Comparison of Clinically Used and Experimental Iron Chelators for Protection against Oxidative Stress-Induced Cellular Injury

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · JUNE 2010

Impact Factor: 3.53 · DOI: 10.1021/tx100125t · Source: PubMed

12 AUTHORS, INCLUDING:

Pavlina Haskova
Charles University in Prague
26 PUBLICATIONS **298** CITATIONS
SEE PROFILE

Anna Jirkovska
Charles University in Prague
18 PUBLICATIONS **229** CITATIONS
SEE PROFILE

Eduard Jirkovský
Charles University in Prague
15 PUBLICATIONS **160** CITATIONS
SEE PROFILE

Katerina Vávrová
Charles University in Prague
73 PUBLICATIONS **899** CITATIONS
SEE PROFILE

Comparison of Clinically Used and Experimental Iron Chelators for Protection against Oxidative Stress-Induced Cellular Injury

Petra Bendova,[†] Eliska Mackova,[†] Pavlina Haskova,[†] Anna Vavrova,[†] Eduard Jirkovsky,[†,‡] Martin Sterba,[‡] Olga Popelova,[‡] Danuta S. Kalinowski,[§] Petra Kovarikova,[†] Katerina Vavrova,[†] Des R. Richardson,*[,§] and Tomas Simunek*[,†]

Faculty of Pharmacy in Hradec Kralove and Faculty of Medicine in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, 500 05 Hradec Kralove, Czech Republic, and Iron Metabolism and Chelation Program, Bosch Institute and Department of Pathology, University of Sydney, Sydney 2006, Australia

Received April 3, 2010

Iron imbalance plays an important role in oxidative stress associated with numerous pathological conditions. Therefore, iron chelation may be an effective therapeutic approach, but progress in this area is hindered by the lack of effective ligands. Also, the potential favorable effects of chelators against oxidative injury have to be balanced against their own toxicity due to iron depletion and the ability to generate redox-active iron complexes. In this study, we compared selected iron chelators (both drugs used in clinical practice as well as experimental agents) for their efficacy to protect cells against model oxidative injury induced by *tert*-butyl hydroperoxide (*t*-BHP). In addition, intracellular chelation efficiency, redox activity, and the cytotoxicity of the chelators and their iron complexes were assayed. Ethylenediaminetetraacetic acid failed to protect cells against *t*-BHP cytotoxicity, apparently due to the redox activity of the formed iron complex. Hydrophilic desferrioxamine exerted some protection but only at very high clinically unachievable concentrations. The smaller and more lipophilic chelators, deferiprone, deferasirox, and pyridoxal isonicotinoyl hydrazone, were markedly more effective at preventing oxidative injury of cells. The most effective chelator in terms of access to the intracellular labile iron pool was di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone. However, overall, the most favorable properties in terms of protective efficiency against *t*-BHP and the chelator's own inherent cytotoxicity were observed with salicylaldehyde isonicotinoyl hydrazone. This probably relates to the optimal lipophilicity of this latter agent and its ability to generate iron complexes that do not induce marked redox activity.

Introduction

Oxidative stress is usually defined as an imbalance between the production of reactive oxygen species (ROS)[1] and the ability of the biological system to detoxify them or readily repair the resulting oxidative damage. Both the brain and the cardiovascular system are particularly susceptible to such redox stress. The important pathophysiological role of oxidative damage is now well-established in a variety of disease states. These include ischemia/reperfusion injury, cardiac arrhythmias, congestive heart failure, myocarditis, atherosclerosis, hypertension, and the toxicity of redox-cycling drugs such as anthracyclines (*1, 2*).

Iron (Fe) is an essential element for virtually all living systems. However, unless appropriately shielded, this metal plays a crucial role in the formation of extremely reactive and toxic hydroxyl radicals (*3*). Given the key catalytic role of free cellular Fe in the production of deleterious hydroxyl radicals, organisms are equipped with specific proteins designed for Fe acquisition, transport, and storage, as well as with sophisticated mechanisms that maintain the intracellular "labile iron pool" (LIP) at an appropriate level. Despite homeostatic mechanisms, under certain pathological states, cells face the threat of Fe toxicity. It has been demonstrated that various reactive oxygen or nitrogen species may severely deregulate cellular Fe homeostasis (*4*). For example, high cellular levels of superoxide and peroxide species, as well as low pH (e.g., during ischemia/reperfusion injury), are able to release Fe from proteins, increase the cytosolic LIP, and thus promote the production of deleterious hydroxyl radicals (*5*). Furthermore, redox-active Fe may also promote the formation of toxic ROS within mitochondria and lysosomes (*6*). Importantly, because free Fe can nonspecifically bind to DNA and proteins, the resulting damage cannot be readily prevented by radical scavengers, because the oxidizing species react very close to the nonspecific Fe-binding sites via the Fenton reaction (*7, 8*).

A considerable amount of work has been invested in Fe chelation therapy to manage systemic Fe overload such as that induced by repeated blood infusions in patients with β-thalassemia (*3, 9*). However, the potential of Fe chelators in pathological conditions associated with oxidative stress unrelated to excessive Fe loading has been much less investigated (*7*). Hence, there is a need for the evaluation of clinically used Fe chelating agents, as well as for assessment of potential new drug candidates. The aim of the present study was to examine and

* To whom correspondence should be addressed. (D.R.R.) Tel: +61-2-9036-6548. Fax: +61-2-9036-6549. E-mail: d.richardson@sydney.edu.au. (T.S.) Tel: +420-495-067-422. Fax: +420-495-512-665. E-mail: Tomas.Simunek@faf.cuni.cz.

[†] Faculty of Pharmacy in Hradec Kralove, Charles University in Prague.

[‡] Faculty of Medicine in Hradec Kralove, Charles University in Prague.

[§] University of Sydney.

[1] Abbreviations: DFO, desferrioxamine; DMEM, Dulbecco's modified Eagle's medium; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; EDTA, ethylenediaminetetraacetic acid; FAC, ferric ammonium citrate; FBS, fetal bovine serum; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein-diacetate; ICL670A, deferasirox; L1, deferiprone; LIP, labile iron pool; NR, neutral red; PIH, pyridoxal isonicotinoyl hydrazone; ROS, reactive oxygen species; SIH, salicylaldehyde isonicotinoyl hydrazone; *t*-BHP, *tert*-butyl hydroperoxide.

10.1021/tx100125t
Published on Web 06/03/2010

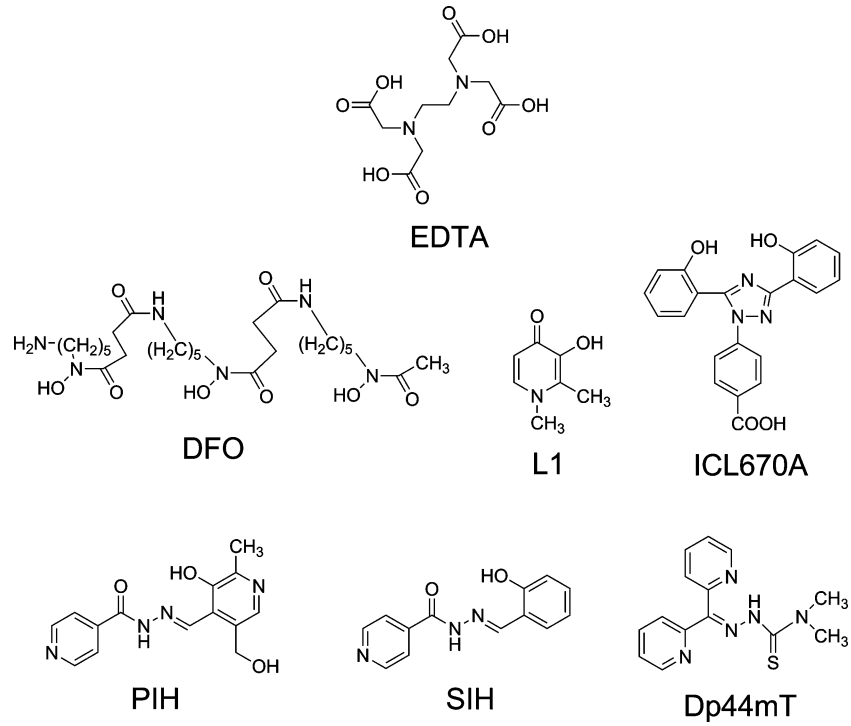

Figure 1. Chemical structures of the studied iron chelators: EDTA, DFO, L1, deferasirox (ICL670A), PIH, SIH, and Dp44mT.

compare seven Fe-chelating agents (Figure 1) for their ability to protect H9c2 cardiac cells against oxidative injury induced by *tert*-butyl hydroperoxide (*t*-BHP). Apart from the unspecific metal chelator, ethylenediaminetetraacetic acid (EDTA), three clinically used Fe chelators, namely, desferrioxamine (DFO), desferasirox (ICL670A), and deferiprone (L1), were compared with three experimental chelators, that is, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT), pyridoxal isonicotinoyl hydrazone (PIH), and salicylaldehyde isonicotinoyl hydrazone (SIH).

