Effects of Combined Chelation Treatment with Pyridoxal Isonicotinoyl Hydrazone (PIH) Analogs and Deferoxamine in Hypertransfused Rats and in Iron-Loaded Rat Heart Cells

Running Head: Combined PIH analog and DFO Chelation Treatment

Heading: Red Cells

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This work was supported by grant DK54199 of the NIDDK,
grant 197/99-2 of the Israel Science Foundation and grants from the Canadian Institutes for Health Research

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Word count: 5,366
ABSTRACT

Although iron chelation therapy with deferoxamine (DFO) results in improved life expectancy of thalassemic patients, compliance with parenteral DFO treatment is unsatisfactory, underlining the need for alternative drugs and innovative ways of drug administration. We examined the chelating potential of pyridoxal isonicotinoyl hydrazone (PIH) analogs alone, or in combination with DFO, employing hypertansfused rats with labeled hepatocellular iron stores and cultured iron-loaded rat heart cells. Our in vivo studies employing two representative PIH analogs 108-o and 109-o, have shown that the PIH analogs given orally are 2.6 to 2.8 times more effective in mobilizing hepatocellular iron in rats, on a weight-per-weight basis, than parenteral DFO administered by i.p. injection. The combined effect of DFO and 108-o on hepatocellular iron excretion was additive and response at a dose range of 25 to 200 mg/kg was linear. In vitro studies in heart cells showed that DFO was more effective in heart cell iron mobilization than all PIH analogs studied. Response to joint chelation with DFO and PIH analogs was similar to an increase in the equivalent molar dose of DFO alone, rather than the sum of the separate effects of PIH analog and DFO. This finding is most likely the result of iron transfer from PIH analogs to DFO, a conclusion supported directly by iron-shuttle experiments employing fluorescent DFO. These findings provide a rationale for the combined, simultaneous use of iron chelating drugs, and may have useful practical implications for designing novel strategies of iron chelation therapy.

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Word count: 245
Running Head: Combined PIH analog and DFO chelation treatment

Abbreviations:

DFO: deferoxamine
L1: deferiprone
PIH: pyridoxal isonicotinoyl hydrazone
ACD: acid citrate dextrose

Keywords: iron overload
iron chelation
iron shuttle
INTRODUCTION

Iron chelation therapy with deferoxamine (DFO) results in a significant improvement in well-being and life expectancy of thalassemic patients with transfusional iron overload (1). This is largely attributed to the prevention of heart disease in the majority of well-treated subjects and the reversal of existing heart disease in some patients by vigorous intravenous DFO therapy (2-4). However, compliance with the rigorous requirements of parenteral DFO treatment is far from satisfactory, and the failure to achieve negative iron balance in a substantial proportion of patients (5) underlines the need for the development of alternative drugs, and innovative ways of drug administration.

Interest in the combined use of chelators has been stimulated by metabolic balance studies performed in thalassemic patients in whom coadministration of deferiprone (L1) and DFO resulted in higher iron excretion rates than with DFO alone (6). The term "shuttle effect" was coined by Grady, implying that the combination of a weak chelator with high cell penetration, and a strong chelator with poor cell penetration but efficient excretion, may result in an improved effect through iron shuttling between the two compounds. In the present studies we wished to explore the mechanism of combined chelation treatment using DFO and a family of orally effective iron chelators, the PIH analogs introduced by Ponka and his associates (7-9). Results of these studies, employing a combination of in vivo (10) and in vitro (11) animal models, may have useful practical implications for designing novel strategies of iron chelation therapy.
MATERIALS AND METHODS

In vivo studies:

Female Wistar rats of the Hadassah strain weighing 170 to 200 g were used throughout. Hypertransfusion was performed by two intravenous injections of 2 mL packed cells per 100 g body weight on days 4 and 1 before storage iron labeling. The mean hematocrit on the first day of study was 69% ± 1% (mean ± 1SD), the serum iron 459 ± 18 µg/dL (mean ± 1SD), and the unsaturated iron binding capacity less than 10 µg/dL. Prelabeling of iron stores was accomplished via intravenous injection of the \(^{59}\)Fe-ferritin label through a tail vein. Animals were killed under ether anesthesia by exsanguination through the abdominal aorta into heparinized syringes.

Chelators: Deferoxamine (DFO) was purchased as the methane-sulfonate salt (Desferal) produced by Novartis, Basel, Switzerland. Prior to use, DFO was dissolved in normal saline to a final concentration of 1%. Three classes of hydrazones of various aldehydes (pyridoxal, salicylaldehyde and 2-hydroxy-1-naphthyl aldehyde) have been studied (Table 1 and Figure 1)

![Figure 1: Structure of pyridoxal isonicotinoyl hydrazone (PIH) and its analogs.](image)
Table 1. Identification and code numbers of PIH analogs

