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STUDIES OF *IN VIVO* IRON MOBILIZATION BY CHELATORS IN THE FERROCENE-LOADED RAT

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Abstract—The oral efficacy of the oral iron chelators 1,2-dimethyl-3-hydroxypyrid-4-one (CP20), 1,2-diethyl-3-hydroxypyrid-4-one (CP94) and desferrioxamine B (DFO) has been compared with intraperitoneal DFO in an experimental model of iron overload with similar biochemical and biophysical characteristics to those observed for human genetic haemochromatosis. The hepatic iron stores in the ferrocene-loaded rat were relatively stable and did not decrease at the end of the loading period. In contrast, the iron dextran rat model showed a rapid depletion of its iron stores 2 weeks after cessation of intraperitoneal injection. When CP20 and CP94 were administered to the ferrocene-loaded rat model in combination with an iron-free diet there were significant decreases in (i) total homogenate iron and (ii) hepatic ferritin iron when compared to the iron-loaded rat receiving the iron-free diet alone. Desferrioxamine, when administered by gavage, only showed chelation of ferritin iron, while intraperitoneal injection of desferrioxamine showed significant depletion of iron both in the total homogenate and ferritin. Subcellular fractionation of the hepatic organelle clearly showed that where there was depletion of homogenate iron there was a net decrease in the lysosomal fraction, while changes in ferritin iron were reflected by decreases in the cytosolic iron content. Although no assessment of net iron excretion was made, we suggest that the use of this animal model should ascertain the site of chelation by iron chelators.

Even though iron overload occurs in several million people [1], the only available treatment is injection of desferrioxamine (Desferal, Ciba-Geigy, Basel, Switzerland), which is both by its cost and its mode of administration unsuitable for use in third world countries. Cheaper oral drugs are currently being synthesized, and their efficacy relates to their partition between lipids and aqueous layers of cells (i.e. partition co-efficient) [2]. It is important to test whether the efficacy of such potential chelators shown *in vitro* is also apparent in experimental animal models of iron overload.

Longueville and Crichton [3] have developed an animal model of iron overload which shows both biochemical, biophysical and histological similarities to human haemochromatosis [4]. In contrast to other animal models used to assess chelator efficacy, this model allows us to investigate both hepatic and tissue mobilization of iron by various chelators, since the pools of iron are relatively stable after cessation of ferrocene (3,5,5-trimethyl hexanoyl ferrocene) [4]. This is in contrast to previous models of iron overload, e.g. carbonyl iron, in which hepatic iron-loading rapidly decreases after cessation of the iron supplementation.

Other animal models of iron overload currently used to test chelator efficacy have not determined whether the hepatic iron stores are relatively stable post iron loading. This is of importance in the rat model before commencing chelator studies since the rat is able to excrete iron through the biliary duct [5], unlike humans which have no such excretory mechanism.

The total hepatic iron stores in iron overload consist of three pools: (i) a small low molecular mass iron pool, approximately 1–2 μmol, which is in equilibrium with both (ii) cytosolic ferritin and lysosomal haemosiderin [6]. As we have pointed out previously [6], current evidence would suggest that the absolute size of the low molecular mass iron pool does not change in iron overload, but the flux through the pool probably increases. Iron chelators will be judged to be effective if they can chelate iron from all three pools, particularly the haemosiderin pool, which is at least 10-fold higher than the ferritin pool.

Quantitative measurement of haemosiderin iron is difficult, since its extraction requires complicated procedures [7]. The strategy that has been adopted, therefore, to assay chelator efficacy, has been to measure both total iron and ferritin protein and iron content, since the purification procedure involved yields both high purity and recoveries of greater than 90% (Crichton, unpublished results). An estimation of iron in the non-ferritin pool (principally haemosiderin) can be calculated from the difference between total iron and total ferritin iron in the liver. In the present study the efficacy of several chelators administered either intraperitoneally or orally to mobilize iron from the liver and its subcellular organelles, and the spleen, heart and brain has been compared.

MATERIALS AND METHODS

Animal models. Ferrocene was synthesized from

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Table 1. Hepatic iron content after iron loading with either iron dextran or ferrocene, after either 4 weeks or 4 weeks + 2 weeks low Fe diet

	Hepatic iron (μg/g)
Iron dextran	
4 weeks	2827 ± 327
4 + 2 weeks	2148 ± 352
Ferrocene	
4 weeks	2507 ± 431
4 + 2 weeks	2633 ± 235
Controls	288 ± 42

Values are means ± SD.

ferrocene and 3,5,6-trimethylhexanoyl chloride by acylation and purified by recrystallation at the Department of Pharmacy, King's College London.