Experimental Procedures

Chemicals. PIH, SIH, and Dp44mT were synthesized and characterized as previously described (*10*−*12*). L1 was provided by Apotex Inc. (Weston, Ontario, Canada), and DFO and ICL670A were purchased from Novartis (Basel, Switzerland). Constituents for various buffers as well as other chemicals (e.g., various Fe salts) were from Sigma-Aldrich (Schnelldorf, Germany), Fluka (Seelze, Germany), Merck (Darmstadt, Germany), or Penta (Prague, Czech Republic) and were of the highest available pharmaceutical or analytical grade.

Cell Culture. The H9c2 cell line, derived from embryonic rat heart tissue using selective serial passages (*13*), was purchased from the American Type Culture Collection (Manassas, VA; catalog #CRL-1446). H9c2 cells provide a suitable model of cardiac myocytes as the response to oxidant stress, and its subsequent rescue by iron chelators is similar to primary neonatal cardiomyocytes (*14, 15*). The H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Lonza), 1% penicillin/streptomycin solution (Lonza), and 10 mM HEPES buffer (Sigma, Germany) in 75 cm^2 tissue culture flasks [Techno Plastic Products (TPP), Switzerland] at 37 °C in a humidified atmosphere of 5% CO_2. Subconfluent cells were subcultured every 3−4 days. For cytotoxicity experiments with neutral red (NR) and experiments with 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) and Calcein Green AM, cells were seeded in 96-well plates (TPP) at a density of 10000 cells/well. For morphology assessments, cells were seeded at a density of 75000 cells/well in 12-well plates (TPP). The cell medium was changed to serum- and pyruvate-free DMEM (Sigma) 24 h prior to conducting experiments.

Cytotoxicity Experiments. The cellular viability was determined using the cytotoxicity assay based on the ability of viable cells to incorporate NR (Sigma). NR is a weak cationic dye that readily penetrates cell membranes by nonionic diffusion, accumulating intracellularly in lysosomes (*16*). After the experimental incubations, half the volume of medium from each well was removed, and the same volume of medium with NR was added (yielding a final NR concentration of 40 μg/mL). After incubation with NR for 3 h/37 °C, the supernatant was discarded, and cells were fixed in 0.5% formaldehyde supplemented with 1% $CaCl_2$ for 15 min/20 °C, washed twice with PBS, and coagulated with 1% acetic acid in 50% ethanol, allowing accumulated NR to be released into the extracellular fluid. The optical density of soluble NR was measured at 540 nm using a Tecan Infinite 200 M plate reader (Tecan, Austria). The viability of experimental groups was expressed as a percentage of the untreated controls (100%).

Oxidative injury was induced in H9c2 cells by a 24 h exposure to 200 μM *t*-BHP. Because of the rapid degradation of *t*-BHP in cells, the actual exposure time of cells to *t*-BHP would clearly be for a shorter period of time. The protective effects of various iron chelators were assayed at different concentrations. The chelators were added to the cells 10 min prior to the addition of *t*-BHP. To dissolve lipophilic chelators, the compounds were dissolved in DMSO and then diluted with cell-culture medium so that the final concentration was 0.1% DMSO (v/v). At this concentration, DMSO had no effect on cellular viability. The inherent cytotoxicity of chelators was evaluated following 24 and 72 h incubations. Lysosomal membrane permeabilization is a slow peroxidative process that continues long after exposure to oxidative stress (*17*), and thus, cellular viability was assessed with NR 24 h after exposure to *t*-BHP. In addition to the assessment of free chelators, the cytotoxic effects of Fe complexes formed at an iron-binding equivalent ratio of 1 (IBE 1 = 1:1 chelator−Fe ratio for the hexadentate chelators, EDTA and DFO; 2:1 chelator−Fe ratio for the tridentate chelators, ICL670A, PIH, SIH, and Dp44mT; and 3:1 chelator−Fe ratio for the bidentate chelator, L1) were studied.

Changes in cellular morphology were evaluated using a Nikon Eclipse TS100 inverted microscope equipped with digital cooled

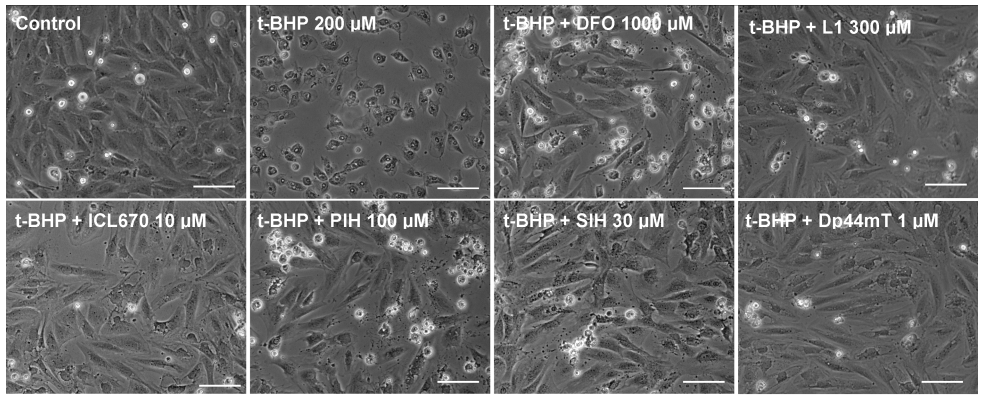

Figure 2. Effects of a 24 h incubation of H9c2 cells with 200 μM *t*-BHP and its combinations with optimal cytoprotective concentrations of iron chelators: DFO (1000 μM), L1 (300 μM), ICL670A (10 μM), PIH (100 μM), SIH (30 μM), or Dp44mT (1 μM) on the cellular morphology of H9c2 cells. Scale bars, 100 μm. The results are typical of three independent experiments.

camera (1300Q, VDS Vosskühler, Germany) and NIS-Elements AR 2.20 software (Laboratory Imaging, Czech Republic). H9c2 cells were seeded in 12-well plates (75000 cells/well) and incubated under identical conditions as described above for NR uptake assays.

Calcein-AM Assay for Assessment of Rate of Cell Membrane Permeation and Access to the Labile Fe Pool. The experiments were performed according to Glickstein et al. (*18*) with slight modifications. H9c2 cells were seeded in 96-well plates (10000 cells per well). Cells were loaded with Fe using the Fe donor, 100 μM ferric ammonium citrate (FAC) (*19*), in serum-free medium 24 h prior to the experiment, and the cells were then washed. To prevent potential interference (especially with regard to various trace elements), the medium was replaced with the ADS buffer (prepared using Millipore water supplemented with 116 mM NaCl, 5.3 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.13 mM NaH_2PO_4, 5 mM glucose, and 20 mM HEPES, pH 7.4). Cells were then loaded with a 1 μM concentration of the cell-permeable calcein green acetoxymethyl ester (Molecular Probes/KRD, Prague, Czech Republic) for 30 min/37 °C and washed. Cellular esterases cleave the acetoxymethyl groups to form the cell membrane-impermeable compound, calcein green, whose fluorescence is quenched by FAC. Intracellular fluorescence ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 530$ nm) was then followed as a function of time (1 min before and 10 min after the addition of chelator) at 37 °C using the plate reader above.

Ascorbate Oxidation Assay for Analysis of Redox Activity of Fe Complexes in Solution. The ascorbate oxidation assay was used to assess the redox activity of the chelator−Fe complexes in buffered solution using an established protocol (*12, 20*). Apart from the redox-cycling ferric/ferrous iron complexes, ascorbate may get oxidized by formed hydroxyl radicals, hydrogen peroxide, or high-valent iron complexes. In brief, ascorbic acid (100 μM) was prepared immediately prior to the experiment and incubated either under control conditions or in the presence of Fe^{III} (10 μM; added as $FeCl_3$), a 50-fold molar excess of citrate (500 μM) and various chelators added at an IBE of 3. An IBE of 3 describes an excess of chelator to Fe and is equal to either three hexadentate (EDTA and DFO), six tridentate (PIH, SIH, and Dp44mT), or nine bidentate (L1) ligands in the presence of one Fe atom. The decrease in absorbance at 265 nm, which is proportional to ascorbate oxidation, was then measured every 10 min for 1 h/20 °C using the plate reader described previously.

The ascorbate assay has been used extensively to assess the redox cycling ability of Fe complexes (*12, 21*−*23*). Moreover, the results from the ascorbate oxidation study have been confirmed by previous benzoate hydroxylation and H_2DCF oxidation assays (*11, 12*).