<table>
<thead>
<tr>
<th>Hydrazine</th>
<th>Aldehyde</th>
<th>2-hydroxy-1-naphthyl aldehyde</th>
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<tbody>
<tr>
<td></td>
<td>pyridoxal</td>
<td>salicyl-aldehyde</td>
</tr>
<tr>
<td>Benzoyl</td>
<td>101</td>
<td>201</td>
</tr>
<tr>
<td>$p$-Hydroxybenzoyl</td>
<td>102</td>
<td>202</td>
</tr>
<tr>
<td>$p$-Methylbenzoyl</td>
<td>103</td>
<td>203</td>
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<tr>
<td>$p$-Nitrobenzoyl</td>
<td>104</td>
<td>204</td>
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<tr>
<td>$p$-Aminobenzoyl</td>
<td>105</td>
<td>205</td>
</tr>
<tr>
<td>$p$-t-Butylbenzoyl</td>
<td>106</td>
<td>206</td>
</tr>
<tr>
<td>$p$-Methoxybenzoyl</td>
<td>107</td>
<td>207</td>
</tr>
<tr>
<td>$m$-Chlorobenzoyl</td>
<td>108</td>
<td>208</td>
</tr>
<tr>
<td>$m$-Fluorobenzoyl</td>
<td>109</td>
<td>209</td>
</tr>
<tr>
<td>$m$-Bromobenzoyl</td>
<td>110</td>
<td>210</td>
</tr>
<tr>
<td>Isonicotinoyl</td>
<td>111</td>
<td>211</td>
</tr>
<tr>
<td>Acetyl</td>
<td>112</td>
<td>212</td>
</tr>
<tr>
<td>2-Pyrindyl</td>
<td>113</td>
<td>213</td>
</tr>
<tr>
<td>2-Furoyl</td>
<td>114</td>
<td>214</td>
</tr>
<tr>
<td>2-Thiophenecarboxyl</td>
<td>115</td>
<td>215</td>
</tr>
</tbody>
</table>
They were synthesized by Schiff base condensation as described previously (12), and the absence of significant amounts of the starting materials in these preparations was confirmed by analytical thin layer chromatography. Identity and purity of the hydrazones were confirmed by $^1$H- and $^{13}$C-NMR. $^1$H spectra were similar to those reported previously (12). Solutions of the chelators were prepared in 0.1 M NaOH in 50% aqueous ethanol, and were immediately used to prevent their base-catalyzed hydrolysis. Control experiments in which stock solutions were prepared in dimethyl sulfoxide yielded similar results (not shown), indicating that any hydrolysis which occurred in the basic solutions was negligible.

**Counting Methods:**

The radioactivity of spleen, kidney, and weighed portions of the liver, and 1 mL samples of blood was determined in an automatic well-type scintillation counter (Auto-Gamma, Model 5360; Packard Instruments Co, Inc. Downer's Grove, IL). Whole body counts were performed in a small-animal counter (Packard Model 446, Armac liquid scintillation detector). To measure the excretion of radioactivity after $^{59}$Fe labeling, the animals were confined in solitary metabolic cages with stainless steel grid bottoms and urine and stool were collected separately. Radioiron excretion was also determined by whole body counts on the first and last days of the study, and corrections were made for decay and differences in geometry. Recovery of radioactivity in excreta compared with the decrease in whole body radioactivity was over 90%.
Preparation of radioiron label:

Soluble ferritin (\(^{59}\text{Fe}-\text{ferritin}\)). In vivo \(^{59}\text{Fe}\)-labeled ferritin was prepared by injecting 100 to 200 \(\mu\text{Ci}\) of \(^{59}\text{Fe}\)-citrate at a citrate to iron ratio exceeding 40:1 into rats that had been given 12 mg of iron dextran the preceding week. The animals were killed 24 h later, and purified radioactive ferritin was prepared by the method of Bjorklid and Helgeland (13). Initial processing of the storage iron label has been described in detail in previous communications (14-17). Total nonheme iron in the liver was determined by the method of Torrance and Bothwell (18).

Design of animal studies:

These studies have been approved by the institutional committe for animal experimentation of the Hebrew University Hadassah Medical School. All studies were performed in hypertransfused animals. Hypertransfused rats were housed in metabolic cages and stool and urine were collected for 7 consecutive days. One hour after intravenous injection of the radioiron-labelled ferritin on day 0, a single 200 mg/kg dose of chelator was administered intraperitoneally (DFO) or orally (PIH analogs). In all experiments, each subgroup of control or treated animals consisted of at least 4 subjects. All animals were killed on day 7 and the organ distribution of radioiron was determined as described above. Whole body radioactivity was determined on day 0 (representing 100% initial radioactivity) and day 7 to determine total radioiron excretion. This measurement was used as an independent confirmation of total radioiron excretion based on the measurement of cumulative fecal and urinary radioiron excretion.