Animal model studies. Male rats (Wistar strain), 50–75 g, received either iron dextran, three i.p. injections per week for 4 weeks, or the ferrocene derivative orally for 4 weeks (see below). At the end of this time-period half of the rats in each group were killed, while the remainder in each group received an iron-free diet for a further 2 weeks before killing.

Male rats (Wistar strain, 100 g) were adapted to a powder diet containing ferrocene (1 g/kg diet) (Fe content 0.35 g Fe/kg) for 4 weeks. Subsequently, they were administered an oral iron chelator every second day for 14 days (i.e. seven administrations) in combination with an iron-free chelator (Fe content 0.003 g Fe/kg).

Iron chelators. Desferrioxamine B methane sulphonate (DFO*) (Ciba-Geigy) was administered either i.p., 10 mg/kg, or by gavage, 30 mg/kg. The two oral chelators 1,2-dimethyl-3-hydroxypyrid-4-one (CP20) (Ciba-Geigy) and 1,2-diethyl-3-hydroxypyrid-4-one (CP94) (British Biotechnology Group) were dissolved in water with gentle warming and administered by gavage (30 mg/kg in a 500 μL dose).

* Abbreviations: DFO, desferrioxamine B; CP20, 1,2-dimethyl-3-hydroxypyrid-4-one; CP94, 1,2-diethyl-3-hydroxypyrid-4-one.

At the end of the treatment period, blood was removed by cardiac puncture for haemotological investigations, the animal was killed by cervical dislocation, and the liver and spleen were removed. Total iron in each tissue was determined by electrothermal atomic absorption [4]. Ferritin was isolated from the liver by the method of Longueville and Crichton [3], and the iron and protein content were determined by reaction with bipyridyl after reduction of ferric iron by Na_2SO_3 and by the method of Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Subcellular fractionation of hepatic organelles was by discontinuous sucrose density ultra-centrifugation. A portion of liver (2 g) was homogenized in 0.25 M sucrose (40 mL) with a Dounce homogenizer centrifuged at 1500 *g* to remove nuclei and cellular debris and then centrifuged at 7500 *g* to yield a supernatant which contained microsomes and cytoplasm, and a pellet containing mitochondria, peroxisomes and lysosomes. The pellet was layered onto a discontinuous sucrose density of 1.17, 1.22 and 1.28 g/mL sucrose and centrifuged at 100,000 *g* for 2 hr. At the end of this time-period the organelles had equilibrated with their appropriate densities, while the iron-loaded lysosomes sedimented through the gradient. The purity of each organelle fraction was assessed by measurement of appropriate marker enzymes for each organelle, i.e. succinate dehydrogenase for mitochondria, *N*-acetyl-β-glucosamindase for lysosomes, catalase for peroxisomes and *N*-α-gluconidase for microsomes. Protein was determined in the subcellular fractions by using a Bio-Rad kit, with bovine serum albumin as standard.

RESULTS

Table 1 shows the changes in hepatic iron content after either loading with ferrocene or iron dextran for 4 weeks or after a further 2 weeks on an iron-free diet. A dramatic decrease in the hepatic iron content was observed 2 weeks after cessation of the rats receiving iron dextran. In contrast, the levels of hepatic iron content remained constant in the ferrocene-loading rat, despite the withdrawal of iron supplementation.

In the chelation studies all treated and control rats gained weight during the 2 weeks of chelation therapy, but at post-mortem the liver weights in each group of rats which received oral chelation were significantly reduced compared to the iron-

Table 2. Total and hepatic weights of rats after chelation therapy

	Total weight (g)	Hepatic weight (g)
Iron overload (4 + 2 weeks)	328 ± 25	15.4 ± 1.4
DFO (10 mg/kg i.p.)	319 ± 10	16.8 ± 2.0
DFO (30 mg/kg gavage)	305 ± 11	11.6 ± 0.8*
CP20 (30 mg/kg gavage)	327 ± 22	12.7 ± 1.0*
CP94 (30 mg/kg gavage)	302 ± 11	10.6 ± 0.8*

* $P < 0.05$.
Values are means ± SD.