Intracellular ROS Generation Measurement Using H_2DCF-DA. The intracellular generation of ROS was assessed using the probe, H_2DCF-DA (Molecular Probes/KRD). Cells were washed with ADS buffer (116 mM NaCl, 5.3 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.13 mM NaH_2PO_4, 5 mM glucose, and 20 mM HEPES, pH 7.4) and loaded with 10 μM H_2DCF-DA solution for 30 min/37 °C. Cellular esterases cleave the acetyl groups to produce the cell membrane-impermeable compound, H_2DCF, which becomes highly fluorescent when oxidized by ROS (particularly hydroxyl radicals and other highly oxidizing species) to DCF (*24*). Following the addition of tested substance(s), changes in intracellular fluorescence intensity ($\lambda_{ex} = 488$ nm; $\lambda_{em} = 530$ nm) were followed every 15 min for 1 h/37 °C using the plate reader above.

Data Analysis. The statistical software, SigmaStat for Windows 3.5 (SPSS, United States), was used in this study. All data are presented as means ± SEMs of a given number of experiments. Statistical significance was determined using Student's *t* test or ANOVA with a Bonferroni posthoc test (comparisons of multiple groups against a corresponding control). Data not displaying a normal distribution were evaluated using nonparametric Mann−Whitney rank sum test or Kruskal−Wallis ANOVA on ranks with Dunn's test. Results were considered to be statistically significant when $p < 0.05$. The chelator concentration inducing 50% viability loss (TC_{50}) and the chelator concentration leading to 50% protection from *t*-BHP (EC_{50}) were calculated using CalcuSyn 2.0 software (Biosoft, Cambridge, United Kingdom).

Results

Cellular Protection by Chelators from *t*-BHP-Induced Toxicity. Incubation of H9c2 cells for 24 h with *t*-BHP at 200 μM resulted in pronounced toxicity. Peripheral membrane blebbing was followed by loss of the spindlelike cell shape and rounding up of cells (Figure 2). Furthermore, severe nuclear condensation was evident. Complete loss of cellular viability was clear from the NR uptake test (Figure 3). Cotreatment with EDTA (3−1000 μM) at all concentrations had no protective effect on cellular toxicity induced by *t*-BHP (Figure 3A). In contrast, the other Fe-chelating agents were able to preserve cellular morphology (Figure 2) as well as to afford a statistically significant increase in cellular viability (Figure 3B−G), although at different concentrations.

The least effective chelator was DFO, with 300 μM being the lowest concentration able to result in a reduction in toxicity (Figure 3B). Additionally, the DFO concentration that yielded 50% protection (EC_{50}) from *t*-BHP exceeded 1000 μM. The more lipophilic chelators, namely, L1, ICL670A, PIH, and SIH, showed maximum protective activity at concentrations ranging from 10 to 300 μM. The order of relative efficacy of the chelators from highest to lowest was ICL670A, SIH, PIH, and L1 (Figure 3C−F). Maximal protection was achieved with ICL670A at a concentration of 10−30 μM, with 100 μM leading to a slight decrease in efficacy, probably due to the cytotoxic effects of the agent (Figure 3D). Dp44mT was the most potent agent at protecting cells against *t*-BHP, with the lowest effective concentration being 0.3 μM (Figure 3G) and the EC_{50} value

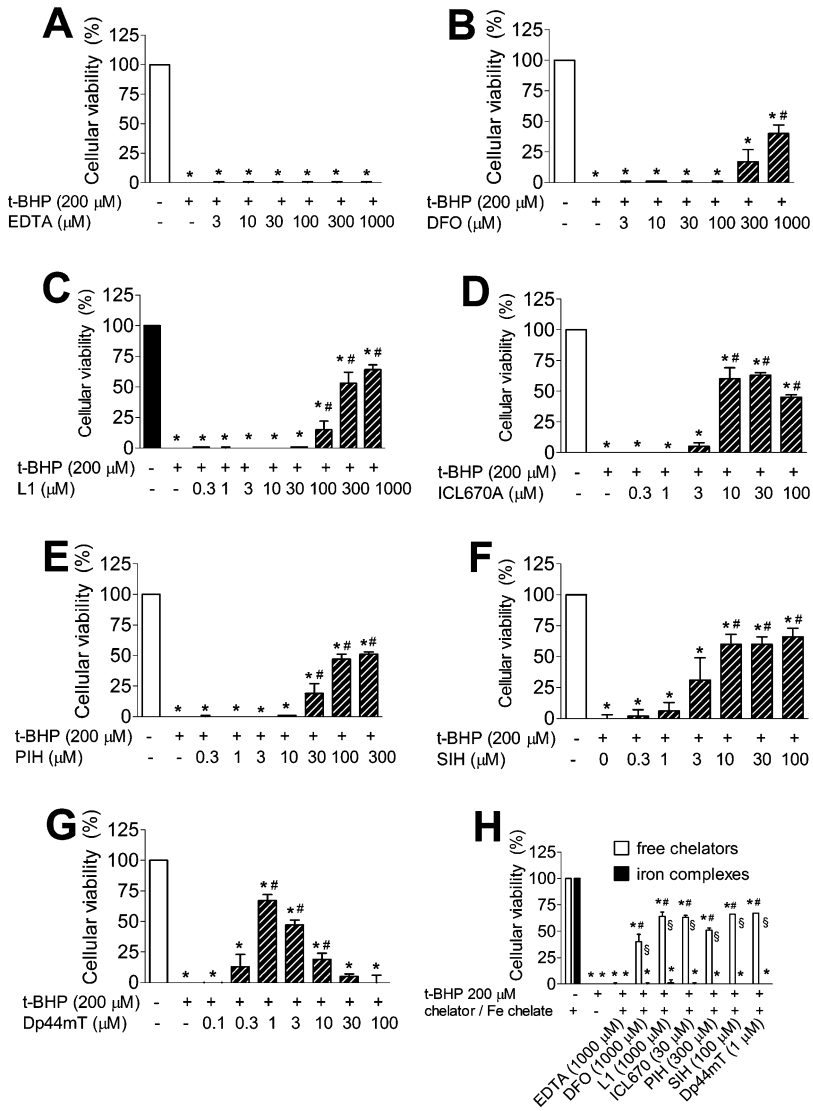

Figure 3. (A−G) Effects of various iron chelators on cellular toxicity induced in H9c2 cells by a 24 h incubation with 200 μM *t*-BHP. (H) Comparison of the protective effects of iron chelators at selected optimal concentrations (white columns) and their iron complexes formed at an appropriate iron binding equivalent (IBE) ratio of 1 (black columns; IBE 1 = 1:1 chelator−Fe ratio for the hexadentate chelators, EDTA and DFO; 2:1 chelator−Fe ratio for the tridentate chelators, ICL670A, PIH, SIH, and Dp44mT; and 3:1 chelator−Fe ratio for the bidentate chelator, L1). Studies were performed using the NR uptake test (see the Experimental Procedures for details), and the results are means ± SEM ($n \geq 4$ experiments). Statistical significance: *, as compared to the control (untreated) group; #, as compared to the 200 μM *t*-BHP group (ANOVA; $p \leq 0.05$); and §, comparison of effects of free chelator and corresponding iron complex (*t* test; $p \leq 0.05$).

equaling 0.69 μM (Table 1). It is important to note that a bell-shaped concentration−response curve was observed with Dp44mT. Indeed, after reaching an optimal cytoprotective concentration of 1 μM, a decrease in cellular viability occurred following further dose escalation, reflecting the marked cytotoxicity of this chelator (Figure 3G). This demonstrated that the narrow therapeutic window that Dp44mT is able to afford cytoprotective activity. Hence, the intracellular ratio of chelator to Fe is a crucial parameter in terms of the outcome with Dp44mT. The cytoprotective action of all of the effective chelators was completely lost after the formation of the Fe complexes (chelator + FAC, mixed at concentrations of appropriate IBE ratio), confirming Fe chelation as a mechanism of protective action (Figure 3H).

Cellular Toxicities of Chelators and Their Fe Complexes. All chelators were compared for their protective effects against *t*-BHP toxicity at a concentration of 100 μM, being the highest level at which the more lipophilic chelators (L1, ICL670A, PIH, SIH, and Dp44mT) could be dissolved. At this concentration, the relative order of efficacy of the chelators against *t*-BHP

Table 1. Protective Efficiencies against Cellular Oxidative Injury Induced by 24 h of Exposure to 200 μM *t*-BHP and Long-Term (72 h) Toxicities of Iron Chelators in H9c2 Cells[a]

chelator	EC_{50} (μM)	EC_{50}/IBE (μM)	TC_{50} (μM)	TC_{50}/IBE (μM)
EDTA	no protection	no protection	182	182
DFO	1350	1350	99	99
L1	375	125	126	42
ICL670A	14.6	7.3	2.9	1.5
PIH	134	67	44	22
SIH	18.2	9.1	29.7	14.9
Dp44mT	0.69	0.35	0.15	0.08

[a] All studies were performed using the NR assay (see the Experimental Procedures for details; mean values are $n \geq 4$ experiments). Abbreviations: EC_{50}, concentration of chelator reducing the toxicity induced by 24 h of incubation with *t*-BHP to 50% of the control (untreated) cells; TC_{50}, chelator concentration inducing 50% viability reduction as compared to control (untreated) cells following a 72 h incubation; IBE, iron-binding equivalent of 1 (IBE 1 = 1:1 chelator−Fe ratio for the hexadentate chelators, EDTA and DFO; 2:1 chelator−Fe ratio for the tridentate chelators, ICL670A, PIH, SIH, and Dp44mT; and 3:1 chelator−Fe ratio for the bidentate chelator, L1).

