Development of potential iron chelators for the treatment of Friedreich's ataxia: ligands that mobilize mitochondrial iron

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Abstract

Friedreich's ataxia (FA) is a crippling neurodegenerative disease that is due to iron (Fe) overload within the mitochondrion. One therapeutic intervention may be the development of a chelator that could remove mitochondrial Fe. We have implemented the only well characterized model of mammalian mitochondrial Fe overload to examine the Fe chelation efficacy of novel chelators of the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH) class. In this model we utilize reticulocytes treated with the haem synthesis inhibitor succinylacetone which results in mitochondrial Fe-loading. Our experiments demonstrate that in contrast to desferrioxamine, several of the PCIH analogues show very high activity at mobilizing 59Fe from 59Fe-loaded reticulocytes. Further studies on these ligands in animals are clearly warranted considering their potential to treat FA. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Friedreich's ataxia (FA) is a severe neurodegenerative condition [1,2]. In 97% of patients the disease is due to a GAA triplet repeat expansion in intron 1 of the FRDA gene resulting in a marked decrease in its expression [1,2]. The protein encoded by this gene is known as frataxin and is located within the mitochondrion [2,3]. Over the last few years evidence has accumulated to suggest that frataxin plays a role in mitochondrial Fe metabolism [2,4–10]. Studies using the yeast cell showed that deletion of the homologous gene (YFH1) resulted in an accumulation of mitochondrial Fe resulting in the loss of mitochondrial DNA, Fe-sulphur cluster-containing enzymes, and respiration [4–6,11]. Like the human FRDA gene, YFH1 encodes a mitochondrial protein (Yfh1p). When YFH1 was reintroduced back into the yeast, mitochondrial Fe was exported back out into the cytosol, suggesting a ‘mitochondrial Fe cycle’ [4,9].

Consistent with the knockout yeast model, Bradley et al. [10] noted reductions in mitochondrial DNA, complex I, complex II/III, and aconitase in the heart of FA patients, observations consistent with mitochondrial damage. In addition, these authors reported increased Fe deposition in the heart, liver, and spleen of FA patients in a pattern consistent with a mitochondrial location [10]. This evidence...
suggesting that FA is caused by mitochondrial Fe overload was supported by work showing Fe deposits within the heart myofibrils [12,13], defective myocardial and skeletal muscle mitochondrial respiration [8,11], and perturbations in the haem synthesis pathway [14].

Since the pathology of FA is linked to mitochondrial Fe overload, new therapies based on these results could provide hope for FA patients. One novel strategy is the use of specific Fe chelators that can permeate the mitochondrion. A trial supported by the National Institute of Health is already investigating the use of the clinically used Fe chelator desferrioxamine (DFO) to treat FA patients [15]. However, DFO cannot efficiently mobilize Fe from cells [16,17], and it is not effective at mobilizing Fe from Fe-loaded mitochondria [18,19].

In contrast to DFO, Ponka and associates [18,20] have demonstrated that another chelator, pyridoxal isonicotinoyl hydrazone (PIH; Fig. 1), shows high activity at mobilizing Fe from an experimental model of mitochondrial Fe overload in reticulocytes. A variety of studies, in vitro, in vivo, and a clinical trial, have shown that PIH and its analogues show high potential for the treatment of Fe overload disease ([18,20-23]; for review see [24]). Unfortunately, the development of PIH was hindered due to the fact that it was not patented, resulting in little interest from the pharmaceutical industry [24]. To overcome this disadvantage we have synthesized a new group

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**Fig. 1.** The structures of the iron chelators assessed in this study: desferrioxamine (DFO), pyridoxal isonicotinoyl hydrazone (PIH), 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH), 2-pyridylcarboxaldehyde m-bromobenzoyl hydrazone (PCBBH), 2-pyridylcarboxaldehyde thiophencarboxyl hydrazone (PCTH), 2-pyridylcarboxaldehyde p-hydroxybenzoyl hydrazone (PCHH), 2-pyridylcarboxaldehyde p-aminobenzoyl hydrazone (PCAH), and 2-furoylcarboxaldehyde isonicotinoyl hydrazone (FIH).
of ligands known as the 2-pyridylcarboxaldehyde iso-
icotinoyl hydrazone (PCIH) analogues (Fig. 1) [25, 26]. These compounds have patent protection and were designed to share structural similarities to PIH (see Fig. 1). Moreover, several PCIH analogues were more active than DFO or PIH at mobilizing $^{59}$Fe from the SK-N-MC neuroepithelioma cell line, and showed low antiproliferative activity [25]. Obviously, both these latter properties are essential for the long-term treatment of Fe overload disease.