Table 3. Haematological indices in rats receiving chelation therapy

	TIBC	Saturation	WCC	Neutrophils	Lymphocytes
Iron overload (4 + 2 weeks)	801 ± 93	26.8 ± 3	6.2 ± 2.6	9.3 ± 4.7	81.8 ± 4.5
DFO (i.p.)	812 ± 57	24.8 ± 3	3.7 ± 1.8	12.5 ± 8.8	84.8 ± 7.9
DFO (gavage)	657 ± 38	40.2 ± 8	6.0 ± 4.5	10.0 ± 7.6	87.4 ± 7.3
CP20	758 ± 94	31.2 ± 5	4.9 ± 1.6	9.3 ± 4.9	88.8 ± 4.8
CP94	685 ± 68	31.5 ± 6	6.0 ± 2.6	9.8 ± 5.0	87.3 ± 5.9

Values are means ± SD.

Table 4. Hepatic iron content after chelation therapy

	Total liver iron (mg)	Ferritin iron (mg)	Ferritin protein (mg)	Excess iron (mg)
Control	3.9 ± 0.45	0.84 ± 0.07	0.9 ± 0.1	3.09 ± 0.41
Ferrocene-loaded	29.6 ± 3.6	3.2 ± 0.3	19.7 ± 0.4	26.4 ± 3.4
DFO i.p.	22.6 ± 2.5*	2.8 ± 0.2*	21.8 ± 0.5	19.6 ± 2.9*
DFO (gavage)	27.4 ± 3.4	2.4 ± 0.2*	16.4 ± 0.5	25.1 ± 3.5
CP20 (gavage)	24.3 ± 4.3*	2.4 ± 0.6*	23.7 ± 0.3	21.9 ± 4.2*
CP94	21.5 ± 7.9*	2.0 ± 0.2*	18.6 ± 0.15	19.2 ± 7.9*

* $P < 0.05$.
Values are means ± SD.

Table 5. Distribution of iron in hepatic subcellular organelles after chelation therapy

	Mitochondria	Peroxisomes	Lysosomes (μg/mg protein)	Cytosol	Microsome
Controls (dextran)	0.51 ± 0.04	0.79 ± 0.28	0.37 ± 0.11	NA	NA
Iron overload	1.83 ± 0.39	6.3 ± 2.6	81.6 ± 4.5	20.8 ± 5.1	NA
CP94	0.99 ± 0.23	5.3 ± 1.4	56.9 ± 8.6*	17.3 ± 4.8	7.6 ± 1.5
CP20	1.2 ± 0.3	5.0 ± 1.5	52.4 ± 20*	12.8 ± 2.7*	7.6 ± 2.9
DFO (i.p.)	1.5 ± 0.26	3.8 ± 0.9	54.3 ± 14.9*	11.7 ± 1.8*	5.9 ± 1.8
DFO (gavage)	2.2 ± 0.7	7.1 ± 2.6	90.9 ± 32	13.3 ± 2.0*	6.7 ± 1.7

NA, not analysed.
* $P < 0.05$.
Values are means ± SD.

loaded control or the group which received DFO intraperitoneally (Table 2). After 2 weeks there were no significant alterations in any of the haematological indices (Table 3).

Table 4 shows the total hepatic iron contents and the ferritin iron and protein contents of the various groups of rats loading with ferrocene for 4 weeks, which then received for a further 2 weeks either a low-iron diet or one of the chelators in combination with a low-iron diet. DFO, when administered i.p., CP20 and CP94 caused a significant depletion of both total hepatic and ferritin iron. By contrast, DFO, when administered by gavage, although causing a decrease in ferritin iron, did not cause any significant depletion of the total hepatic iron. There were no consistent changes in the ferritin protein associated with successful chelation therapy.

Analysis of the iron content of the subcellular organelles showed a significant decrease in both the cytosolic and lysosomal fractions after chelation with DFO (i.p.), CP20 or CP94 (Table 5). However, DFO by gavage showed only a significant fall in the cytosolic fraction, the lysosomal fraction being similar in iron concentration to that of the overloaded rat. Neither mitochondrial or peroxisomal fractions showed any significant alterations after chelation therapy (Table 5). DFO (i.p.) and CP20 caused a significant reduction in both ferritin iron and protein content of the spleen, by approximately 25%, although there was little change in the total iron content (Table 6). However, neither the brain nor heart showed any significant decrease in iron content after ferrocene loading.