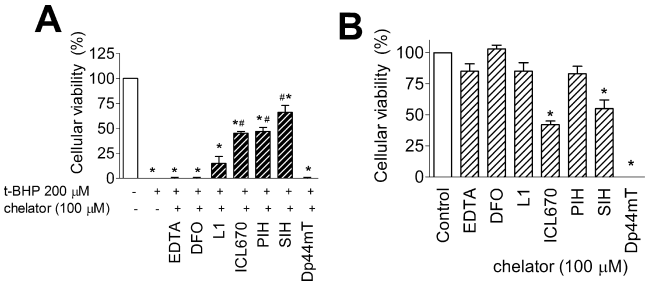

Figure 4. (A) Protective effects of various iron chelators at 100 μM against cellular injury induced in H9c2 cells by a 24 h incubation with 200 μM *t*-BHP. (B) Effects of a 24 h incubation with these chelators at 100 μM on cellular viability. Studies were performed using the NR uptake test (see the Experimental Procedures for details), and the results are means ± SEMs ($n \geq 4$ experiments). Statistical significance: *, as compared to the control (untreated) group; and #, as compared to the 200 μM *t*-BHP group (ANOVA; $p \leq 0.05$).

cytotoxicity was SIH > PIH = ICL670A > L1, while DFO, Dp44mT, and EDTA had no protective effect (Figure 4A). To assess the toxic effects of the chelators on their own, cells were incubated with these agents for 24 h/37 °C in the absence of *t*-BHP (Figure 4B), that is, the same time period as implemented in the cytoprotection experiments (Figures 2 and 3). As seen in Figure 4B, DFO at 100 μM showed no sign of toxicity. EDTA, L1, and PIH decreased the number of viable cells only slightly and insignificantly, while ICL670A and SIH led to a significant reduction in viability to approximately 50% of the control value. The most cytotoxic chelator was Dp44mT, which at the 100 μM concentration induced complete viability loss. The pronounced cytotoxic effects of Dp44mT agreed with previous studies, demonstrating its marked antiproliferative activity against a range of tumor cell types (*11, 25, 26*).

Given the marked differences in cytotoxicity and limited solubility of the lipophilic agents, to obtain TC_{50} values, a longer treatment period was necessary, and the next set of experiments was performed using a 72 h incubation period (Figure 5). All chelators reduced cellular viability in a concentration-dependent manner, and the TC_{50} values are summarized in Table 1. EDTA, L1, and DFO were the least toxic agents, and these were followed in order of increasing cytotoxicity by PIH, SIH, and ICL670A. Dp44mT induced a marked and significant reduction in cellular viability at the lowest concentration of 0.1 μM (Figure 5G). Although Dp44mT is a known topoisomerase II inhibitor (*27*), it is unlikely that this factor played a role in the observed cytotoxicity as the cells were incubated in serum-free medium and did not display significant proliferation during the experiment. Indeed, it has already been well-characterized in a variety of studies that the antiproliferative effects of Dp44mT are due to iron binding and the generation of ROS (*11, 12*).

In addition to the parent chelators, toxicities of their Fe complexes were also determined. The Fe complexes were prepared by mixing free chelators with appropriate concentrations of FAC to form complexes according to the IBE ratio of each chelator (i.e., at an IBE of 1 = 1:1 chelator−Fe ratio for the hexadentate chelators, EDTA and DFO; 2:1 chelator−Fe ratio for the tridentate chelators, ICL670A, PIH, SIH, and Dp44mT; and 3:1 chelator−Fe ratio for bidentate L1). As seen in Figure 5A, EDTA was the only agent where the Fe complex was more toxic than the free ligand at concentrations greater than 30 μM. On the contrary, the cytotoxicities of all other Fe complexes, except Dp44mT, were lower in comparison to their parent free ligands. The most pronounced difference was observed with DFO, where even the Fe complex (ferrioxamine) at 1000 μM did not show any signs of toxicity, whereas free

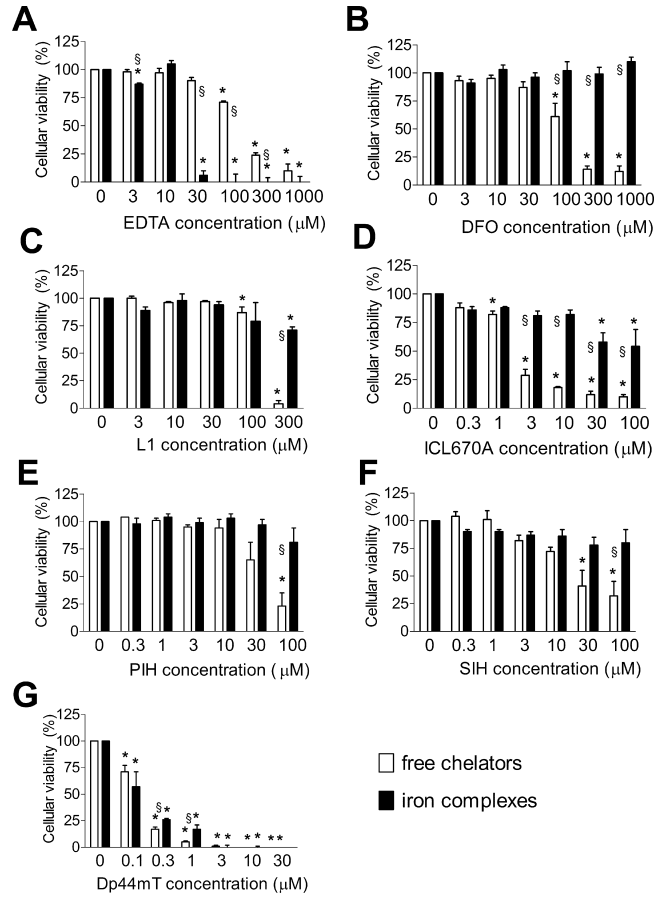

Figure 5. Effects of a 72 h incubation with various iron chelators or the corresponding iron complexes on cellular viability of H9c2 cells. Left (white) columns, free chelators; right (black) columns, iron complexes formed at the iron-binding equivalent (IBE) ratio of 1. Studies were performed using the NR uptake test (see the Experimental Procedures for details), and the results are means ± SEMs ($n \geq 4$ experiments). Statistical significance: *, as compared to the control (untreated) group (ANOVA; $p \leq 0.05$); and §, comparison of effects of the chelator and Fe complex at corresponding concentrations (Student's *t* test; $p \leq 0.05$).

DFO was significantly toxic above 100 μM (Figure 5B). The other Fe complexes were significantly less toxic than the free ligands, although for all of them some concentration-dependent cellular viability reductions occurred. The concentration−response profile of the Dp44mT−Fe (2:1) complex resembled that of the free chelator (Figure 5G), suggesting that the Fe complex was cytotoxic, as found previously (*12*). These results indicate that the mechanism of action of Dp44mT not only involves the chelation and mobilization of intracellular Fe but also the generation of redox-active Fe−Dp44mT complexes that catalyze the formation of cytotoxic ROS (*12*).

A number of previously published studies have shown the redox cycling ability of the Fe−Dp44mT complexes through a diverse range of techniques, including electrochemical potentials of the Fe−Dp44mT complexes (*12*), benzoate hydroxylation (*12*), ascorbate oxidation (*12*), and H_2DCF-DA assay (*11*). In fact, two of these methods, namely, the benzoate hydroxylation and H_2DCF oxidation assays, directly measure the production of ROS generated by the redox cycling of the Fe−Dp44mT complex (*11, 12*). Collectively, these results demonstrate the redox-cycling capability of the Fe−Dp44mT complex. Also, it should be noted that we have performed electron paramagnetic resonance studies to assess the redox activity of Fe−Dp44mT, and in contrast to results of others (*28*), we have conclusively

