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# Protection Against Hydrogen Peroxide-Mediated Cytotoxicity in Friedreich's Ataxia Fibroblasts using Novel Iron Chelators of the PCIH Class.

**Lim, C. K., Kalinowski, D. S. and Richardson, D.R.**

*Iron Metabolism and Chelation Program, Department of Pathology, Blackburn Building (D06), and Bosch Institute, University of Sydney, Sydney, New South Wales, 2006 Australia*

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***Running Title:*** Iron chelation and hydrogen peroxide-mediated cytotoxicity

***Corresponding Author:*** Dr. D.R. Richardson, Iron Metabolism and Chelation Program, Department of Pathology, University of Sydney, Sydney, New South Wales, 2006, Australia. Email: d.richardson@med.usyd.edu.au; Ph: +61-2-9036-6548; FAX: +61-2-9036-6549.

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***Abbreviations:*** BPS, bathophenanthroline disulphonate; Cat, catalase; DFO, desferrioxamine; DP, dipyridyl; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; FA, Friedreich's ataxia; L1, deferiprone; PCAH, 2-pyridylcarboxaldehyde *p*-aminobenzoyl hydrazone; PCBH, 2-pyridylcarboxaldehyde benzoyl hydrazone; PCBBH, 2-pyridylcarboxaldehyde *m*-bromobenzoyl hydrazone; PCHH, 2-pyridylcarboxaldehyde *p*-hydroxybenzoyl hydrazone; PCIH, 2-pyridylcarboxaldehyde isonicotinoyl hydrazone; PCTH, 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone; PIH, pyridoxal isonicotinoyl hydrazone; ROS, reactive oxygen species; RS, radical scavengers; SOD, superoxide dismutase; Tf, transferrin; TfR1, transferrin receptor-1.

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**Abstract**

Iron (Fe)-loading diseases remain an important problem due to the toxicity of Fe-catalyzed redox reactions. Iron-loading occurs in the mitochondria of Friedreich's ataxia (FA) patients and may play a role in its pathogenesis. This suggests that Fe chelation therapy could be useful. Previously, we developed lipophilic Fe chelators known as the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH) ligands and identified 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazone (PCTH) as the most promising analog. Hence, this study assessed the efficacy of PCTH and other PCIH analogs in comparison to various chelators including deferiprone and desferrioxamine (DFO). Age- and sex-matched control and FA fibroblasts were pre-incubated with Fe chelators and subsequently challenged with hydrogen peroxide ($H_2O_2$; 50 μM) for up to 24 h. The current study demonstrates an interesting structure-activity relationship amongst the closely related PCIH series of ligands, with only PCTH being highly effective at preventing $H_2O_2$-induced cytotoxicity. PCTH increased FA fibroblast cell viability by up to 70%, while DFO rescued viability by 1-5% only. Hence, PCTH, which was well-tolerated by cells was far more effective than DFO at preventing oxidative stress. Significantly, kinetic studies demonstrated that PCTH rapidly penetrated cells to induce $^{59}Fe$ efflux, while DFO, PCIH, PCBH and PCBBH were far slower, indicating it is the rate of chelator permeation that is crucial for protection against $H_2O_2$. Additionally, PCTH was found to be as, or more effective, than classical radical scavengers or the clinically-trialed anti-oxidant, idebenone, at protecting cells against $H_2O_2$-mediated cytotoxicity. These findings further indicate the potential of PCTH for treatment of Fe-overload.

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## Introduction

Development of novel orally effective iron (Fe) chelators is vital for the treatment of Fe-loading diseases including β-thalassemia major (Kalinowski and Richardson, 2005). This remains an important research area since the clinically used chelator, desferrioxamine (DFO; Figure 1), suffers several problems that are due, in part, to its hydrophilicity (Kalinowski and Richardson, 2005). This leads to poor absorption from the gut and poor penetration of cell membranes that prevents access to intracellular Fe pools. Due to its short half-life, DFO requires subcutaneous administration for 12-24 h/day, 5-6 times/week to achieve a negative Fe balance (Hershko et al., 2003). Together, these disadvantages lead to poor patient compliance (Kalinowski and Richardson, 2005).

Over the past 25 years, development of orally-effective Fe chelators has been on-going (Kalinowski and Richardson, 2005). The most successful of these agents, deferiprone (L1; Figure 1), is a small molecular weight chelator that is available in Europe for Fe overload treatment (Kalinowski and Richardson, 2005). However, its safety remains controversial due to conflicting studies reporting liver fibrosis (Richardson, 2001). Recently, Novartis announced the development of the triazole, ICL670 (deferasirox; Figure 1), which gained FDA approval as an orally-active Fe chelator (Kalinowski and Richardson, 2005). However, the Fe chelation efficacy and safety of ICL670 remains unclear. Hence, the development of new, efficient and safe orally-active Fe chelators remains an important aim.