DISCUSSION

Hepatocellular iron mobilization by the three drugs CP20, CP94 and DFO has been evaluated in these present studies by the measurement of total hepatic iron and iron content of hepatic ferritin of rats overloaded with ferrocene after oral drug administration, and compared to that by DFO after

Table 6. Total splenic and ferritin iron after chelation

	Total iron (μg/g)	Ferritin iron (mg)	Ferritin protein (mg)
Iron overload (4 + 2 weeks)	368 ± 126	0.35 ± 0.14	4.80 ± 1.78
DFO (i.p.)	303 ± 88	0.26 ± 0.04*	2.64 ± 0.76*
DFO (gavage)	367 ± 89	0.29 ± 0.05	3.78 ± 1.18
CP20	297 ± 44	0.26 ± 0.02*	1.74 ± 0.26*
CP94	484 ± 86	0.39 ± 0.10	3.05 ± 0.61

* $P < 0.05$.
Values are means ± SD.

intraperitoneal injection. Both CP20 and CP94 when given orally showed efficacy to remove iron from ferritin and haemosiderin. It is noteworthy that when DFO was administered by gavage there was a net decrease in both the ferritin and cytosolic iron content, but no overall depletion in hepatic content. Similarly, Gyparaki *et al.* [8] and Smith [9] concluded that there was little mobilization of iron when DFO was administered orally by the lack of increase in urinary ^{59}Fe excretion. However, the problem with such radio-tracer methods [10, 11] used to assess efficacy is that they represent a very indirect approach for the measurement of iron mobilization by chelation, since the radio-label may not behave in a manner which is representative of the bulk iron storage pool. Furthermore, we have shown in this present study that the iron stores of the iron dextran model are rapidly depleted on cessation of iron supplementation such that equilibrium of the radio-labelled iron with such iron stores would be difficult.

Therefore, the use of the ferrocene-loaded rats for chelation studies has particular advantages over iron dextran models. Most importantly, the hepatic iron overload in this model in the time-frame during which iron loading is established is essentially parenchymal [4, 12]. Secondly, the levels of hepatic iron are comparable to both genetic and secondary haemochromatosis and haemosiderin is the principle iron storage protein [13]. Finally, levels are maintained at the cessation of supplementation and even increase [3]. After iron loading the majority of the hepatic iron was located within the lysosomes and was assessed to be haemosiderin by both histological methods and its biochemical extraction [4]. It is noteworthy that after 2 weeks of successful chelation therapy the lysosomal iron content and, to a lesser degree, the iron content of the cytosolic fraction had decreased while the iron content of the subcellular compartments, mitochondria and peroxisomes remained constant. Similarly, histological studies of the livers of genetic haemochromatosis patients after venesection showed a reduced number of enlarged lysosomes, indicating mobilization of iron from this organelle [14].

Porter *et al.* [15] evaluated the chelator efficacy of several hydroxypryridone-4-one iron chelators by measurement of tissue iron content after their intraperitoneal injection to iron dextran-loaded rats for 60 days at high toxic doses. The drugs were poorly tolerated at these high doses, CP20 inducing leucopenia. Similarly, we showed in this present study poor tolerance of CP20 and CP94, by decreased liver weight, although no significant changes in haematological indices were apparent at the low doses used (30 mg/kg). However, both drugs showed good chelator efficacy in the mobilization of iron. Such results should commend CP20 for clinical use, but initial studies in human patients, although showing efficacy to chelate iron [16], have indicated the occurrence of leucopenia [17–19]. Such toxicity may deter the use of CP20 in the treatment of iron overload. Furthermore, the fact that CP20 is rapidly glucuronidated *in vitro*, which diminishes its ability to chelate iron, will necessitate higher doses with known toxicity. In contrast, the products of metabolism of DFO retain their capacity for chelation (Singh S, personal communication).

We would confirm that in our model CP94 is as effective as CP20 [15] and might, therefore, represent a better candidate for chelation studies in man.

In conclusion, this animal model is particularly appropriate for the screening of new oral iron chelators due to its short iron-loading time and its similarities to human genetic haemochromatosis disease.

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