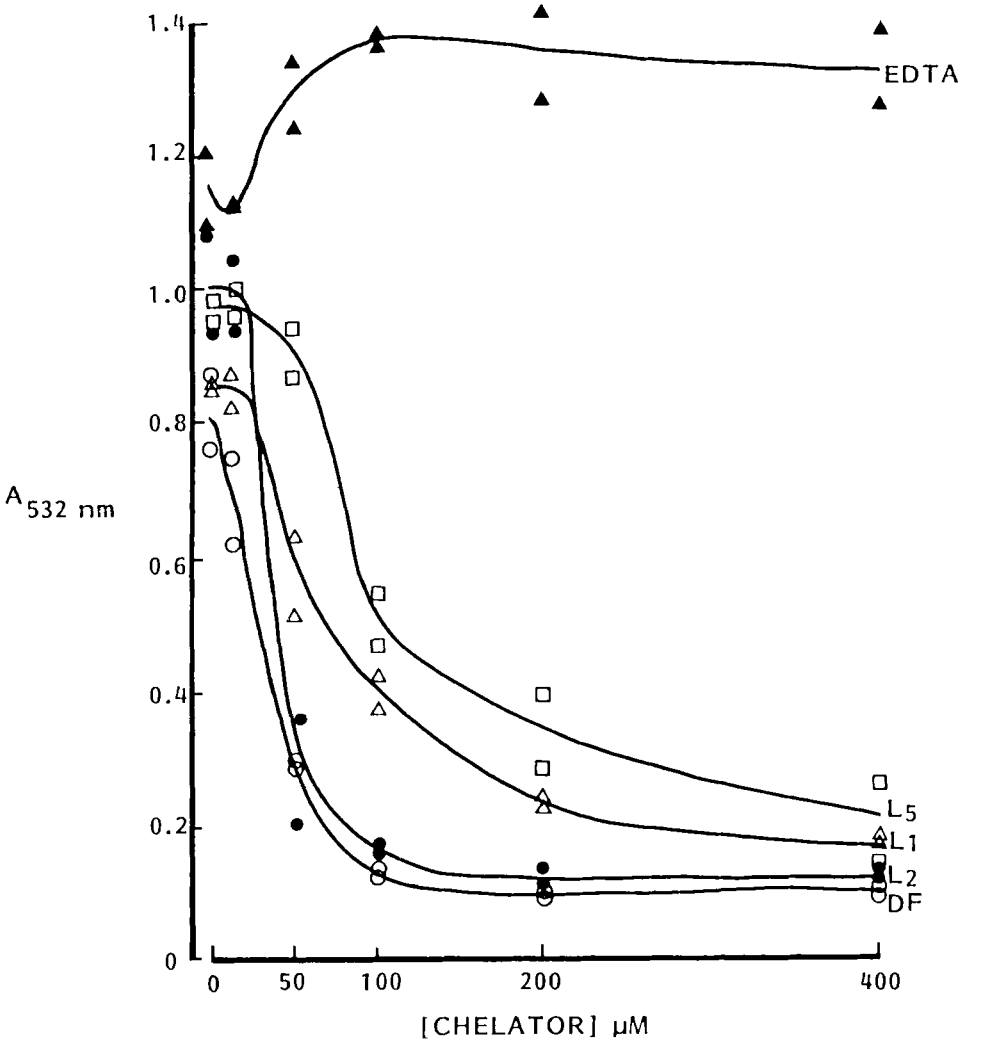

FIGURE 2 *The effect of chelators on thiobarbituric acid reacting substances formed from the breakdown of deoxyribose by free radicals.*

