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Cellular Uptake and Release of Two Contrasting Iron Chelators

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Abstract

Desferrioxamine and CP94 (1,2-diethyl-3-hydroxypyridin-4-one) are metal chelators used or proposed for use in the clinical treatment of iron overload. Recent data on their capacity to deplete intracellular iron led to the conjecture that the differences observed arose from the different membrane-penetration properties of the two compounds.

The time-course of accumulation and subsequent release of [^{14}C]CP94 by the rat visceral yolk sac in-vitro was compared with that of [^{14}C]desferrioxamine and for ^{125}I-labelled poly(vinylpyrrolidone), a marker for fluid-phase endocytosis. The results indicate that [^{14}C]CP94 crosses the plasma and lysosome membranes rapidly whereas [^{14}C]desferrioxamine and ^{125}I-labelled poly(vinylpyrrolidone) are effectively incapable of crossing these membranes, entering cells only by endocytosis.

It is concluded that although CP94 readily enters and leaves cells, desferrioxamine has the potential to accumulate to high concentration in the lysosomes and complex with intralysosomal iron. The results support and extend the proposed correlation between pharmacological activity and capacity for membrane penetration.

β-Thalassaemia major patients, who can be kept alive only by regular blood transfusions, require iron-chelation therapy as an essential adjunct treatment. Desferrioxamine is an iron chelator that has been in clinical use for over 30 years but is effective only if administered parenterally. A 20-year search for orally effective iron-chelators has led to the identification of the 3-hydroxypyridin-4-ones as particularly promising candidate drugs with relatively low toxicity and good iron-chelating ability (Hider et al 1996; Smith et al 1997).

1,2-Diethyl-3-hydroxypyridin-4-one (CP94) is one member of this chemical class of iron-chelator. In a recent study desferrioxamine and CP94 were compared for their capacity to deplete intracellular iron in cultured cells from man, and in consequence inhibit the iron-dependent enzyme ribonucleotide reductase (Cooper et al 1996). Cells were exposed to chelator for different times and the extent of enzyme inhibition and of intracellular iron-chelate formation was measured. The results showed that CP94 inhibited the enzyme more rapidly than did desferrioxamine. The CP94 chelate reached maximum levels within 20 min, whereas the desferrioxamine chelate took 4 h (approx.) to reach the same level, although by 24 h it had reached levels twice those reached by CP94. In other experiments cells were incubated with ^{59}Fe-labelled transferrin for 3 h and then incubated with chelator. CP94 extracted ^{59}Fe from the cells twice as rapidly as desferrioxamine. The authors interpreted these data in terms of differential permeability of CP94 and desferrioxamine across cell membranes, pointing out that the octanol–water partition coefficient of CP94 is two to three orders of magnitude greater than that of desferrioxamine.

Some years ago we measured the kinetics of accumulation of [^{14}C]desferrioxamine by mammalian cells (Lloyd et al 1991). The results indicated that uptake is mainly by fluid-phase endocytosis, rather than by permeation across membranes. That conclusion is concordant with the low oil–water partition coefficient of desferrioxamine and also implies that desferrioxamine might be concentrated within cell lysosomes rather than within the cytosol, as had been postulated previously (Laub et al 1985).

We propose the following hypothesis to explain the contrasting behaviour of desferrioxamine and CP94 in the experiments of Cooper et al (1996). CP94 and its iron-chelate, owing to their relatively high oil–water partition coefficients, can enter and leave cells rapidly across the plasma membrane. In

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contrast desferrioxamine slowly enters the endosome and then the lysosome compartment, where it accumulates and can chelate intralysosomal iron. Because the iron chelate of desferrioxamine has an oil–water partition coefficient 10 times greater than that of the unconjugated ligand (Cooper et al 1996), it slowly leaves the lysosome compartment, reaching the cytosol and subsequently the extracellular fluid.

If this hypothesis is correct, the uptake and release kinetics of CP94 by cells should be very different from those of desferrioxamine. Because we had access to radiolabelled CP94 and desferrioxamine, we were able to test this inference experimentally. We used the 17·5-day visceral yolk sac maintained in organ culture as an experimental model, as this model has been found particularly suitable for quantitative studies of endocytosis (see Lloyd 1990 for a review).

Materials and Methods

[^{14}C]Desferrioxamine and [^{14}C]CP94 were gifts from Ciba Geigy. ^{125}I-Labelled poly(vinylpyrrolidone) (PVP) was from Amersham International. Other chemicals and reagents were from Sigma.

Uptake of radiolabelled solutes by the 17·5-day rat visceral yolk sac was measured as described by Roberts et al (1977). Yolk sacs were incubated individually at 37°C in Erlenmeyer flasks containing medium 199 (10 mL) and the labelled solute. After the appropriate time yolk sacs were washed three times in ice-cold 1% (v/v) NaCl and dissolved in 1 M NaOH. Radioactivity was measured by liquid scintillation counting (Roberts et al 1977) and protein content by the method of Lowry et al (1951). In some experiments yolk sacs were incubated for a further hour, in radiotracer-free medium 199 (10 mL) at 37°C, before washing and processing.