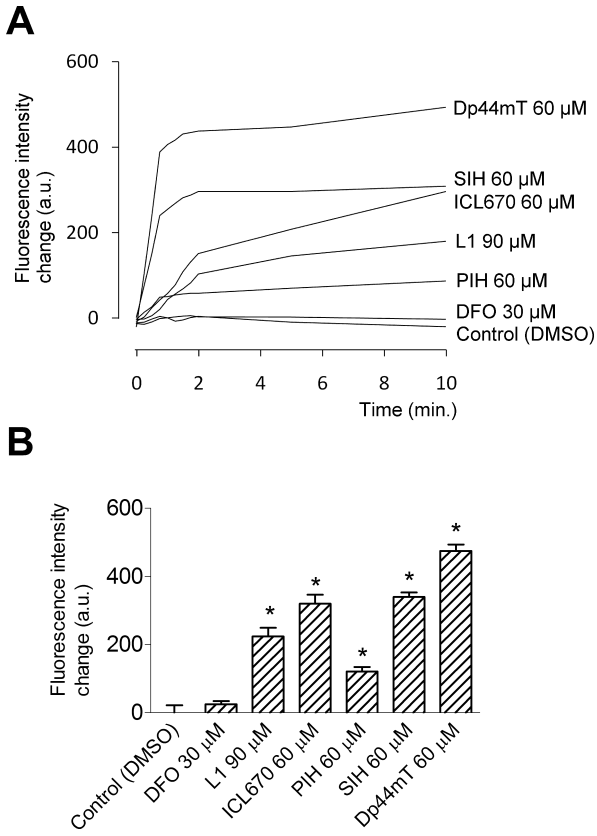

Figure 6. Intracellular iron chelation efficiencies of various iron chelators. Increase in free calcein green fluorescence upon displacement of intracellular labile iron from its complex with calcein in H9c2 cells following the extracellular addition of various Fe chelators at an iron-binding equivalent (IBE) concentration of 1. (A) Typical experiment showing the time course of fluorescence change following the addition of chelators. (B) Mean changes in fluorescence intensity following a 10 min incubation with the chelators. Results are presented as the mean ± SEM ($n = 4$ experiments). *, Statistically significant difference as compared to the control group (ANOVA; $p \leq 0.05$).

shown that this complex is redox-active (Jansson, P., Hawkins, C., and Richardson, D. R. Manuscript in preparation).

Intracellular Fe Chelation Efficiencies of Various Chelators. Considering the protective activity of the chelators against *t*-BHP-induced oxidative stress and that the studies above indicated that Fe binding was key to this activity, the ability of the compounds to bind intracellular Fe was vital to assess. The well-described calcein-AM assay (*11, 29*) was employed to compare the ability of various chelators to access the poorly characterized intracellular LIP in H9c2 cells. Changes in intracellular fluorescence intensity were recorded during a 10 min incubation of cells with various chelators at concentrations that compensate for their different IBEs. The increase in fluorescence following the addition of chelator to culture medium is proportional to the removal of Fe from the intracellularly trapped complex with calcein, resulting in decreased levels of quenching.

As shown in Figure 6, Dp44mT was both the fastest as well as the most efficient intracellular Fe chelator. In fact, near complete dequenching was achieved in less than 60 s following its addition to culture medium. SIH showed a similar pattern of chelation efficacy, although at 10 min the fluorescence intensity was lower than Dp44mT. In contrast, ICL670A, L1, and PIH were less effective and slower at dequenching intracellular calcein, but all induced a significant increase in intracellular fluorescence intensity. A 10 min incubation with DFO resulted in only very slight and insignificant fluorescence

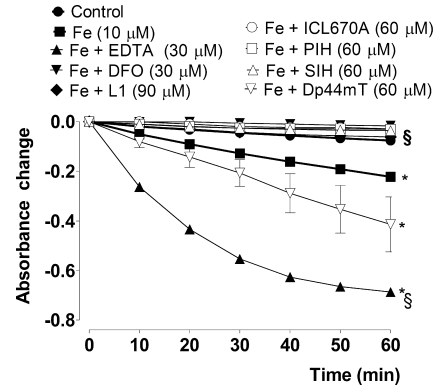

Figure 7. Effects of iron chelators on iron-induced oxidation of ascorbic acid in buffered solution (pH 7.4). Various chelators at an iron-binding equivalent (IBE) ratio of 3 were incubated in the presence of ferric citrate (10 μM) and ascorbate (100 μM). The UV absorbance at $\lambda = 265$ nm was recorded as a function of time. The absorbance decrease is proportional to ascorbate oxidation. Results are presented as the mean ± SEM ($n = 3$ experiments). Statistical significance (ANOVA; $p \leq 0.05$): *, as compared to the control group (spontaneous ascorbate oxidation without Fe); and §, as compared to the Fe citrate group.

increase (Figure 6), which is consistent with its poor membrane permeability (*30*).

Effects of Chelators on Fe-Induced Ascorbate Oxidation in Solution. Considering the results above showing the ability of the Fe complexes of EDTA and Dp44mT to induce cytotoxicity (Figure 5A,G), it is notable that Fe chelation by a ligand may result in the prevention, preservation, or even facilitation of the redox activity of Fe. These consequences are dependent on the chemical properties of the chelator; hence, the design of the compound can be manipulated to induce or prevent these effects (*31*). Spontaneous oxidation of ascorbic acid is markedly accelerated in the presence of redox-active free Fe ions, and this assay has been used extensively to examine the effects of chelators to inhibit, preserve, or stimulate this reaction (*12, 20*). Hence, this method was implemented in the current study to examine the redox activity of the Fe complexes.

As shown in Figure 7, 10 μM ferric citrate significantly increased the rate of oxidation of 100 μM ascorbate, evident from the decreased ascorbate absorbance at 265 nm. Effects of all Fe chelators were assayed at an IBE ratio of 3. EDTA markedly and significantly enhanced the rate of Fe-catalyzed ascorbate oxidation and was an appropriate positive control (*12, 20*). Although Dp44mT was less effective than EDTA, it also increased the oxidation rate, and at 60 min, there was a significant increase relative to the control (Figure 7). All of the other studied agents (DFO, L1, ICL670A, PIH, and SIH) significantly reduced ascorbate oxidation to a rate even lower than the control (Fe-free) ascorbate solution (Figure 7). In addition to the results with the ligands in excess to Fe (i.e., an IBE of 3), studies examining IBE ratios of 1 yielded similar results (data not shown). Collectively, these results indicated that EDTA and Dp44mT generate redox-active complexes that enhance the oxidation of ascorbate by Fe, while the other chelators inhibited this effect.

Intracellular ROS Production. To assess ROS generation inside H9c2 cells, measurement of H_2DCF-DA oxidation was used. This nonfluorescent reagent diffuses passively through the plasma membrane into the cell, where the acetate groups are cleaved by intracellular esterases (*11*). The compound can then be oxidized by ROS formed within the cell (particularly by hydroxyl radicals and other highly oxidizing species) to form fluorescent 2′,7′-dichlorofluorescein (DCF). The fluorescence

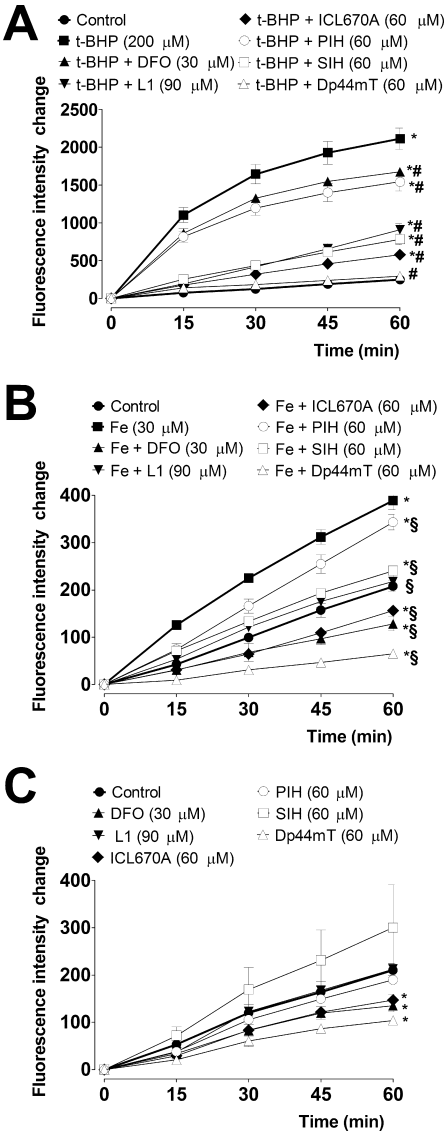

Figure 8. Intracellular ROS production in H9c2 cells loaded with H_2DCF-DA and exposed for 60 min to *t*-BHP, FAC, and/or various Fe chelators at their iron-binding equivalent (IBE) concentrations. Following the intracellular acetate group cleavage, an increase in fluorescence intensity ($\lambda_{ex} = 488$; $\lambda_{em} = 520$ nm) is proportional to probe oxidation by ROS to green fluorescent DCF. (A) Effects of chelators on oxidative burst induced by incubation with 200 μM *t*-BHP; (B) effects of iron chelators on probe oxidation induced by incubation with 30 μM FAC; and (C) effects of Fe chelators alone. Results are presented as the mean ± SEM ($n = 4$ experiments). Statistical significance (ANOVA; $p \leq 0.05$): *, as compared to the control group (spontaneous H_2DCF oxidation); #, as compared to the *t*-BHP group; and §, as compared to the FAC group.

intensity is proportional to the ROS concentration, leading to a quantitative measurement of intracellular ROS (*11*).