We synthesized new aroylhydrazone Fe chelators known as the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH; Figure 1) analogs (Becker and Richardson, 1999). These chelators are tridentate (Bernhardt et al., 2007) and were derived from the highly effective pyridoxal isonicotinoyl hydrazone ligand (PIH; Figure 1) (Ponka et al., 1979). The latter compound showed marked activity *in vitro*, *in vivo* and in a clinical trial (Richardson and

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Ponka, 1998). However, the unfortunate failure to patent PIH led to a lack of commercial interest and the necessity to develop the PCIH series of ligands that maintain its optimal characteristics. Some of these chelators demonstrated high Fe mobilization efficacy and effectively prevented Fe uptake after it has been released from the Fe transport protein, transferrin (Tf), *in vitro* (Becker and Richardson, 1999). Of the PCIH class, 2-pyridylcarboxaldehyde benzoyl hydrazone (PCBH; Figure 1), 2-pyridylcarboxaldehyde *m*-bromobenzoyl hydrazone (PCBBH; Figure 1) and 2-pyridylcarboxaldehyde thiophenecarboxyl hydrazone (PCTH; Figure 1) were most effective at mobilizing intracellular Fe and preventing Fe uptake (Becker and Richardson, 1999).

In order for a ligand to be ideal for the treatment of Fe overload disease, it must not exhibit marked redox activity or anti-proliferative effects. Studies with the PCIH analogs examined this and demonstrated that these ligands did not potentiate ascorbate oxidation or benzoate hydroxylation, nor did they damage DNA in intact mammalian cells (Chaston and Richardson, 2003). Furthermore, these chelators had little anti-proliferative activity ($IC_{50}$: 40-50 μM) *in vitro* against the SK-N-MC neuroepithelioma cell line, with an effect similar to or less than DFO (Becker and Richardson, 1999). Another *in vitro* study using a model of mitochondrial Fe overload illustrated that several PCIH analogs were highly effective at increasing Fe release from the Fe-loaded mitochondrion, which could be an important factor in the treatment of the neuro-degenerative and cardio-degenerative disease, Friedreich's ataxia (FA) (Richardson et al., 2001).

Considering its appropriate properties, an *in vivo* mouse trial investigated the Fe chelation efficacy and tolerability of PCTH. This study demonstrated the ligand was well tolerated in mice at 100 mg/kg/bd and could induce substantial Fe excretion when administered orally (Wong et al., 2004). In fact, the efficacy of PCTH was comparable to L1 or PIH at the same

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dose (Wong et al., 2004). Collectively, these data suggested the PCIH series and particularly PCTH demonstrated properties suitable for treatment of Fe overload disease.

In this study, we investigated the activity of the PCIH class of chelators at inhibiting hydrogen peroxide ($H_2O_2$)-induced cytotoxicity in fibroblasts from FA patients compared to sex- and age-matched controls. The study demonstrated an interesting structure-activity relationship amongst the structurally-related PCIH series of chelators with only PCTH being highly effective at preventing $H_2O_2$-induced cytotoxicity in fibroblasts from either control or FA patients. Importantly, PCTH was found to rapidly penetrate cells to effectively induce $^{59}Fe$ efflux, while DFO, PCIH, PCBH and PCBBH were significantly slower. This indicates it is the rate of permeation of the chelator which is a vital factor for protection against $H_2O_2$-mediated cytotoxicity. Also, PCTH was as or more effective than classical radical scavengers or the anti-oxidant, idebenone, at protecting fibroblasts against $H_2O_2$-mediated cytotoxicity. These results confirm the potential of PCTH as an agent for the treatment of Fe-loading diseases.

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## Materials and Methods

### *Reagents.*

All commercial reagents were used without further purification. Desferrioxamine (DFO) was purchased from Novartis, Basel, Switzerland. The PCIH analogs and PIH were prepared using standard procedures (Bernhardt et al., 2007). Deferiprone was a gift from Prof. Roger Dean (Heart Research Institute, Sydney, Australia). Idebenone was obtained from Smart Nutrition (San Diego, CA, USA). Bathophenanthroline disulphonate (BPS), catalase (Cat), diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), dipyridyl (DP), MnTBAP and superoxide dismutase (SOD) were from Sigma (St. Louis, Mo, USA).

### *Cell Culture.*

Human FA fibroblasts and lymphoblasts and normal age- and sex-matched control fibroblasts and lymphoblasts were obtained from Corielle (Camden, NJ, USA). Human SK-N-MC neuroepithelioma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown as described (Richardson and Baker, 1992; Richardson et al., 1995).