For the statistical evaluation of differences between treatment groups, the Student's t-test has been used.
In vitro studies:

Cell cultures: Cultures from 1-day-old rats (Hebrew University strain) were obtained by methods described in detail in our previous studies (11,19-23). The combined fractions of heart cells were resuspended in growth medium into a sterile 250 ml flask (Nunc; Nunclon Delta, Herlev, Denmark) through a sterile mesh, to exclude explants. To reduce the number of fibroblasts and increase the proportion of myoblasts a preplating method was used exploiting the faster attachment of fibroblasts to the dish surface (24). After 1 h preplating at 37°C in 100 mm diameter Petri dishes, the pooled cells were diluted in growth medium to a density of 9 x 10^5 to 1 x 10^6 cells/ml and seeded into a 35 mm diameter Petri dish (Falcon 3001; Falcon Labware, Oxnard, Calif.). This concentration yielded after 24 to 36 hours an almost confluent layer of beating heart cells at a final density of about 2x10^6 cells. Cultures were kept at 37°C in an atmosphere of 5% CO₂ and 95% air. Experiments were performed at 5 days of culture when over 80% of the cells were beating myocardial cells. Continued viability of cultured iron-loaded cells was documented by supravital dye exclusion and by the absence of enzyme (lactate dehydrogenase) leakage into the culture medium after 24-hour iron loading. For the chelating studies, DFO or PIH analogs were dissolved in serum-free medium to a final concentration of 0.25 % or less.

Radioactive iron labeling: ^{59}Fe-citrate (specific activity 3 to 20 µCi/µg; Amersham Radiochemical Centre, Amersham, England) was mixed with sufficient sterile ferric ammonium citrate (BDH Fine Chemicals Ltd., Poole, England) to provide a concentration of 100 µg/ml elemental iron. In all studies a final concentration of 160 µM iron was used for iron-loading and radioiron labeling of heart cell cultures. To terminate incubations the culture plates were washed twice with 1 ml cold culture
medium. The cells were scraped and transferred into counting tubes by means of a rubber policeman and resuspended in 0.5 ml culture medium. $^{59}$Fe activity was determined in an automatic well scintillation counter (Auto-Gamma model 5360, Packard Instrument Co., Inc., Downers Grove, Ill.).

**Iron shuttle studies:** Fluorescein-DFO (Fl-DFO, F-6877) was purchased from Molecular Probes Inc., Eugene, OR. Plastic 96-well plates with clear, flat bottom were F96 Maxisorp from Nunc, Roskilde, Denmark. Heps-buffered-saline (HBS: 150 mM NaCl, 20 mM Heps pH 7.3). The fluorescence of Fl-DFO was determined in a multiwell plate reader (BMG LabTechnologies, Offenburg, Germany) with excitation/emission filters of 485/538 nm and gain of 20. Complexes of 108-o:Fe were formed by mixing FeNTA (5 mM ferrous ammonium sulfate : 35 mM NTA) and 108-o in HBS to yield solutions containing 10 $\mu$M Fe and increasing concentrations of 108-o up to 500 $\mu$M, followed by incubation for 1 hr at room temperature. At 0 min., 25 $\mu$l of each chelator-Fe complex was mixed with 100 $\mu$l of 2.5 $\mu$M Fl-DFO in HBS, and fluorescence was monitored over time in a Fluorescence Plate Reader. In our previous studies we have shown that DFO and its fluorescein derivative Fl-DFO have similar Fe-binding affinities and, that Fl-DFO provides a valid model for native DFO (25).

**Evaluation of co-treatment:** Quantitative analysis of the dose response profiles of a combination of drugs was based on the methods described by Poch et al. (26-28) The parameter values of dose response curves were calculated by fitting the sigmoidal four parameter logistic function $Y = ((a-d)/(1+(x/c)^b))+d$, where $y$ represents the response (in %) for a given concentration $x$ of the drug, $d=$ the basal response and $a$ the maximal response, $b=$slope of the response, and $c=$ ED50, the concentration of drug that elicits 50% response. Curves of dose-additive combinations were obtained
by calculating the dose $x$ of the drug A with which a fixed dose applied was equi-effective. According to the definition of dose-additivity, the effects of curve A alone are to be expected at the doses of A minus x, thus providing the y-values for the simulation of the corresponding additive action of the drugs. Curves of independent combinations were calculated on the basis of the effects E of drug A and drug B alone, whereby $EA+B = EA + AB-(EA*EB)$ and the effects E are expressed by the fraction of maximum response. Statistical comparison of the simulated (i.e. theoretical) dose response profiles with the experimental profile was done by using 95% confidence intervals as described by Holzmann et al (28). The analysis was carried out on the basis of a program package kindly provided by Prof. G. Pöch (Gratz, Austria), which consisted of user-defined worksheet, curve fits and transforms adapted for SigmaPlot, version 5.0 for Windows 2000 (Jandel).
RESULTS

Studies in hypertransfused rats:

DFO or PIH-class compounds alone.