In the first set of experiments performed, ROS formation was induced by 200 μM *t*-BHP, and this resulted in a marked and statistically significant increase in intracellular H_2DCF oxidation (Figure 8A). Co-incubations of *t*-BHP with all chelators (compared at concentrations that compensate for their different IBEs) resulted in a statistically significant reduction in fluorescence intensity. DFO was the least effective agent, while Dp44mT was the most potent, yielding a fluorescence intensity change comparable to that of untreated control cells.

In the next series of studies, the cells were incubated with the Fe donor, FAC (30 μM) (*19*), either alone or in combinations with various chelators at their IBE ratio of 1. As demonstrated in Figure 8B, in cells incubated with FAC, after 60 min, the ROS production significantly increased as compared to control. Co-incubations with all chelators resulted in significant reduction in fluorescence intensity as compared to uncomplexed Fe. Interestingly, incubation of cells with the Fe complexes of ICL670A, DFO, and Dp44mT resulted in even lower levels of fluorescence than in control Fe-free cells (Figure 8B).

In Figure 8C, effects of chelators on spontaneous cellular ROS production were examined. Only SIH showed a trend to increase the fluorescescence intensity, but this did not reach statistical significance. All other agents suppressed spontaneous cellular ROS production, and this reduction was significant for ICL670A, DFO, and Dp44mT. The effect of Dp44mT was of interest as previous studies have shown that this compound increases intracellular ROS as judged using the DCF method (*11*). However, as discussed above, it is the relative ratio of ligand to cellular Fe that is probably important in determining its overall net effect on ROS concentration. For instance, Dp44mT may at low concentrations lead to the removal of redox-sensitive cellular Fe pools that can mediate H_2DCF-DA oxidation, and their depletion leads to decreased ROS. In contrast, at higher levels of chelator, the redox activity of the Fe complex could predominate, leading to increased H_2DCF-DA oxidation. Of note, a recent study revealed that DCF-dependent fluorescence largely reflected relocation of lysosomal Fe and/or mitochondrial cytochrome *c* to the cytosol (*32*). The marked reduction of DCF fluorescence seen in our study following incubation of cells with Dp44mT could therefore predominantly reflect its marked access to the LIP (*11*), rather than its antioxidant action.

Discussion

For many years, chelators have been successfully used in the clinics to promote Fe excretion in conditions associated with systemic Fe overload (*3*). Nevertheless, the concept of Fe chelation may also be useful to prevent Fe-mediated aggravation of oxidative stress, which can occur in the presence of normal Fe stores, or when Fe is redistributed to lead to Fe-loading in sensitive compartments, for example, mitochondria (*33*). In the present study, an in vitro model of oxidative injury was induced by a 24 h incubation of the H9c2 cardiac cells with the organic peroxide, *t*-BHP. Once inside cells, *t*-BHP gradually generates *tert*-butoxy radicals, which induce various toxic effects including peroxidation of lipids, depletion of intracellular reduced glutathione, modification of protein thiols, and disturbance of calcium homeostasis (*8, 34*).

Our experiments have revealed that except for the membrane-impermeable molecule, EDTA (*35*), all of the tested Fe-specific chelators could afford significant cytoprotection, which indicates that intracellular Fe is essential for mediating the cytotoxicity of *t*-BHP. However, strikingly variable concentrations of chelators were required for protection. In fact, their EC_{50} values differed by 3 orders of magnitude, from millimolar to submicromolar levels. Protection from *t*-BHP-mediated oxidative stress by Dp44mT, and to a far lesser extent, ICL670A, decreased with increasing dose.

The use of Fe chelators in states without Fe overload is associated with the risk of toxicity due to removing or withholding Fe from critical Fe-containing proteins. In this study, cytotoxicity of the tested chelators was determined following 24 and 72 h incubations. However, because of the limited solubility of the more lipophilic agents, only the latter time period allowed for the determination of TC_{50} values.

EDTA is often used as a universal metal chelating agent in the chemical industry or laboratory applications. In our experi-

ments, EDTA did not protect H9c2 cells against the *t*-BHP oxidative challenge. This may be due to several factors, including (i) the inability of EDTA to effectively permeate cell membranes to bind intracellular Fe pools (*35*); (ii) the lack of selectivity of EDTA for Fe; and/or (iii) the redox activity of the EDTA−Fe complex, which may result in cytotoxicity itself (*36*). In fact, EDTA was the only chelating agent that exhibited higher toxicity when complexed with Fe than when incubated with cells as a free ligand. Clearly, our results indicate that EDTA has no potential in the prevention of Fe-mediated oxidative injury.

DFO has been successfully used for the treatment of Fe-overloading diseases (*3, 9*) and, probably due to its commercial availability, has been most commonly used in experimental studies examining the potential of Fe chelation in pathological states with normal Fe stores. Although DFO has been shown to significantly reduce ischemia−reperfusion-associated injury in various tissues including the heart (*37*), in numerous other studies, it failed to afford significant protection (*38, 39*). A plausible explanation for its limited efficiency is its known poor ability to permeate cells due to its relatively high hydrophilicity (*30*). In our study, DFO showed some potential to prevent *t*-BHP toxicity. However, the effective DFO concentrations ($\geq 300\ \mu M$) largely exceed the highest DFO plasma levels seen in clinical settings (*40*).

L1 and ICL670A are two orally effective Fe-chelating agents in clinical practice for the treatment of Fe overload disease (*3*). In comparison to DFO, our results have shown better efficacies of both L1 and ICL670A in protecting cells against *t*-BHP, with a 10- or even 100-fold decrease in EC_{50} values (Table 1). Even though the TC_{50} values of L1 and ICL670A were also lower, the increase in protective potential more than compensates for this. Moreover, both of the latter agents were shown to significantly reduce cellular LIP and reduce cellular oxidative stress induced by either *t*-BHP or FAC. Only a limited number of other studies with L1 or ICL670A have been published examining their antioxidant potential in situations without Fe overload. L1 protected neuronal cells as well as fibroblasts against H_2O_2-induced cell death in vitro (*33, 41*), and it also protected rat hearts against the ischemia−reperfusion injury (*42*). L1 also exerted protection of isolated cardiomyocytes against the toxicity of the anthracycline, doxorubicin (*43*), but was ineffective in vivo (*44*). On the contrary, ICL670A has been reported not to protect cardiomyocytes against doxorubicin (*45*).

Aroylhydrazones, which include PIH and SIH, represent another class of Fe chelators primarily developed as orally active agents for the management of Fe overload (*46*). Previously, micromolar concentrations of PIH were shown to protect plasmid DNA from damage by Fe^{2+} and H_2O_2 (*47*). Furthermore, SIH has been shown to be highly effective in protecting guinea pig cardiomyocytes, H9c2 cells, as well as other cell types against H_2O_2-induced cell injury, apparently due to the preservation of mitochondrial function and/or lysosomal integrity (*14, 48−50*). In vivo, PIH prevented ischemia−reperfusion injury of retina in a neonatal piglet model where it was markedly more effective than DFO (*51*). In the current study, both PIH and SIH exerted significant protection against *t*-BHP. Interestingly, while PIH and SIH have been previously shown as comparably effective in inducing ^{59}Fe release from macrophages or reticulocytes (*52*), SIH displayed much higher efficiency in protecting cells against *t*-BHP.

While PIH was protective at concentrations approximately half that of L1, the protective action of SIH was similar to ICL670A. Nevertheless, SIH was less toxic than ICL670A both during the 24 h but especially during the 72 h incubation. Of note, SIH has a very favorable in vivo toxicological profile (*53*) and was effective at preventing cardiotoxicity induced by the anthracycline, daunorubicin, both in vitro and in vivo (*15, 54*). These data clearly indicate that aroylhydrazone chelators (in particular the salicylaldehyde-derived analogues) merit further investigation as potentially useful antioxidant agents with low toxicity. Although tens of different aroylhydrazone chelators have been synthesized, they have only been thoroughly screened for their ability to bind intracellular Fe and/or their antiproliferative activity (*46, 55*). Hence, structure−activity studies are needed to identify structural features necessary for optimal protective action against cellular oxidative injury. Of note, aroylhydrazone pro-chelators have recently been developed where the Fe-binding groups are chemically masked and the active chelators PIH and SIH are generated only at sites of ROS production (*56−58*).

Finally, Dp44mT is a thiosemicarbazone chelator that demonstrates potent antiproliferative activity against a variety of tumor cells both in vitro and in vivo (*25, 59*). In this study, Dp44mT was the most effective agent at protecting cells against *t*-BHP-induced cytotoxicity. Importantly, in contrast to the other chelators, the ability of Dp44mT to prevent *t*-BHP cytotoxicity was markedly dependent on concentration (Figure 3G). At a concentration of 1 μM, Dp44mT induced a pronounced rescue of cellular viability, while higher concentrations led to decreased viability. Hence, the initial protective effect at low Dp44mT concentrations was probably due to the high Fe chelation efficacy of the ligand that was shown in the current (Figure 6) and in previous investigations (*11, 60*). In contrast, when the ligand concentration was increased, the inherent cytotoxicity of the compound then becomes predominant. The mechanism of the toxicity has previously been shown to be due to the ability of the chelator to bind cellular Fe that is essential for proliferation and the redox cycling of the Dp44mT−Fe complex that then leads to decreased viability (*12*). On the other hand, hence, the ability of Dp44mT to protect cells against oxidative injury is concentration-dependent.