A mixture containing deoxyribose (4 mM), H_2O_2 (4 μM), ascorbic acid (4 μM), Iron II (10 μM) and chelators (0-400 μM) of total volume of 1 ml in phosphate buffer (0.05 M, pH 7.4) was incubated at 26 C for 30 min, treated with trichloroacetic acid (1 ml, 2.8%) and thriobarbituric acid (0.5 ml, 1% in 0.5 M Na OH) and heated in boiling water for 10 min. The absorbance of the reactive mixture was measured at 532 nm at room temperature. The curve of each chelator is drawn through the mean absorbance at 532 nm, of two samples for each chelator concentration (▲) EDTA, (□) Maltol (L_5), (△) 1,2-dimethyl-3-hydroxypyrid-4-one (L_1), (●) 3-hydroxy-1-methylpyrid-2-one (L_2), (○) desferrioxamine (DF).

chelator concentrations (200–400 μM), in the case of EDTA and purpurogallin the activation was only up to 20% at the same range of concentrations. The amount of thiobarbituric acid reactive substances formed during incubations with duplicate samples, (Fig. 2) varied significantly. Similar variations in the results of Table 1 are also expected but the overall effects seem to be characteristic for each chelator.

The percentage inhibition of induced fluorescence on IgG following UV irradiation by chelators at 5, 20 and 100 μM concentration is shown on Table 2. The following chelators inhibited the fluorescence at all concentrations L_1, L_5, DF, 8-hydroxyquinoline, pyrogallol, purpurogallin, catechol and 2,3-DHB. The catechol containing chelators showed the highest inhibition; e.g. 45% by catechol (at 5 μM and 20 μM) and 31.5% by 2,3-DHB at 100 μM. Although greater concentrations of some chelators, e.g. Maltol, pyrogallol, purpurogallin and 2,3-DHB caused proportional decrease in fluorescence, in other chelators variable degree of inhibition occurred in relation to the concentration e.g. L_1, DF, 8-hydroxyquinoline, catechol, caffeic acid. An increase in fluorescence at high chelator concentration (100 μM) accompanied by a decrease at low concentration (5 μM and 20 μM) was observed in the case of L_2, L_3 and kojic acid while L_6 had the reverse effect. Overall there was a great variability in the effects of chelators on IgG fluorescence. These effects do not seem to be related to their chelation properties only, but also to other forms of interaction.

The thiobarbituric acid — reactive substances produced by the muscle homogenate is shown in Table 3. At the concentration of 500 μM all the chelators examined significantly inhibited lipid peroxidation. In particular, the chelators L_1, L_2, L_3, catechol, 2,3-DHB and caffeic acid caused almost complete inhibition. 8-Hydroxyquinoline and D-penicillamine only had a minor effect.

TABLE 2
The effect of chelators on fluorescence of UV irradiated IgG

Chelator	Chelator concentration		
	5 μm	20 μm	100 μm
	% Inhibition of IgG Fluorescence		
L_1	11.3	19.5	4.5
L_2	0.3	15.0	88.5[a]
L_3	2.0	16.5	31.5[a]
L_4	14.3	25.0	3.5[a]
L_6	2.4	1.5[a]	33.0
Maltol (L_5)	3.0	16.5	21.7
Kojic Acid	0.0	0.0	11.0[a]
Catechol	47.5	47.5	30.0
2,3-DHB	10.8	32.0	81.5
Pyrogallol	6.2	10.7	54.8
Caffeic Acid	0.0	0.0	10.5
Purpurogallin	12.5	21.7	48.5
8-Hydroxyquinoline	19.2	20.0	20.0
DF	45.0	45.0	12.0

a — Activation

b — % Inhibition of IgG fluorescence is expressed in comparison to IgG fluorescence in buffer with no added chelator, and is corrected for chelator fluorescence using chelators in buffer with no IgG. Results are means of three separate experiments with 5–10% variation.