In the present study we have utilized the only well characterized cellular model of mitochondrial Fe overload, namely reticulocytes treated with the specific haem synthesis inhibitor, succinylacetone (SA) [27–31]. It is well established that in erythroid cells Fe is targeted to the mitochondrion for haem synthesis [31, 32]. Succinylacetone is a competitive inhibitor of the enzyme δ-aminolevulinic acid dehydratase and prevents protoporphyrin production [27]. Hence, in the presence of SA, Fe cannot be incorporated into protoporphyrin to form haem and results in an accumulation of non-haem mitochondrial Fe [28–33]. This provides a convenient model for assessing the ability of chelators to permeate this organelle and remove Fe [18–20, 33]. The results of the present investigation demonstrate that several PCIH analogues are very effective at mobilizing mitochondrial Fe. Consequently, we suggest that these ligands deserve further vigorous investigation as agents for the potential treatment of FA.

2. Materials and methods

2.1. Chelators

DFO was purchased from Novartis Pharmaceuticals (Switzerland). The PCIH analogues and PIH (Fig. 1) were synthesized by Schiff base condensation and characterized by elemental analysis, infrared spectroscopy, $^1$H-NMR, and X-ray crystallography as described previously [18, 20, 25, 26].

2.2. Reticulocytes

Reticulocytosis was induced in CD1 mice by injecting phenylhydrazine [33] using a protocol approved by the McGill University Animal Care Com-

mittee. Mice were injected with phenylhydrazine once per day for 3 days and the animals sacrificed 4 days after the last injection. Blood was obtained by cardiac puncture using a heparinized needle. Reticulocytes were identified based on staining with new methylene blue, and cell counts were determined using an improved Neubauer counting chamber. All preparations consisted of 30–35% reticulocytes and all samples used were free of haemolysis. Cell viability was assessed by microscopic examination of the reticulocytes for lysis and the detection of released haemoglobin into the supernatant.

2.3. Labelling of transferrin

Apotransferrin (Sigma, St. Louis, MO, USA) was prepared and labelled with $^{59}$Fe (as ferrie chloride in 0.1 M HCl, Dupont NEN, MA, USA) to produce $^{59}$Fe$_2$-transferrin ($^{59}$Fe-Tf) using established methods [34].

2.4. Mobilization of $^{59}$Fe from $^{59}$Fe-loaded reticulocytes

Reticulocytes were obtained as described above and incubated with 1 mM SA (Sigma) in minimum essential medium (MEM; Gibco, Grand Island, NY, USA) to inhibit haem synthesis [27–31]. After a 30 min preincubation at 37°C in the presence of SA, $^{59}$Fe-Tf (10 μM) was added and incubated with the cells for 1 h at 37°C in a shaking water bath. A 30 min incubation with SA has been shown previously to result in marked mitochondrial Fe accumulation and that sufficient $^{59}$Fe is incorporated after a 1 h label with $^{59}$Fe-Tf [28, 35].

The reticulocytes were subsequently washed three times with ice-cold PBS to remove non-specifically bound $^{59}$Fe-Tf. The washed $^{59}$Fe-labelled reticulocytes (30–35 μl) were then incubated with shaking at 37°C in MEM (500 μl final volume) with the ligands (10–200 μM) for 15–240 min. The SA (1 mM) was present in all incubations with the chelators to prevent the utilization of non-haem $^{59}$Fe for haem synthesis [35]. The $^{59}$Fe was measured in washed reticulocytes and in the medium, and the $^{59}$Fe release (counts per minute; cpm) was calculated as a percentage of the total number of cpm originally found in the cells [18, 20]. Radioactivity was measured using
a γ-counter with background correction (LKB 1282 Compugamma, Finland).