In conclusion, the present study suggests further investigation of Fe chelation in diseases where oxidative stress is involved. However, different chelators have shown strikingly distinct efficacies in protecting cells from oxidative injury. Many previous cellular or animal studies could have severely underestimated the protective potential of Fe chelation by using inappropriate chelators (such as DFO) and/or wrong chelator doses or concentrations. The lipophilicity of the compound and the rate of chelator access to the cellular LIP are the most important determinants of protective action, as found previously (*33*). However, there is apparently no clear relation between chelator efficacy in inducing excretion of excessive Fe in disease, such as β-thalassemia, and the management of oxidative stress in conditions without Fe overload. Although all small molecular and lipophilic ligands (L1, ICL670A, PIH, SIH, and Dp44mT) have shown much greater cytoprotective efficacy as compared to DFO, SIH was observed to have the best ratio of protective efficiency and inherent toxicity.

Acknowledgment. We thank Dr. John Connelly (ApoPharma Inc.) for providing L1. This study was supported by the Charles University Grant Nos. 124307/C/2007 and SVV/2010/261/003. D.R.R. thanks the National Health and Medical Research Council for a Senior Principal Research Fellowship and Project

Grant support. D.S.K. thanks the Cancer Institute NSW for an Early Career Development Fellowship.

References

(1) Griendling, K. K., and FitzGerald, G. A. (2003) Oxidative stress and cardiovascular injury: Part I: Basic mechanisms and in vivo monitoring of ROS. *Circulation 108*, 1912–1916.

(2) Simunek, T., Sterba, M., Popelova, O., Adamcova, M., Hrdina, R., and Gersl, V. (2009) Anthracycline-induced cardiotoxicity: Overview of studies examining the roles of oxidative stress and free cellular iron. *Pharmacol. Rep. 61*, 154–171.

(3) Kalinowski, D. S., and Richardson, D. R. (2005) The evolution of iron chelators for the treatment of iron overload disease and cancer. *Pharmacol. Rev. 57*, 547–583.

(4) Mladenka, P., Simunek, T., Hubl, M., and Hrdina, R. (2006) The role of reactive oxygen and nitrogen species in cellular iron metabolism. *Free Radical Res. 40*, 263–272.

(5) Reif, D. W. (1992) Ferritin as a source of iron for oxidative damage. *Free Radical Biol. Med. 12*, 417–427.

(6) Terman, A., and Brunk, U. T. (2006) Oxidative stress, accumulation of biological 'garbage', and aging. *Antioxid. Redox Signaling 8*, 197–204.

(7) Galey, J. B. (2001) Recent advances in the design of iron chelators against oxidative damage. *Mini Rev. Med. Chem. 1*, 233–242.

(8) Halliwell, B., and Gutteridge, J. M. C. (2007) *Free Radicals in Biology and Medicine*, 4th ed., Oxford University Press, Oxford, New York.

(9) Olivieri, N. F., and Brittenham, G. M. (1997) Iron-chelating therapy and the treatment of thalassemia. *Blood 89*, 739–761.

(10) Edward, J. T., Gauthier, M., Chubb, F. L., and Ponka, P. (1988) Synthesis of new acylhydrazones as iron-chelating compounds. *J. Chem. Eng. Data 33*, 538–540.

(11) Yuan, J., Lovejoy, D. B., and Richardson, D. R. (2004) Novel di-2-pyridyl-derived iron chelators with marked and selective antitumor activity: In vitro and in vivo assessment. *Blood 104*, 1450–1458.

(12) Richardson, D. R., Sharpe, P. C., Lovejoy, D. B., Senaratne, D., Kalinowski, D. S., Islam, M., and Bernhardt, P. V. (2006) Dipyridyl thiosemicarbazone chelators with potent and selective antitumor activity form iron complexes with redox activity. *J. Med. Chem. 49*, 6510–6521.

(13) Kimes, B. W., and Brandt, B. L. (1976) Properties of a clonal muscle cell line from rat heart. *Exp. Cell Res. 98*, 367–381.

(14) Simunek, T., Boer, C., Bouwman, R. A., Vlasblom, R., Versteilen, A. M., Sterba, M., Gersl, V., Hrdina, R., Ponka, P., de Lange, J. J., Paulus, W. J., and Musters, R. J. (2005) SIH—a novel lipophilic iron chelator—protects H9c2 cardiomyoblasts from oxidative stress-induced mitochondrial injury and cell death. *J. Mol. Cell Cardiol. 39*, 345–354.

(15) Simunek, T., Sterba, M., Popelova, O., Kaiserova, H., Adamcova, M., Hroch, M., Haskova, P., Ponka, P., and Gersl, V. (2008) Anthracycline toxicity to cardiomyocytes or cancer cells is differently affected by iron chelation with salicylaldehyde isonicotinoyl hydrazone. *Br. J. Pharmacol. 155*, 138–148.

(16) Fotakis, G., and Timbrell, J. A. (2006) In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol. Lett. 160*, 171–177.

(17) Antunes, F., Cadenas, E., and Brunk, U. T. (2001) Apoptosis induced by exposure to a low steady-state concentration of H2O2 is a consequence of lysosomal rupture. *Biochem. J. 356*, 549–555.

(18) Glickstein, H., El, R. B., Link, G., Breuer, W., Konijn, A. M., Hershko, C., Nick, H., and Cabantchik, Z. I. (2006) Action of chelators in iron-loaded cardiac cells: Accessibility to intracellular labile iron and functional consequences. *Blood 108*, 3195–3203.

(19) Richardson, D., and Baker, E. (1992) Two mechanisms of iron uptake from transferrin by melanoma cells. The effect of desferrioxamine and ferric ammonium citrate. *J. Biol. Chem. 267*, 13972–13979.

(20) Mladenka, P., Kalinowski, D. S., Haskova, P., Bobrovova, Z., Hrdina, R., Simunek, T., Nachtigal, P., Semecky, V., Vavrova, J., Holeckova, M., Palicka, V., Mazurova, Y., Jansson, P. J., and Richardson, D. R. (2009) The novel iron chelator, 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone, reduces catecholamine-mediated myocardial toxicity. *Chem. Res. Toxicol. 22*, 208–217.

(21) Kalinowski, D. S., Sharpe, P. C., Bernhardt, P. V., and Richardson, D. R. (2007) Design, synthesis, and characterization of new iron chelators with anti-proliferative activity: Structure-activity relationships of novel thiohydrazone analogues. *J. Med. Chem. 50*, 6212–6225.

(22) Kalinowski, D. S., Sharpe, P. C., Bernhardt, P. V., and Richardson, D. R. (2008) Structure-activity relationships of novel iron chelators for the treatment of iron overload disease: The methyl pyrazinylketone isonicotinoyl hydrazone series. *J. Med. Chem. 51*, 331–344.

(23) Richardson, D. R., Kalinowski, D. S., Richardson, V., Sharpe, P. C., Lovejoy, D. B., Islam, M., and Bernhardt, P. V. (2009) 2-Acetylpyridine thiosemicarbazones are potent iron chelators and antiproliferative agents: Redox activity, iron complexation and characterization of their antitumor activity. *J. Med. Chem. 52*, 1459–1470.

(24) Wang, H., and Joseph, J. A. (1999) Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biol. Med. 27*, 612–616.

(25) Whitnall, M., Howard, J., Ponka, P., and Richardson, D. R. (2006) A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics. *Proc. Natl. Acad. Sci. U.S.A. 103*, 14901–14906.

(26) Noulsri, E., Richardson, D. R., Lerdwana, S., Fucharoen, S., Yamagishi, T., Kalinowski, D. S., and Pattanapanyasat, K. (2009) Antitumor activity and mechanism of action of the iron chelator, Dp44mT, against leukemic cells. *Am. J. Hematol. 84*, 170–176.

(27) Rao, V. A., Klein, S. R., Agama, K. K., Toyoda, E., Adachi, N., Pommier, Y., and Shacter, E. B. (2009) The iron chelator Dp44mT causes DNA damage and selective inhibition of topoisomerase IIalpha in breast cancer cells. *Cancer Res. 69*, 948–957.

(28) Hasinoff, B. B., and Patel, D. (2009) The iron chelator Dp44mT does not protect myocytes against doxorubicin. *J. Inorg. Biochem. 103*, 1093−1101.

(29) Tenopoulou, M., Kurz, T., Doulias, P. T., Galaris, D., and Brunk, U. T. (2007) Does the calcein-AM method assay the total cellular 'labile iron pool' or only a fraction of it? *Biochem. J. 403*, 261–266.