### *Effect of $H_2O_2$ in the Presence and Absence of Chelators on Fibroblast Viability.*

Fibroblasts were sub-cultured from culture flasks to 96 well plates and allowed to grow for 24 h at 37$^{o}$C. The growth medium was then removed and the cells preincubated with control medium in the absence of FCS or this medium containing the agent(s) to be tested (i.e., chelators and/or anti-oxidants) for 30 min or 12 h at 37$^{o}$C. Medium alone or medium containing $H_2O_2$ (50 μM) was then added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37$^{o}$C. This $H_2O_2$ concentration was chosen after preliminary experiments in fibroblasts assessing its cytotoxicity. Viability was examined

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using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay, as described (Richardson et al., 1995). MTT color formation was directly proportional to the number of viable cells measured by direct cell counts using Trypan blue staining (Richardson et al., 1995).

***Preparation of $^{59}$Fe-Transferrin.***

Human transferrin (Tf) (Sigma) was labeled with $^{59}$Fe (Dupont NEN, MA, USA) to produce $^{59}Fe_2$-Tf ($^{59}$Fe–Tf), as previously described (Richardson and Baker, 1990; Richardson and Baker, 1992). Briefly, apo-Tf was labeled with Fe using the ferric-nitrilotriacetate complex at a ratio of 1 Fe to 10 NTA. This complex was prepared in 0.1 M HCl and then this solution adjusted to pH 7.4 using 1.4% $NaHCO_3$. This solution was added to apo-Tf and then incubated for 1 h at 37$^o$C. Unbound Fe was removed by exhaustive vacuum dialysis against 0.15 M NaCl adjusted to pH 7.4 using 1.4% $NaHCO_3$. The saturation of Tf with Fe was monitored by UV-Vis spectrophotometry with the absorbance at 280 nm (protein) being compared with that at 465 nm (Fe-binding site). In all studies, fully saturated diferric Tf was used.

***$^{59}$Fe Efflux Assay from Fibroblasts.***

Iron efflux experiments examining the ability of various chelators to mobilize $^{59}$Fe from sex- and age-matched control and FA fibroblasts were performed using established techniques (Baker et al., 1992; Richardson et al., 1995). Briefly, to enable comparison to studies assessing the effects of $H_2O_2$ and chelators on viability, an analogous incubation protocol was followed. Fibroblasts were initially prelabeled with $^{59}$Fe-Tf (0.75 μM) for 30 h at 37$^o$C and then washed four times with ice-cold PBS. The cells were then incubated with either control medium or medium containing the chelator (50 μM) for 12 h at 37$^o$C. After this, $H_2O_2$ (50

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μM) was then added to the medium in the presence and absence of the chelator for 24 h at 37°C. Subsequently, the overlying supernatant containing released $^{59}$Fe was then separated from the cells using a pasteur pipette and placed in a γ-counting tube. The cells were removed from the plate in 1 mL of PBS using a plastic spatula and added to a γ-counting tube. Radioactivity was measured in both the cell pellet and supernatant using a γ-scintillation counter (Wallac Wizard 3, Turku, Finland). In these studies, the novel ligands were compared to the well characterized chelators, DFO and PIH (positive controls).

***Statistical Analysis.***

Data were compared using the Student's *t*-test. Results were considered statistically significant when $p < 0.05$.

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## Results

### *PCTH Rescues $H_2O_2$-Mediated Cytotoxicity in Fibroblasts from Control and Friedreich's Ataxia Patients*

Previous studies demonstrated that the orally effective Fe chelator, PCTH, has high Fe chelation efficacy *in vitro* (Becker and Richardson, 1999; Richardson et al., 2001) and *in vivo* (Wong et al., 2004) and possessed properties suitable for the treatment of Fe-loading diseases (Kalinowski and Richardson, 2005). FA is a condition where Fe-loading occurs in the mitochondrion and this could potentially play some role in the pathogenesis of this disease (Boddaert et al., 2007). Moreover, fibroblasts from FA patients compared to controls were shown to be more sensitive to redox stress (Sturm et al., 2005; Wong et al., 2000). Considering this, the current study was designed to assess the ability of PCTH and other chelators to prevent $H_2O_2$-mediated toxicity in age- and sex-matched fibroblasts from control and FA patients.