After the incubation with the chelators, the efflux medium was removed and the $^{59}$Fe-labelled reticulocytes were lysed by incubating reticulocytes for 15 min with 200 µl of ice-cold doubly distilled water. The proteins were then precipitated with 1 ml of ice-cold 95% ethanol. The resulting suspension was then centrifuged (13,000 rpm/30 min/4°C) on an IEC Micromax microcentrifuge (IEC, Canada) to result in an ethanol-soluble fraction containing $^{59}$Fe-bound to the chelators, and an ethanol-precipitated fraction containing protein-bound $^{59}$Fe [33,36]. Previous studies have demonstrated that this method results in the precipitation of $^{59}$Fe in ferritin and Tf while $^{59}$Fe bound to chelators remains in a soluble form [33, 36]. An increase of $^{59}$Fe in the ethanol-soluble fraction shows that the chelator can cross the cell membrane and form intracellular $^{59}$Fe complexes which are released with limited efficiency [36].

3. Results

In this investigation we have examined the Fe chelation efficacy of the PCIH analogues using the only well characterized model of mitochondrial Fe overload, that is, reticulocytes loaded with mitochondrial non-haem $^{59}$Fe [18–20,28–33]. In all studies PIH was used as our reference compound as this chelator has been characterized in previous studies to effectively deplete the non-haem mitochondrial Fe pool [18, 20,33].

In initial studies the effect of reincubation time on $^{59}$Fe release from Fe-loaded reticulocytes was assessed (Fig. 2). In these experiments cells were labelled with $^{59}$Fe-Tf for 1 h at 37°C, washed, and then reincubated for up to 240 min in the presence and absence of the chelators (200 µM). Of the eight compounds examined, PCIH was the most effective at increasing cellular $^{59}$Fe release as a function of incubation time (Fig. 2). Indeed, PCIH was more effective than PIH during incubation periods from 15 to 120 min, but had similar activity after a 240 min reincubation. The high activity of PCIH was evident after only 15 min incubation with $^{59}$Fe-loaded reticulocytes, at which point the compound had mobilized 21 ± 1% (three determinations) of cellular $^{59}$Fe (Fig. 2). The amount of $^{59}$Fe released by PCIH after 15 min was more than that mobilized by PCBBH, PCAH, PCHH, or FIH after 240 min of incubation, viz. 17%, 15%, 6%, and 4% respectively (Fig. 2). Desferrioxamine was the least effective chelator examined in terms of its ability to mobilize $^{59}$Fe from $^{59}$Fe-loaded reticulocytes, being only slightly more effective than control medium (Fig. 2). In all
experiments, the $^{59}$Fe released was ethanol-soluble, indicating that it is a low molecular weight complex that is not bound to protein.

None of the chelators had any effect on cellular viability over all incubations tested, and the $^{59}$Fe release was not due to the chelators inducing cell lysis. Examination of the ethanol-soluble intracellular $^{59}$Fe after incubation with the chelators revealed that this only increased in the presence of FIH (Fig. 3), suggesting the possible accumulation of its $^{59}$Fe complex within the cell. We did not see any accumulation of the $^{59}$Fe-PCIH complex in the ethanol-soluble fraction (Fig. 3), probably because the chelator quickly diffuses into the cell and then the complex rapidly exits out into the supernatant. In the case of PCIH and some of the highly active chelators (e.g. PIH and PCTH), the process of entry and exit from the cell is probably rapid, with a steady-state low intracellular ligand concentration becoming established. The appropriate lipophilicity of these compounds is probably an important criterion in this regard [25].

In further studies the effect of chelator concentration was assessed on $^{59}$Fe mobilization from $^{59}$Fe-loaded reticulocytes. In these experiments the cells were labelled with $^{59}$Fe-Tf for 1 h at 37°C, washed, and then reincubated for 1 h at 37°C in the presence and absence of the chelators (Fig. 4). Again, PCIH was the most active chelator. At a concentration of 200 μM, PCIH released 31 ± 1% (three determinations) of cellular $^{59}$Fe compared to PIH that mobilized 18 ± 1% (three determinations) (Fig. 4). The compound PCTH had similar Fe chelation efficacy as PIH, while the remaining ligands were substantially less efficient (Fig. 4). As described previously [18,19], DFO even at high concentrations up to 5 mM had little effect on mobilizing $^{59}$Fe, having an activity similar to that observed with control medium. Similar to our previous studies using the SK-N-MC neuroepithelioma cell line [25], both FIH and PCHH showed very low activity at mobilizing intracellular $^{59}$Fe (Fig. 4). As illustrated in Fig. 3, an increase in ethanol-soluble intracellular $^{59}$Fe was only observed with FIH, and this increased as the concentration was raised up to 200 μM (data not shown).