In untreated controls, total body radioactivity 7 days after labeling was 88.9 ± 1.8 % (mean ± 1SD) of the injected dose. Of this, 79.9 ± 9.4 were in the liver, 1.5 ± 0.7% in circulating RBC, 0.5 ± 0.1 % in the spleen and 7.0 ± 0.3 % in residual tissues. The injection of a single dose of 200 mg/kg DFO resulted in a decrease in whole body radioactivity from 88.9 ± 1.8 % to 69.8 ± 3.8 % (p< 0.001) and after an identical oral dose of 108-o to 37.5 ± 2.1 % and of 109-o to 33.8 ± 7.5 % (p< 0.001 ). This was accompanied by a decrease in liver radioactivity from 79.9 ± 9.4  to 55.5 ± 5.5% (p<0.001) following DFO, to 30.6 ± 5.5% (mean ± 1SD) following 108-o and to 26.4 ± 4.2 following 109-o (p< 0.001). Thus, practically all chelator-induced radioiron excretion could be accounted for by the loss of hepatic radioactivity. Recovery of radioactivity in the stool collected over 7 days accounted for 97.1 ± 4.3 % of radioiron excretion. By contrast, urinary radioiron excretion was less than 1 % in all experiments performed. Response to both 108-o and 109-o, as manifested in a decrease in both whole body radioactivity and liver radioactivity was clearly greater with 108-o and 109-o than with DFO (p< 0.001). Variations in blood, spleen and residual radioactivity in the chelator-treated groups compared with untreated controls were minimal.
**Dose response and combined therapy.**

In the second part of our *in vivo* studies in hypertransfused ⁵⁹Fe-ferritin-tagged animals, we explored the effect of increasing doses of *oral* 108-o administered separately, or in combination with a fixed dose (100 mg/kg) of *parenteral* DFO (Figure 2 A and B).

A

![Figure 2A: Effect of 108-o dose on radioiron excretion (A) and on residual hepatic radioactivity (B) with or without the addition of a constant (100 mg/kg) dose of DFO. Results expressed as net 7 day measurements (% of injected radioactivity). Each treatment group consisted of 4 animals (mean ±1SD).](image)

108-o-induced radioiron excretion was linearly dose-related and ranged from 2.3± 1.8 % at 25 mg/kg to 42.6± 7.7 % at 200 mg/kg (mean ±1SD) (Figure 2A). By comparison, net radioiron excretion following a single 100 mg/kg dose of DFO was 17.5 ± 1.6 %. A mirror image of this response can be seen in Figure 2B describing the decrease in residual hepatic radioactivity.
Combined 108-o and DFO treatment was more efficient than each drug given alone. This effect was most prominent (p< 0.001) at the highest 108-o dose levels studied namely, 200 mg/kg for net radioiron excretion and 100 to 200 mg/kg for the decrease in residual hepatic radioactivity. The magnitude of this combined response was roughly equal to the combined effects of each drug given alone, supporting an additive effect of combined treatment.

Studies in cultured heart cells:

The ability of DFO and PIH analogs (identified by their numbers in Table 1) to inhibit the uptake of radioiron by cultured heart cells is described in Table 2. Five-days old rat heart cell cultures were exposed for 24 h to 160 µM iron supplied as ferric ammonium citrate alone (controls), or in the presence of various chelators, at concentrations ranging from 0 to 320 µM. Washed control cells had a radioiron uptake of 29.6±2.6 % (mean ± 1SD) of the total radioactivity in the iron-loading culture medium. In the case of compounds 108, 109 and 208, both ortho, and para substituted isomers have been studied.
Table 2. Inhibition of radioiron uptake (% of untreated controls)

<table>
<thead>
<tr>
<th>Chelator concentration µM</th>
<th>(n)</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFO</td>
<td>(6)</td>
<td>79.3±0.5</td>
<td>75.9±5.9</td>
<td>32.4±8.8</td>
<td>0.7±0.2</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>101 (PBH)</td>
<td>(4)</td>
<td>96.1±10.7</td>
<td>109.0±12.2</td>
<td>80.1±9.0</td>
<td>58.1±11.1</td>
<td>26.9±5.0</td>
</tr>
<tr>
<td>108-o</td>
<td>(4)</td>
<td>92.8±12.1</td>
<td>87.2±8.6</td>
<td>78.4±5.9</td>
<td>49.5±4.2</td>
<td>7.8±0.8</td>
</tr>
<tr>
<td>108-p</td>
<td>(4)</td>
<td>99.5±6.3</td>
<td>97.9±15.9</td>
<td>83.7±9.7</td>
<td>121.3±8.5</td>
<td>153.7±0.8</td>
</tr>
<tr>
<td>109-o</td>
<td>(4)</td>
<td>99.4±14.0</td>
<td>106.6±10.1</td>
<td>84.6±12.9</td>
<td>76.2±10.0</td>
<td>50.8±4.9</td>
</tr>
<tr>
<td>109-p</td>
<td>(4)</td>
<td>107.8±4.9</td>
<td>96.1±1.9</td>
<td>87.8±8.2</td>
<td>63.9±6.0</td>
<td>25.8±1.6</td>
</tr>
<tr>
<td>111 (PIH)</td>
<td>(4)</td>
<td>89.8±7.0</td>
<td>76.7±30.7</td>
<td>73.2±1.2</td>
<td>36.1±1.1</td>
<td>4.8±0.4</td>
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<tr>
<td>208-o</td>
<td>(4)</td>
<td>100.8±8.4</td>
<td>115.4±11.9</td>
<td>110.6±10.8</td>
<td>106.6±2.2</td>
<td>41.9±5.8</td>
</tr>
<tr>
<td>208-p</td>
<td>(4)</td>
<td>106.8±5.4</td>
<td>101.4±10.1</td>
<td>112.5±11.8</td>
<td>124.4±2.9</td>
<td>126.6±11.0</td>
</tr>
<tr>
<td>211 (SIH)</td>
<td>(4)</td>
<td>91.0±6.8</td>
<td>86.6±10.6</td>
<td>74.6±10.4</td>
<td>63.9±2.8</td>
<td>10.3±10.7</td>
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<tr>
<td>301</td>
<td>(4)</td>
<td>104.5±2.9</td>
<td>103.3±19.3</td>
<td>88.6±11.9</td>
<td>125.1±5.2</td>
<td>132.5±10.9</td>
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<tr>
<td>308</td>
<td>(4)</td>
<td>94.41.4</td>
<td>107.5±1.6</td>
<td>86.1±13.6</td>
<td>108.8±9.2</td>
<td>106.5±11.0</td>
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<tr>
<td>309</td>
<td>(4)</td>
<td>86.2±2.0</td>
<td>97.9±3.8</td>
<td>100.3±5.3</td>
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<td>(4)</td>
<td>86.5±5.9</td>
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<tr>
<td>315</td>
<td>(4)</td>
<td>84.1±2.7</td>
<td>97.0±3.3</td>
<td>97.9±4.6</td>
<td>101.9±3.8</td>
<td>91.3±1.2</td>
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</tbody>
</table>