Results

The uptake of ^{125}I-labelled poly(vinylpyrrolidone) (PVP) has been shown to be a reliable measure of fluid-phase endocytosis in the rat visceral yolk sac system (Lloyd 1990). Figure 1 shows the kinetics of uptake of ^{125}I-labelled PVP by yolk sacs cultured in-vitro. Uptake is expressed as a clearance, to normalize data for inter-experiment variation in yolk-sac protein content and in the amount of radioactivity added to the culture medium (Roberts et al 1977). Units of clearance are μL (mg tissue-protein)$^{-1}$. Over a 4-h uptake period PVP accumulated steadily. The clearance rate was

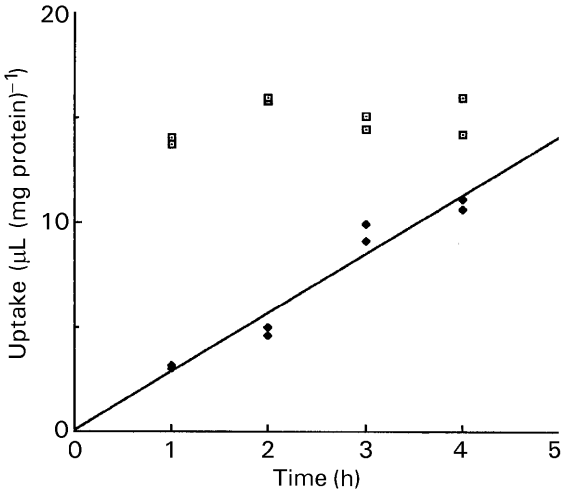

Figure 1. Uptake of [^{14}C]CP94 (⊡) and ^{125}I-labelled PVP (◆) by 17·5-day rat visceral yolk sacs cultured in vitro for 1 to 4 h. Each point represents data from a single yolk sac. Uptake of ^{125}I-labelled PVP appears linear with time (correlation coefficient of the regression line 0·94), with a slope of 2·81 μL (mg protein)$^{-1}$ h^{-1}.

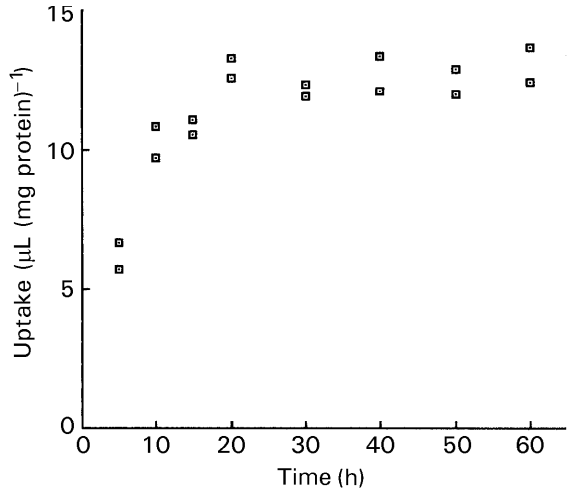

Figure 2. Uptake of [^{14}C]CP94 by 17·5-day rat visceral yolk sacs cultured in-vitro for 5 to 60 min. Each point represents data from a single yolk sac.

2·81 μL (mg protein)$^{-1}$ h^{-1}. This is similar to values obtained in similar experiments with [^{14}C]sucrose and [^{14}C]desferrioxamine as substrates (Lloyd et al 1991).

Figure 1 shows that the uptake pattern for [^{14}C]CP94 was quite different—the tissue level was already maximal by the first time-point. Figure 2 shows the uptake kinetics of [^{14}C]CP94 over the first hour of incubation. Uptake was maximal by 20 min, but values between 5 and 20 min show progressive accumulation.

Table 1 shows the radioactivity present in the tissue after 1 h and 4 h incubation with [^{14}C]desferrioxamine or [^{14}C]CP94. The table also shows the effect on these values of incubation for a further

Table 1. Accumulation and retention of desferrioxamine and CP94 by 17·5-day rat visceral yolk sacs.

Substrate	Incubation time (h)	Tissue radioactivity (μL (mg protein)$^{-1}$)	
		Without wash-out period	With wash-out period
[^{14}C]Desferrioxamine	1	8·4 ± 0·4	6·4 ± 0·2
	4	20·4 ± 1·1	19·0 ± 1·6
[^{14}C]CP94	1	11·8 ± 0·5	2·7 ± 0·6
	4	14·8 ± 0·6	3·6 ± 0·5

Yolk sacs were incubated with the radiolabelled drug for the period indicated. Tissue radioactivity was measured immediately in one series of experiments, and after a further 1-h incubation in medium without radiotracer in the second series. Tissue radioactivity is expressed as a clearance value, the volume of culture medium whose content of radioactivity is found in the tissue, and further normalized to the protein content of each individual yolk sac. Values are means ± standard deviation for 4–6 individual measurements.

hour in (isotope-free) culture medium before counting. After uptake of [^{14}C]CP94, the tissue released most of its radioactivity during the wash-out period, but little was released after uptake of [^{14}C]desferrioxamine.