4. Discussion

At present there is no treatment which totally prevents the pathogenesis of FA. The exciting finding that mitochondrial Fe accumulation may play an important role in its pathogenesis [2,4-10] suggests that one possible therapeutic intervention may be Fe chelation therapy. This study identifies some of the PCIH class of chelators as highly effective ligands for mobilizing mitochondrial non-haem Fe from reticulocytes. This latter model was used as it is the only well characterized system of mitochondrial Fe overload in cells [28-33]. Previous studies from our laboratories and others have shown that incubation
of reticulocytes with haem synthesis inhibitors such as SA results in a very marked accumulation of mitochondrial non-haem $^{59}$Fe [18,19,28–31,33,35]. This mitochondrial Fe has been shown to be bound by some chelators of the PIH class [18,19,33,35], and this obviously provides an appropriate model to test potential ligands for the treatment of FA. It is relevant to note that reticulocytes treated with $^{59}$Fe-Tf in the absence of haem synthesis inhibitors do not accumulate mitochondrial non-haem $^{59}$Fe, as it is efficiently incorporated into haem that cannot be accessed and mobilized by the chelators [18,19].

The ability of PCIH and PCTH to mobilize mitochondrial Fe pools overcomes the disadvantage of DFO that cannot effectively deplete Fe from this compartment [18,19]. These studies complement our previous work demonstrating the high chelation efficacy and low toxicity of the PCIH group of ligands in the SK-N-MC neuroepithelioma cell line [25]. Indeed, some of these compounds were far more efficient than DFO at increasing Fe mobilization from cells and preventing Fe uptake from Tf [25].

The PCIH class of chelators was specifically designed based upon our previous studies on a wide range of compounds of the PIH class [35,37,38]. From these studies structural features critical for high chelation efficacy and low toxicity were chosen to optimize the use of these ligands as agents to treat Fe overload disease. Indeed, our design strategy has been successful, as it has resulted in chelators that show higher activity than the parent compound PIH (this study and [25]).

The strategy to design new chelators derived from PIH was based upon the advantageous properties of this ligand. These are: (1) oral effectiveness; (2) near optimal hydrophilic-lipophilic balance; (3) high specificity and selectivity for Fe; (4) predominantly neutral at physiological pH; (5) economical and simple to synthesize; and (6) high chelation efficacy both in vitro and in vivo (for review see [24]). These new patented chelators were essential to synthesize, as the development of ligands as therapeutic agents requires commercial intervention. Unfortunately, PIH was never patented, which made it an unattractive candidate for development by the pharmaceutical industry.

The potential for the use of Fe chelators as agents to treat FA is suggested by a recent clinical trial examining the effect of DFO in these patients [15]. Although this trial is vital to perform, it should be noted that DFO is quite a hydrophilic drug that does not efficiently permeate plasma membranes to bind...
cytosolic Fe [16,17]. Hence, DFO may not be able to efficiently permeate mitochondrial membranes, and this is a question that requires further investigation. Certainly, in this study (Figs. 2 and 4) and previous investigations [18,19], DFO had little effect at mobilizing mitochondrial Fe, even at very high clinically unachievable concentrations (5 mM) [18,19]. The other problems with DFO therapy, namely its poor intestinal absorption that necessitates long s.c. infusion (12–24 h/day, 5–6 days/week) and high cost, are serious disadvantages that necessitate development of an orally effective chelator [39].

The reason for the ability of some of the PCIH ligands to mobilize mitochondrial Fe may be their much higher lipophilicity in comparison to DFO. Indeed, to permeate the mitochondrion and chelate Fe, three lipid membranes need to be transversed, viz., the plasma membrane and the inner and outer mitochondrial membranes. Obviously, a lipophilic chelator that rapidly permeates membranes and targets mitochondrial Fe will be far more effective than a hydrophilic compound such as DFO. This may be a critical property of the ligand, since the Fe loading in FA is not as pronounced as that found in untreated β-thalassaemia. Hence, only very short durations of therapy may be possible or necessary in order to prevent overall body Fe depletion. Under these conditions, specific targeting of mitochondrial Fe pools will be a vital property. Indeed, perhaps combination of Fe chelators with antioxidants such as idebenone [40] may be a successful strategy in the effective treatment of FA.

In summary, the present study has identified several members of the PCIH class of ligands, particularly PCIH and PCTH, that are highly efficient at mobilizing mitochondrial Fe pools. Further studies on these compounds in animal models are clearly warranted considering their potential to treat FA.

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