TABLE 3
Effect of chelators on production of thiobarbituric acid-reacting substances (malonaldehyde) by skeletal muscle homogenates

Chelator[a]	Malonaldehyde[b] Production	% Inhibition
L_1	40.1 ± 6.8	99.0
L_2	44.5 ± 10.7	96.2
L_3	48.4 ± 8.0	93.7
L_4	86.3 ± 13.4	69.3
L_6	98.0 ± 3.5	53.2
Maltol (L_5)	72.8 ± 5.3	79.4
Kojic Acid	84.6 ± 3.8	67.1
Catechol	42.6 ± 5.4	97.4
2,3-DHB	50.8 ± 2.4	92.2
Pyrogallol	77.6 ± 6.7	74.1
Caffeic Acid	69.1 ± 2.6	83.0
Purpurogallin	57.6 ± 3.8	95.1
8-Hydroxyquinoline	132.7 ± 8.0	17.2
D-Penicillamine	128.8 ± 2.9	21.2

a All chelators were added at a concentration of 500 μm.
b Production of thiobarbituric acid-reacting substances is expressed as the malonaldehyde content of homogenates. The extinction coefficient of the malonaldehyde — thiobarbituric acid product at 532 nm was taken as 1.56×10^5 for all calculations. The results are the mean of six experiments ± standard error of the mean.

DISCUSSION

Evidence is now accumulating to show that several pathological conditions are associated with increased amounts of "free" iron in certain sites. It has been hypothesised that this iron may be toxic via catalysis of the production of oxygen radicals[20,21]. In order to devise efficacious therapy for these disorders, it is necessary to be able to examine agents for both their ability to remove iron from the body and for their ability to do this without enhancing the production of oxygen-free radicals. The preferred agent would therefore be a non-toxic, cheap, orally active chelator able to remove iron from the body and able to inhibit iron catalysed free radical production. Techniques for examining the ability of chelators to remove iron from the body are well described using animal models[7,8,22,23,24,25] but no single test is accepted as the method of choice for examination of the ability of a compound to inhibit free radical reactions. We have therefore tested the ability of several well-known iron chelators to inhibit free radical processes in three separate systems, i.e. free radical-mediated damage to sub-cellular components (deoxyribose and IgG) and a whole tissue system.

In the deoxyribose model of screening, it was shown that almost all the chelators inhibited ferrous iron induced damage (due to hydroxyl radicals) at a high chelator (200–400 μM) to iron (II) (10 μM) concentration ratios (Fig. 2). The mechanism of inhibition may be via the oxidation of iron (II) to iron (III) followed by the formation of strong iron (III) complex which does not catalyse the formation of oxygen activated products. This mechanism is supported by the previous observation that DF, L_1, L_2 and maltol form almost instantaneous iron (III) complexes when mixed with iron (II) at pH 7.4[26]. When the chelator concentrations were less than the 3 chelator to 1 iron

(II) molar ratio which is a minimum requirement for a complex formation with iron at pH 7.4 (except in DF), there was little or no inhibition (Fig. 2). It seems that in this model excess chelator is needed if it is to inhibit the effects of iron (II). In contrast to the other chelators, EDTA and purpurogallin enhanced by 20% the breakdown of deoxyribose, and their iron complexed structures are suspected to increase the iron catalysed formation of $OH^{\cdot}$ under these incubation conditions.

Immunocomplexs of IgG have been previously detected in inflammation, and similar IgG polymers could be produced using UV irradiation through a process of free radical activity[18]. In this work a decrease in the UV induced fluorescence of IgG was observed with many chelators — especially those containing the catechol ring which could scavenge the $OH^{\cdot}$ production[27]. Since most chelators bind iron through the oxygen atoms of their hydroxyl groups (Fig. 1) and this hydroxyl group could possibly trap free radicals by forming an orthoquinone, it could be assumed that the inhibition of free radical damage in IgG by chelators may be caused by trapping free radicals and also by inhibiting the catalytic formation of oxygen activated products from traces of iron present in the medium. The increase of IgG fluorescence in some cases, which was also previously observed with the sulphydryl compounds,[18] indicates that other possible forms of interaction between some of the chelators and the side chains of IgG, may increase free radical production and IgG polymerisation. Additional studies are required to further characterise the effect of chelators and other compounds on IgG free radical damage.