Discussion

Fluid-phase endocytosis has been shown to be the principal mode of entry of several highly hydrophilic substances into the 17·5-day rat visceral yolk sac cultured in-vitro. PVP and sucrose are two such substrates (Lloyd 1990), and our previous investigation (Lloyd et al 1991) showed that desferrioxamine is another. In an early study (Roberts et al 1977) we showed that ^{125}I-labelled PVP and [^{14}C]sucrose are efficiently retained within the tissue after endocytic uptake, an indication that they do not easily cross the lysosome and plasma membranes. We now demonstrate that this is also true for desferrioxamine—when yolk sacs that had accumulated [^{14}C]desferrioxamine were re-incubated for 1 h in radiotracer-free medium most of the radioactivity was retained in the tissue. Because desferrioxamine is not metabolized by the enzymes of the lysosome and is only very slowly modified when incubated with cell extracts (Laub et al 1985), this radioactivity can confidently be identified as unchanged desferrioxamine.

In sharp contrast to ^{125}I-labelled PVP (these experiments), [^{14}C]sucrose and [^{14}C]desferrioxamine (Lloyd et al 1991), which progressively accumulate in the yolk sac over 4 h, CP94 reached its maximum concentration in the tissue within 20 min. Radioactivity was equally rapidly lost during re-incubation in radiotracer-free medium. Although CP94 is metabolized by rats in-vivo to a hydroxylated derivative (Singh et al 1992), metabolites are likely to comprise an insignificant fraction of the radiolabelled material released from the yolk sac, owing to the extreme rapidity of the release. These results indicate that CP94 efficiently traverses the plasma membrane, in either direction. The amount retained after 4-h incubation followed by 1-h wash-out was much lower than the value for desferrioxamine, an indication that CP94 can also traverse the lysosome membrane. If this were not so, the CP94 inevitably captured by fluid-phase endocytosis would be retained during the wash-out period.

The maximum uptake of CP94 is 15 μL (approx.) (mg protein)$^{-1}$. This value seems to indicate a steady-state tissue/medium concentration ratio greater than unity, assuming the protein content of the visceral yolk sac is greater than 7% w/v. CP94, like other weak bases (De Duve et al 1974), might become concentrated in acidic organelles.

Our results support and amplify the contention (Cooper et al 1996) that CP94 and desferrioxamine differ sharply in their tendency to cross biological membranes. The disparity is consistent with the chemical structures of the chelators. CP94 exists principally in the uncharged form at neutral pH and its structure includes few groups capable of forming hydrogen-bonds, a molecular feature that correlates well with permeability across plasma and lysosome membranes (Stein 1967; Lloyd 1996). In contrast desferrioxamine is a charged primary amine that contains two secondary amide and three hydroxamate groups, and would be predicted to penetrate biological membranes very slowly. Although the octanol–water partition coefficient of desferrioxamine is some 500 times less than that of CP94 (Cooper et al 1996), this might underestimate the difference between their permeabilities across biological membranes, which correlate better with hexadecane–water partition coefficients (Walter & Gutknecht 1986). Hexadecane–water partition coefficients have not been reported for desferriox-

amine and CP94, but they might differ more than octanol–water coefficients, because hexadecane is a more hydrophobic solvent than octanol.

If, as we propose, desferrioxamine enters intact cells only by endocytosis, its intracellular location will be exclusively the endosome-lysosome complex. Like many other compounds that cannot readily permeate membranes, desferrioxamine will steadily accumulate in the lysosomes as long as the cell is exposed to the drug (Lloyd 1973). Thus the concentration of desferrioxamine in the lysosomes could reach high levels. This might explain why, despite the slow accumulation of desferrioxamine compared with that of CP94, its ability to complex intracellular iron outstrips that of CP94, after longer periods of exposure (Cooper et al 1996).

In conclusion, the data in this and our previous paper (Lloyd et al 1991) indicate clearly that quantitative differences in hydrophobicity can lead to a significant qualitative difference between the intracellular distribution of iron-chelators. Whereas a molecule such as CP94 enters and leaves cells rapidly, a more hydrophilic molecule such as desferrioxamine has the capacity to accumulate in the endosome-lysosome system. This disparity might be an important determinant of capacity to remove intracellular iron. We believe that future work on novel iron-chelators should pay great attention to their subcellular distribution.

Finally we note that, although CP94 is itself unlikely to be useful in the clinical treatment of iron overload, owing to its rapid metabolism to an inactive glucuronide (Porter et al 1993) and some cardiac and hepatic toxicity (Carthew et al 1994), the drug has recently emerged as having potential in the photodynamic treatment of cancer. CP94 enhances the 5-aminolaevulinic acid-induced accumulation of protoporphyrin IX, presumably by inhibiting its further conversion into haem, and thus increases the photosensitivity of affected cells (Bech et al 1997; Chang et al 1997; Smith et al 1997). This finding offers the prospect of a novel and unexpected therapeutic role for orally active iron chelators.

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