As shown in Table 2, some of the analogs had no ability at all to inhibit radioiron uptake. These included: para-108, para-208, and compounds 301,308, 309, 311 and 315. All other compounds showed a dose-related inhibition of heart cell iron uptake, with DFO being the most effective. As DFO is a hexadentate chelator combining with iron at a 1:1 molar ratio, whereas all others are
tridentate, requiring 2 M of the chelator to bind one M of iron, a better comparison of chelating ability is offered by presenting results according to iron-binding equivalents (Figure 3). This has shown that prevention of iron uptake was complete, or near complete for all effective chelators at an iron binding equivalent concentration of 160 µM, i.e. identical with the concentration of iron present in the incubation mixture.

Figure 3: Effect of treatment on radioiron uptake by cultured heart cells. 5 day old cultures were incubated with 160 µM ⁵⁹Fe-labelled ferric ammonium citrate for 24 h in the presence of PIH analogs at concentrations ranging from 0 to 160 µM iron binding equivalents. Results expressed as percentage of total cellular radioactivity in untreated controls. Each point represents the mean ±1SD of 6 cultures.

The ability of DFO and PIH analogs to remove iron from iron-loaded heart cells was studied next. Heart cell cultures were exposed for 24 h to 160 µM iron supplied as ferric ammonium citrate, washed and treated subsequently for an additional 24 h with DFO or a PIH analog, at concentrations ranging from 0 to 320 µM (Figure 4).
As shown in Figure 4, the ability of DFO to remove heart cell radioiron was substantially better than that of PIH and its analogs at all concentrations studied. This advantage of DFO over the other compounds remained significant (p<0.001) even when allowance was made for the difference in iron binding capacity (i.e. PIH analogs at twice the molar concentration of DFO).

The effect of combined DFO and PIH analog treatment performed in the same experimental system is shown in Figure 5.
The effect of combined DFO and PIH analog treatment performed in the same experimental system is shown in Figure 5. Increasing concentrations of the PIH analogs 108-0 have been used in Figure 5: Effect of increasing PIH analog and DFO concentrations on radioiron mobilization from cultured, iron-loaded heart cells. DFO+analog I: The molar concentration of mixed DFO+analog was identical with the sum of the corresponding single drugs, i.e. twice the molar concentration of each single drug. DFO+analog II: The total molar concentration of mixed DFO+analog was identical with the corresponding single drug and consisted of equal parts of the two compounds at half the molar strength. Results expressed as % or total cellular radioactivity in untreated controls. Each point represents the mean ±1SD of 6 cultures.
As shown previously (Figure 4), in all cases, DFO was more effective than the corresponding PIH analog, at equimolar concentrations ranging from 40 to 320 µM. Co-treatment with both chelators (DFO + analog I) resulted in a response that was significantly (p< 0.001) greater than either drug alone, over the concentration range of 80 to 320 µM. However, such co-treatment involved the use of twice the molar concentration of each drug used separately. Hence, a more appropriate comparison was to examine the effect of combined treatment consisting of equal parts of both chelators at half the molar concentration of the single drug (DFO + analog II). This method of comparison revealed an interesting phenomenon: At molar concentrations of 160 to 320 µM, a crossover effect has been obtained with all analogs studied, wherein the effectiveness of DFO + analog became equal to or even exceeded that of DFO. This finding, encountered with all analogs studied, did not support a simple additive effect in which the expected result should be intermediate between the effect of each chelator alone.