The production of free radical products by tissue homogenates may have some relevance to tissue damage in pathological conditions such as thalassaemia[2,3], muscular dystrophy,[28] cancer,[29] and rheumatoid arthritis[4]. In these diseases "free" non-transferrin bound iron may be present or released which may give rise to oxygen activated products. Lipid peroxidation in skeletal muscle homogenates has been shown to be initiated by iron present within the homogenate[19]. The source of this iron is unclear, but it may be derived by disruption of intracellular storage materials during tissue homogenisation, or from iron contamination of laboratory reagents. This system has previously been used to examine the ability of amphiphilic "membrane-active" agents to inhibit lipid peroxidation,[30]. The hydroxypyridone derivatives (L_1, L_2 and L_3) and the catecholic chelators were the most effective inhibitors of lipid peroxidation in this system (Table 3), while the lipophilic chelators, L_4, L_6, 8-hydroxy-quinoline, and the α-ketohydroxy pyrone chelators, maltol and kojic acid were much less effective. D-penicillamine, which is primarily a chelator of copper and zinc was also of limited use in this system.

The inhibition of iron induced formation of oxygen activated products by some chelators may be of therapeutic importance[20], however the problems associated with screening likely substances for beneficial properties are illustrated by the results presented here, the chelators being found to have variable effects depending on the nature of the test system. Furthermore, the complexity surrounding the source and the site of free radical-formation in pathological conditions and also the mechanisms involved in tissue damage make the selection of a site specific chelator difficult.

Our results suggest that the catecholic chelators are a promising group which may inhibit free radical damage but unfortunately these chelators are easily oxidised at physiological pH and their use may be limited *in vivo*. Similar effects to the catecholic chelators were also shown by L_2. Kojic acid and maltol the two naturally occurring α-ketohydroxypyrone chelators tested also inhibited free radical production. This result is particularly important for studies of maltol because its iron complex may have a use

in the treatment of iron deficiency anaemia[8]. Although at certain concentrations the N-hydroxypyrid-2-one derivatives caused exacerbation of the free radical damage on IgG, their inhibitory free radical effects in the other two models and the oral activity of L_3 in increasing iron excretion in mice[8] increases their prospects in treating "free" iron induced inflammation. As previously shown, DF was effective in inhibiting free radical activity (Tables 1 and 2). This suggests that the toxic side effects observed with DF *in vivo* such as ocular changes in thalassaemia patients[31], vomiting and nausea in rheumatoid arthritis patients[6,33] and *in vitro*, such as the oxidation of haemoglobin (G.J. Kontoghiorghes, unpublished work), are not caused by increased iron catalysis of free radical production.

The most promising chelator with potential clinical use from this study is L_1 which inhibited free radical formation products in all three models. L_1 has also been shown to be orally active in mobilising iron from iron overloaded[8] and non-iron overloaded animals[7] to equivalent levels to those caused by parenteral DF. It has also been shown to mobilise iron from transferrin[32] and ferritin[26] *in vitro*. Further evaluation of the anti-inflammatory effects of L_1 and its analogues is in progress.

In summary, certain chelators may inhibit free radical formation caused by iron, UV irradiation and in tissue homogenates. Although the complexity of the free radical mechanisms and the differences in the properties of the chelators make the task of chelator selection difficult, use of the techniques described here should allow effective screening to find specific and effective iron chelators, like L_1, which could minimise iron toxicity and therefore be of therapeutic value.

Acknowledgements

This work was supported by the UK Thalassaemia Society. We would like to thank Professor A.V. Hoffbrand, Professor E.R. Huehns, Drs. C.C. Winderbourn, M.T. Wilson, R.C. Hider and J. Charalambous for helpful discussions and the provision of facilities. We also thank Miss J. Martin and Ms. C. Pericleous for the typing.