(30) Richardson, D., Ponka, P., and Baker, E. (1994) The effect of the iron(III) chelator, desferrioxamine, on iron and transferrin uptake by the human malignant melanoma cell. *Cancer Res. 54*, 685–689.

(31) Kalinowski, D. S., and Richardson, D. R. (2007) Future of toxicology—Iron chelators and differing modes of action and toxicity: The changing face of iron chelation therapy. *Chem. Res. Toxicol. 20*, 715–720.

(32) Karlsson, M., Kurz, T., Brunk, U. T., Nilsson, S. E., and Frennesson, C. I. (2010) What does the commonly used DCF-test for oxidative stress really show? *Biochem. J. 428*, 183–190.

(33) Lim, C. K., Kalinowski, D. S., and Richardson, D. R. (2008) Protection against hydrogen peroxide-mediated cytotoxicity in Friedreich's ataxia fibroblasts using novel iron chelators of the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone class. *Mol. Pharmacol. 74*, 225–235.

(34) Sardao, V. A., Oliveira, P. J., Holy, J., Oliveira, C. R., and Wallace, K. B. (2007) Vital imaging of H9c2 myoblasts exposed to tert-butylhydroperoxide--characterization of morphological features of cell death. *BMC Cell Biol. 8*, 11.

(35) Richardson, D. R., and Baker, E. (1994) Two saturable mechanisms of iron uptake from transferrin in human melanoma cells: The effect of transferrin concentration, chelators, and metabolic probes on transferrin and iron uptake. *J. Cell Physiol. 161*, 160–168.

(36) Graf, E., Mahoney, J. R., Bryant, R. G., and Eaton, J. W. (1984) Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. *J. Biol. Chem. 259*, 3620–3624.

(37) Bolli, R., Patel, B. S., Zhu, W. X., O'Neill, P. G., Hartley, C. J., Charlat, M. L., and Roberts, R. (1987) The iron chelator desferrioxamine attenuates postischemic ventricular dysfunction. *Am. J. Physiol. 253*, H1372–1380.

(38) Reddy, B. R., Wynne, J., Kloner, R. A., and Przyklenk, K. (1991) Pretreatment with the iron chelator desferrioxamine fails to provide sustained protection against myocardial ischaemia-reperfusion injury. *Cardiovasc. Res. 25*, 711–718.

(39) Watanabe, B. I., Limm, W., Suehiro, A., Suehiro, G., Premaratne, S., and McNamara, J. J. (1993) Failure of deferoxamine to reduce myocardial infarct size in a primate model of ischemia-reperfusion injury. *J. Surg. Res. 55*, 537–542.

(40) Summers, M. R., Jacobs, A., Tudway, D., Perera, P., and Ricketts, C. (1979) Studies in desferrioxamine and ferrioxamine metabolism in normal and iron-loaded subjects. *Br. J. Hamaetol. 42*, 547–555.

(41) Molina-Holgado, F., Gaeta, A., Francis, P. T., Williams, R. J., and Hider, R. C. (2008) Neuroprotective actions of deferiprone in cultured cortical neurones and SHSY-5Y cells. *J. Neurochem. 105*, 2466–2476.

(42) van der Kraaij, A. M., van Eijk, H. G., and Koster, J. F. (1989) Prevention of postischemic cardiac injury by the orally active iron chelator 1,2-dimethyl-3-hydroxy-4-pyridone (L1) and the antioxidant (+)-cyanidanol-3. *Circulation 80*, 158–164.

(43) Barnabe, N., Zastre, J. A., Venkataram, S., and Hasinoff, B. B. (2002) Deferiprone protects against doxorubicin-induced myocyte cytotoxicity. *Free Radical Biol. Med. 33*, 266–275.

(44) Popelova, O., Sterba, M., Simunek, T., Mazurova, Y., Guncova, I., Hroch, M., Adamcova, M., and Gersl, V. (2008) Deferiprone does not protect against chronic anthracycline cardiotoxicity in vivo. *J. Pharmacol. Exp. Ther. 326*, 259–269.

(45) Hasinoff, B. B., Patel, D., and Wu, X. (2003) The oral iron chelator ICL670A (deferasirox) does not protect myocytes against doxorubicin. *Free Radical Biol. Med. 35*, 1469–1479.

(46) Buss, J. L., Hermes-Lima, M., and Ponka, P. (2002) Pyridoxal isonicotinoyl hydrazone and its analogues. *Adv. Exp. Med. Biol. 509*, 205–229.

(47) Hermes-Lima, M., Nagy, E., Ponka, P., and Schulman, H. M. (1998) The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) protects plasmid pUC-18 DNA against *OH-mediated strand breaks. *Free Radical Biol. Med. 25*, 875–880.

(48) Kurz, T., Gustafsson, B., and Brunk, U. T. (2006) Intralysosomal iron chelation protects against oxidative stress-induced cellular damage. *FEBS J. 273*, 3106–3117.

(49) Horackova, M., Ponka, P., and Byczko, Z. (2000) The antioxidant effects of a novel iron chelator salicylaldehyde isonicotinoyl hydrazone in the prevention of H(2)O(2) injury in adult cardiomyocytes. *Cardiovasc. Res. 47*, 529–536.

(50) Lukinova, N., Iacovelli, J., Dentchev, T., Wolkow, N., Hunter, A., Amado, D., Ying, G. S., Sparrow, J. R., and Dunaief, J. L. (2009) Iron chelation protects the retinal pigment epithelial cell line ARPE-19 against cell death triggered by diverse stimuli. *Invest. Ophthalmol. Vis. Sci. 50*, 1440–1447.

(51) Bhattacharya, M., Ponka, P., Hardy, P., Hanna, N., Varma, D. R., Lachapelle, P., and Chemtob, S. (1997) Prevention of postasphyxia electroretinal dysfunction with a pyridoxal hydrazone. *Free Radical Biol. Med. 22*, 11–16.

(52) Ponka, P., Richardson, D., Baker, E., Schulman, H. M., and Edward, J. T. (1988) Effect of pyridoxal isonicotinoyl hydrazone and other hydrazones on iron release from macrophages, reticulocytes and hepatocytes. *Biochim. Biophys. Acta 967*, 122–129.

(53) Klimtova, I., Simunek, T., Mazurova, Y., Kaplanova, J., Sterba, M., Hrdina, R., Gersl, V., Adamcova, M., and Ponka, P. (2003) A study of potential toxic effects after repeated 10-week administration of a new iron chelator—Salicylaldehyde isonicotinoyl hydrazone (SIH) to rabbits. *Acta Med. (Hradec Kralove) 46*, 163–170.

(54) Sterba, M., Popelova, O., Simunek, T., Mazurova, Y., Potacova, A., Adamcova, M., Guncova, I., Kaiserova, H., Palicka, V., Ponka, P., and Gersl, V. (2007) Iron chelation-afforded cardioprotection against chronic anthracycline cardiotoxicity: A study of salicylaldehyde isonicotinoyl hydrazone (SIH). *Toxicology 235*, 150–166.

(55) Richardson, D. R., Tran, E. H., and Ponka, P. (1995) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents. *Blood 86*, 4295–4306.

(56) Yiakouvaki, A., Savovic, J., Al-Qenaei, A., Dowden, J., and Pourzand, C. (2006) Caged-iron chelators a novel approach towards protecting skin cells against UVA-induced necrotic cell death. *J. Invest. Dermatol. 126*, 2287–2295.

(57) Charkoudian, L. K., Dentchev, T., Lukinova, N., Wolkow, N., Dunaief, J. L., and Franz, K. J. (2008) Iron prochelator BSIH protects retinal pigment epithelial cells against cell death induced by hydrogen peroxide. *J. Inorg. Biochem. 102*, 2130–2135.

(58) Charkoudian, L. K., Pham, D. M., and Franz, K. J. (2006) A prochelator triggered by hydrogen peroxide inhibits iron-promoted hydroxyl radical formation. *J. Am. Chem. Soc. 128*, 12424–12425.

(59) Yu, Y., Kalinowski, D. S., Kovacevic, Z., Siafakas, A. R., Jansson, P. J., Stefani, C., Lovejoy, D. B., Sharpe, P. C., Bernhardt, P. V., and Richardson, D. R. (2009) Thiosemicarbazones from the old to new: Iron chelators that are more than just ribonucleotide reductase inhibitors. *J. Med. Chem. 52*, 5271–5294.

(60) Kalinowski, D. S., Yu, Y., Sharpe, P. C., Islam, M., Liao, Y. T., Lovejoy, D. B., Kumar, N., Bernhardt, P. V., and Richardson, D. R. (2007) Design, synthesis, and characterization of novel iron chelators: Structure-activity relationships of the 2-benzoylpyridine thiosemicarbazone series and their 3-nitrobenzoyl analogues as potent antitumor agents. *J. Med. Chem. 50*, 3716–3729.

TX100125T