In order to examine in a more comprehensive manner whether the interaction between DFO and the PIH analogs was additive or independent, additional studies have been performed using constant (20 or 40 µM) concentrations of DFO in the presence of various concentrations of the PIH analog 108-o, ranging from 0 to 160 µM (Figure 6). The dose response profiles of the combination of drugs was subjected to quantitative analysis based on the methods described by Poch and associates (26-28).
Figure 6:
Dose-response curves of chelator 108 in the absence (open symbol) and presence of either 20 µmol/L (upper) or 40 µmol/L (lower) deferoxamine=DFO (filled symbols). The symbols and bars represent experimental mean values of triplicate samples (±SEM). Sigmoidal fits to the experimental points are shown by solid lines, and the theoretical dose response curves by dashed lines (dose-additivity) and dotted lines (independent).
5 day old cultures were first incubated with 160 µM $^{59}$Fe-labelled ferric ammonium citrate for 24 h, washed and then exposed for an additional 24 h to 20 or 40 µM deferoxamine.
(DFO), and 0 to 160 µM 108-o. Effect described as percent of initial radioactivity in control iron-loaded cells.

As shown in Figure 6, a significant deviation from dose-additivity was obtained for the two combinations of 108-o and DFO, using the 95% confidence limit with upper and lower boundaries, as described by Holzmann et al. (28).

In order to document the ability of DFO to remove iron bound to PIH analogs (the shuttle effect), we have exploited the phenomenon of fluorescence quenching when iron is bound to fluorescein-DFO (Fl-DFO). Fl-DFO (2 µM) was added to pre-formed analog-Fe complexes containing a fixed Fe concentration (2 µM) with increasing concentrations of the PIH analog 108-o. The PIH analog:Fe ratios ranged from 1.56:1 to 50:1. The transfer of iron from 108-o to DFO at an 108-o concentration of 3.12 µM was compete within less than 10 minutes, as indicated by the quenching of fluorescence (Figure 6). Although at higher molar ratios 108-o was able to slow down the transfer of Fe to Fl-DFO in a concentration dependent manner, it was unable to prevent it even when the molar ratio of 108-o to Fl-DFO was 50:1.
Figure 7: Effect of 108-o concentration on iron exchange between 108-o-Fe and fluorescein-DFO (Fl-DFO). Complexes of 108-o:Fe were preformed by mixing Fe:NTA (5 mM Ferrous Ammonium Sulfate : 35 mM Nitrilotriacetate) with 108-o in HBS to yield solutions containing 10 µM Fe and increasing concentrations of 108-o (0, 15.6, 31.3, 62.5, 125, 250 and 500 µM), followed by incubation for 1 hr at room temperature. At 0 min., 25 µl of each chelator-Fe complex was mixed with 100 µl of 2.5 µM Fl-DFO in HBS, and the fluorescence was monitored over time in a Fluorescence Plate Reader. The final concentrations of both Fe and Fl-DFO were 2 µM in each system, while the final concentration of 108-o varied as shown: from 0 through 100 µM. The fluorescence of Fl-DFO without added chelator or Fe is indicated by ‘Fl-DFO alone’.

**DISCUSSION**

In view of the failure of iron chelation treatment with DFO to achieve the goals of iron depletion in a substantial proportion of thalassemic patients (5), alternative methods of treatment have been explored using other, orally active chelators. Studies employing the oral chelator deferiprone (L1) have indicated (6) that combined DFO and L1 treatment may be more effective than either drug alone and, in addition, it may decrease their toxic side effects, some of which are dose-related.
However, the use of deferiprone is associated with an increased risk of agranulocytosis and other complications (29-31). Hence it would be interesting to validate the concept of increased efficacy of combined chelation treatment using other orally effective chelators of potential clinical value.

Pyridoxal isonicotinoyl hydrazone (PIH) was first introduced by Ponka et al in 1979, demonstrating its ability to mobilize iron from $^{59}$Fe-labelled reticulocytes (7). PIH is a tridentate chelator with a molecular weight of 287 (Figure 1). At physiologic pH, PIH is mainly in its neutral form which allows access across cell membranes and absorption from the gut. At pH 7.4 and a ligand concentration of 1 mmol/L, the pM value of PIH is 27.7 which is less than 28.6 for DFO (8,9,35). The selectivity of PIH for iron is comparable with that of DFO. Studies in rats (32,33) have shown that PIH is able to remove parenchymal and RE iron and, that practically all chelated iron is excreted through the bile (33). Its in vivo chelating efficiency in rats was equal to, or slightly better than DFO and there was no evidence of toxicity at doses up to 500 mg/kg/d. A variety of PIH analogues have been prepared and evaluated by Richardson and Ponka (9,35). Some of these, such as the pyridoxal-benzoyl hydrazones are up to 280% more effective than PIH, a feature largely attributed to their greater lipophilicity.

Unfortunately, studies in patients with iron overload treated with PIH at a dose of 30 mg/kg/d have shown only a modest net iron excretion of $0.12 \pm 0.07$ mg/kg/d (34), which is much less than the mean value of 0.5 mg/kg/d required to achieve negative iron balance in most cases. Although the results of this pilot study in thalassemic patients were generally regarded as evidence for the limited value of PIH in the treatment of thalassemia, several arguments have been raised in favour of PIH: First, the dose of 30 mg/kg used in the above study was much less than the effective dose of 125 to 500 mg/kg employed in experimental animals. Second, PIH was given to patients after calcium
carbonate as a powder in gelatin capsules. This may have prevented acid hydrolysis of PIH, but also could drastically limit its absorption because of the low solubility of PIH in aqueous solution at a neutral pH (35,36).