References

1. J.B. Neilands in Iron in Biochemistry and Medicine II, ed A. Jacobs and M. Worwood (Academic Press, London and New York, 1980) p. 529.
2. A. Jacobs, *Brit. J. Haem.* **34**, 1 (1976).
3. E.A. Rachmilewitz, B.H. Lubin and S.B. Shohet, *Blood*, **47**, 495 (1976).
4. D.R. Blake, N.D. Hall, P.A. Bacon, P.A. Dieppe, B. Halliwell and J.M.C. Gutteridge, *Lancet*, **ii**, 1142 (1981).
5. Bulletin of the World Health Organisation, Community control of Hereditary Anaemias. Memorandum from a WHO Meeting, **63(i)**, 63 (1983).
6. R.J. Polson, A. Jawed, A. Bomford, H. Berry and R. Williams. *Brit. Med. J.* **291**, 448 (1985).
7. G.J. Kontoghiorghes, *Lancet* **i**, 817 (1985).
8. G.J. Kontoghiorghes, *PhD Thesis*, University of Essex (1982).
9. A. Stefanovic, J. Havel and L. Sommer, *Collection Czechoslov. Chem. Commun.* **33**, 4198 (1968).
10. Y. Murakami and J. Inorg, *Nucl. Chem.* **24**, 679 (1962).
11. R.L.N. Harris, *Aust. J. Chem.* **29**, 1329 (1976).
12. M. Yasue, N. Kawamura and J. Sakakibara, *Yakugaku Zasshi* **90**, 1222 (1970).
13. T. Severin and A. Loidl., *Z. Lebensm. Unters-Forsch* **161**, 119 (1976).
14. H. Mohrle and H. Weber, *Tetrahedron* **26**, 3779 (1970).
15. S. Mizukami, E. Hirai, M. Morimoto, *Ann. Rept. Shionogi Res. Lab.* **16**, 29 (1966).
16. Z. Talik, *Roczniki Chem.* **35**, 475 (1961).
17. B. Halliwell and J.M.C. Gutteridge, *FEBS Lett.* **128**, 347 (1981).

18. D.G. Wickens, A.G. Norden, J. Lunec and T.L. Dormandy, *Biochim. Biophys. Acta* **742**, 607 (1983).
19. M.J. Jackson, D.A. Jones, R.H.T. Edwards, *Bioscience Reports* **3**, 609 (1983).
20. Editorial *Lancet* **i** 143 (1985).
21. B. Halliwell and J.M.C. Gutteridge, *Biochem. J.* **219**, 1 (1984).
22. T. Hoy, J. Humphrys, A. Jacobs, A. Williams and P. Ponka, *Brit. J. Haem.* **43**, 443 (1979).
23. C. Hershko, *Blood* **51**, 415 (1978).
24. M.J. Pippard, D.K. Johnson, C.A. Finch, *Blood* **58**, 685 (1981).
25. J.H. Graziano, R.W. Grady, A. Cerami, *J. Pharmac. Exp. Ther.* **190**, 570 (1974).
26. G.J. Kontoghiorghes, *Biochem J.* **233**, 299 (1986).
27. A. Cerami, R.W. Grady and C.M. Peterson in Chelation therapy in chronic Iron overload, ed E.C. Zaino and R.H. Roberts (Symposia Specialists, Miami, Florida, USA, 1977) p. 37.
28. I.A. Clark, *Medical Hypotheses* **13**, 153 (1984).
29. R.L. Willson in Free radicals, lipid peroxidation and Cancer, ed D.C.H. McBrien and T.F. Slater (Academic Press, London, New York, Paris, San Diego, San Francisco, Sao Paolo, Sydney, Tokyo, Toronto 1982) p. 275.
30. M.J. Jackson, D.A. Jones, E.J. Harris, *Bioscience Reports* **4**, 581 (1984).
31. S.G. Davies, R.E. Marcus, J.L. Hungerford, M.H. Miller, G.B. Arden and E.R. Huehns, *Lancet* **ii**, 181 (1983).
32. G.J. Kontoghiorghes and R.W. Evans, *FEBS Lett.* **189**, 141 (1985).
33. D.R. Blake, P. Winyard, J. Lunec. et al. *Quartern. J. Medicine,* **56**, 345 (1985).
34. J. Lunec, D.R. Blake, S.J. McCleary et al. *J. Clin. Invest.* **76**, 2084 (1985).

Accepted by Dr. B. Halliwell