In view of the uncertainty regarding the ability of PIH to induce satisfactory iron excretion as a single drug, we wished to explore the possible advantage of combined chelation treatment consisting of PIH analogs and DFO using in vivo and in vitro animal models. In these studies, we wished to determine whether or not the coadministration of PIH analogs and DFO may result in an additive, or an even stronger effect.

In our in vivo studies we have selectively labeled hepatocellular iron stores with $^{59}$Fe-ferritin to document the effect of iron chelation on storage iron mobilization. The aim of hypertransfusion was to minimize the recycling of storage iron onto circulating transferrin. These studies comparing the in vivo effects of DFO and two representative PIH analogs 108-o and 109-o, allow the following conclusions to be drawn: (a) The PIH analogs given orally are 2.6 to 2.8 times more effective in rats, on a weight-per-weight basis, than parenteral DFO in promoting the excretion of storage iron from parenchymal iron stores. (b) The combined effect of DFO and 108-o on hepatocellular storage iron excretion was additive. (c) The dose-response obtained with 108-o at a dose range of 25 to 200 mg/kg was linear, with no suggestion of a diminishing response at the highest dose studied.

The effect of combined chelation treatment was further explored in cultured iron loaded heart cells. Unlike the studies performed in intact animals, this in vitro model offers accurate control of experimental conditions such as the degree of iron overload and extracellular drug concentrations.
These studies have shown that the chelators studied, including DFO and a large number of PIH analogs, are equally effective in preventing the uptake of non-transferrin bound iron from the culture medium. This effect was directly related to their iron binding capacity requiring a 2:1 molar ratio of drug to iron for all tridentate PIH analogs, and a 1:1 ratio for the hexadentate chelator DFO. However, when iron loading preceded chelating treatment in order to study the mobilization of intracellular iron, DFO was much more effective than any of the PIH analogs. This difference cannot be attributed to better cell penetration of DFO since previous studies in hepatocytes and reticulocytes have documented the excellent ability of the small lipophilic PIH class molecules to enter cells compared to the more limited cellular penetration of DFO (8,9,37). An alternative, and more likely explanation based on previous studies in labeled reticulocytes, macrophages and hepatocytes (38) is that some PIH derivatives form membrane-impermeable iron complexes within the cell, limiting their ability to promote the exit of intracellularly chelated iron. This may not be the case in the liver which has an alternative in vivo route of excretion through the bile (4).

In line with our previous in vivo experiments, in vitro cotreatment of heart cells with DFO and PIH analogs had an improved iron-mobilizing effect. In order to analyze the dose-response profiles of the drug combinations employed, we have used the methods described by Poch et al (26-28). The use of model combination effects for the delineation of sites and mechanisms of action requires some understanding of the underlying bases of these models. The dose-additivity model describes the combination effects of agents which share the same molecular site of action. Agents which compete for the same binding site are then expected to exhibit a dose-additive combination, which can be seen as a special case of a competitive interaction. Applying this model for the evaluation of chelating agents
means that two agents acting at the same molecular site of action should behave according to the model of dose-additivity. On the other hand, any significant deviation from this model suggests either different sites of action of the compounds tested in combination or a dual action of one component or both agents. An interaction of a dual action compound with another agent possessing one of the two components is likely to result in a combination effect which is different from a pure competitive action.

Since the combined effect of the two chelators was significantly greater than dose-additive, we wished to explore the possibility of a shuttle effect wherein iron first chelated by PIH analogs is transferred subsequently to DFO. Our study employing fluorescent DFO (Fl-DFO) provides direct evidence for the transfer (shuttling) of iron from 108-o to DFO, manifested in the quenching of fluorescence when iron prebound to 108-o is transferred to Fl-DFO. This transfer was rapid and complete within less than 10 minutes in the presence of roughly equal molar concentrations of the two chelators. Although this transfer did slow down in a concentration-dependent manner, it was not prevented even by an 108-o to Fl-DFO ratio of 50:1.

In attempting to evaluate the implications of the present findings, one should take account of the limitations of the experimental systems employed:

(a) *Dose and delivery of the two chelators*: Although both compounds were given at an identical dose of 200 mg/kg in vivo, the molar dose of DFO (M.W. 657) was less than one half that of PIH (M.W. 287). However, on a weight per weight basis the iron binding equivalent of the tridentate PIH analog chelators was roughly identical with the hexadentate chelator DFO. Although DFO was
given parenterally, whereas the PIH analogs were administered by gastric gavage, these methods of
delivery and a dosimetry relying on weight per/kg rather than molarity, are identical with the
methods accepted in clinical practice for evaluating chelating drugs. However, the single i.p.
injection of DFO may have been less effective than continuous s.c. infusion as used in clinical
practice.

(b) Use of selective storage iron labels: This allowed identification of specific chelatable storage
iron pools interacting with the chelators. However this method may overlook other chelatable pools
such as non-transferrin-bound plasma iron (NTBI) derived largely from reticuloendothelial (RE) cells
(4). In the present studies we did not employ a selective RE iron label such as heat damaged RBC,
because our previous study of PIH has already shown that labelled RE iron does not contribute
greatly to PIH-induced iron excretion (33). Conversely, first-pass interaction of oral PIH –class
chelators with hepatocellular iron stores via the portal circulation may have overestimated their
efficacy, compared with parenteral DFO of which only a fraction may reach the liver. This
difference, however, is identical with the clinical setting wherein oral PIH analogs (unlike DFO) are
first presented to hepatocytes through the portal circulation. Thus, our estimates of the relative
efficacy of PIH analogs compared with DFO may be distorted by the methodology used and should
be interpreted with caution. First, we did not document DFO-induced iron excretion derived from
RBC catabolism in the RE system, a major source of chelated iron in the clinical context. Second, a
single i.p. injection of DFO may have been less effective than continuous s.c. infusion as employed in
clinical practice.

(c) In vitro studies in cultured heart cells: Results of these studies showing a lower efficiency of
PIH analogs appear to conflict with the results of our in vivo experiments in which 108-o and 109-o
were more effective than DFO on a weight-per-weight basis. This may reflect a genuine difference in
the organ specificity of DFO as compared with PIH analogs. Previous studies in macrophages, reticulocytes and hepatocytes have shown that hepatocytes are particularly responsive to pyridoxal hydrazone chelators (38). This may be explained by the unique ability of hepatocytes to secrete chelated iron through the bile, instead of relying on the limited ability of the intracellularly chelated iron complex to cross the plasma membrane.

Having considered all of these caveats it is still possible to arrive at some significant conclusions on the joint effects of DFO and PIH analogs, based on results of the present studies. Simultaneous administration of DFO and PIH analogs results in an increase in chelating effect which is additive in hepatic iron excretion, but higher than additive in respect to myocardial iron mobilization. In the rat heart cell model employed by us, the magnitude of this joint effect is similar to an increase in the equivalent molar dose of DFO alone, rather than the sum of the separate effects of PIH analog and DFO. This finding is most probably the result of a transfer of chelated iron from PIH analogs to DFO, a conclusion which is supported directly by the results of our experiments employing fluorescent DFO. Our conclusion is also supported by the results of previous studies showing that at physiologic pH 7.4 the pM value of DFO is higher than that of PIH (39). Our in vivo findings are also consistent with clinical observations in patients receiving combined treatment with DFO and the oral chelator L1 indicating that combined chelation treatment improves iron excretion in thalassemic patients and results in a decrease in serum ferritin in patients previously failing to respond to standard L1 treatment (40).

A number of theoretical arguments may be considered in favor of employing several chelating drugs simultaneously to improve response to treatment: (a) Since the toxic side-effects of the two
compounds are different and some of them are dose-dependent, it has been claimed that cotreatment may allow dose reduction and diminish dose-related drug toxicity. However, this expectation has not been confirmed by early experience gained in clinical studies of combined DFO and deferiprone administration, and in vitro studies of non-heme iron enzyme inhibition have shown synergistic toxicity when the two drugs were employed simultaneously (41). (b) DFO has a limited access to intracellular iron stores but may be able to interact indirectly with chelated iron thanks to the shuttling effect of the small lipophilic PIH analog. Such an interaction has already been demonstrated in the context of antimalarial activity of combined DFO and PIH analog treatment (37). Such combined action may also be able to prevent the production of the toxic NTBI originating from RBC hemoglobin breakdown in the RE system (4). Finally: (c) if the shuttling concept is validated, it may be utilized for the coadministration of other drug combinations. For example, Grady et al have already shown that the coadministration of deferiprone and HBED greatly enhances the oral effectiveness of HBED (42). Likewise, our recent studies in animal models have shown a favorable interaction between ICL670A, a new orally active iron chelator designed by Novartis, and DFO (43), which is most probably explained by an exchange of chelated iron between these two compounds.

Although the present study offers new insights into the mechanism of combined chelation therapy, the implications of these findings to the practical management of transfusional iron overload have yet to be shown. The most serious obstacle to the introduction of new chelators for clinical use is drug safety. In this regard, the PIH analogs still require formal detailed animal toxicity studies pending their application for treating patients. Other obstacles to the introduction of combined treatment with PIH analogs and DFO include the technical difficulty of quantitating iron excretion with PIH analogs, as all such excretion is limited to the bile. In view of recent experience with ICL670, another oral
chelator with exclusive biliary effect, SQUID measurement of hepatic iron concentrations may be a preferred method for documenting a negative iron balance induced by long-term chelation therapy (44).

Current efforts aimed at the development of new orally effective iron chelators such as improved hydroxypyridone derivatives (45), PIH analogs (35) and bishydroxyphenyl triazoles (43,44) may allow better protection from the harmful effects of hemosiderosis and offer new directions in the management of transfusional iron overload. As shown in the present studies, the efficacy of such new drugs may be enhanced by innovative strategies of drug administration and delivery.
REFERENCES


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Effects of combined chelation treatment with pyridoxal isonicotinoyl hydrazone (PIH) analogs and deferoxamine in hypertransfused rats and in iron-loaded rat heart cells

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