Initial studies examined the susceptibility of control or FA fibroblasts to a 1 min – 24 h incubation with $H_2O_2$ (50 μM) after preincubation for 30 min (Figure 2A, B) or 12 h (Figure 2C, D) at 37°C with control medium or a variety of Fe chelators including DFO, PIH or PCTH (50 μM). In this case, DFO and PIH were used as relative controls to judge the importance of membrane permeability. DFO is relatively hydrophilic and shows limited permeability and low Fe chelation efficacy (Richardson et al., 1994; Richardson and Ponka, 1994), while PIH is considerably more lipophilic, permeating cells rapidly and having high activity at binding intracellular Fe (Ponka et al., 1979; Richardson and Baker, 1991). Preincubation of control or FA fibroblasts with control medium for 30 min followed by the addition of $H_2O_2$ for up to 24 h led to a marked decrease in viability to less than 5% of the control after a 24 h incubation (Figure 2A, B). As shown by others (Sturm et al., 2005), fibroblasts from FA patients were more sensitive to effects of $H_2O_2$ than control fibroblasts,

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the decrease in viability being more rapid in the former (Figure 2A, B). For instance, after a 30 min preincubation with control medium followed by a subsequent 2 h reincubation with $H_2O_2$, viability of control and FA fibroblasts was equal to 49 $\pm$ 3 % ($n$ = 3) and 29 $\pm$ 3 % ($n$ = 3), respectively (Figure 2A, B).

Irrespective of the preincubation period, cells incubated with DFO, PIH or PCTH alone in the absence of $H_2O_2$ had no effect on the viability of the fibroblasts relative to control medium (Figure 2). This clearly demonstrates the low anti-proliferative activity of these chelators which confirms our earlier work (Becker and Richardson, 1999). Examining cells preincubated with DFO or PIH for 30 min or 12 h, the addition of $H_2O_2$ led to a marked decrease in the viability of fibroblasts from control or FA patients. While both chelators were not markedly effective at preventing $H_2O_2$-mediated cytotoxicity, PIH was significantly ($p$ < 0.05) more active than DFO at inhibiting the $H_2O_2$-mediated reduction of viability after a 12 h preincubation (Figure 2C, D). This may be related to the greater lipophilicity and membrane permeability of PIH relative to DFO (Richardson and Baker, 1991).

When cells were preincubated with PCTH for 30 min (Figure 2A, B) or 12 h (Figure 2C, D), the subsequent cytotoxic effects of $H_2O_2$ were markedly and significantly ($p$ < 0.0025) less pronounced than when cells were exposed to $H_2O_2$ alone or when cells were preincubated with DFO or PIH. The preincubation of cells with PCTH for 12 h (Figure 2C, D) was significantly ($p$ < 0.0025) more effective than that for 30 min (Figure 2A, B) at preventing the decrease in viability of fibroblasts from both control and FA patients. Considering this, a 12 h preincubation was used in all other experiments examining the effects of chelators on $H_2O_2$-mediated cytotoxicity. While PCTH was effective at rescuing the cytotoxicity imparted by $H_2O_2$, the protective effect was less marked in fibroblasts from FA patients than controls. For instance, after a 12 h preincubation of control or FA fibroblasts with PCTH, the viability after

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a 24 h incubation with $H_2O_2$ was equal to 86 $\pm$ 5 % ($n$ = 3) and 71 $\pm$ 1 % ($n$ = 3), respectively (Figure 2C, D).

To determine if the effect of PCTH at rescuing $H_2O_2$-mediated cytotoxicity was not just a property of fibroblasts, similar experiments were repeated with age- and sex-matched lymphoblasts from control and FA patients after a preincubation of 12 h with the chelators (Figure 3A and B). In these studies, similar results were obtained to those shown with fibroblasts (Figure 2C, D), with PCTH being significantly more effective than DFO and PIH at preventing $H_2O_2$-mediated toxicity in lymphoblasts (Figure 3A and B). Again, the chelators were less effective at preventing the decrease in viability of lymphoblasts from FA than control patients (Figure 3A and B). PIH or PCTH added in the absence of $H_2O_2$ showed no significant effect on the viability of control FA lymphoblasts. PCTH also showed similar efficacy at preventing the detrimental effects of $H_2O_2$ on the viability of human SK-N-MC neuroepithelioma cells as that found in fibroblasts and lymphoblastoid cells from control and FA patients (data not shown).

In summary, these experiments demonstrated that PCTH was markedly more effective than either DFO or PIH at preventing $H_2O_2$-mediated cytotoxicity of fibroblasts and lymphoblasts from control and FA patients.

***PCTH, But Not Other Closely Related PCIH Analogs, Effectively Rescues $H_2O_2$-Mediated Cytotoxicity***

To further investigate the effects of Fe chelators on $H_2O_2$-mediated cytotoxicity, we examined the effect of 5 other chelators of the PCIH class which show high structural similarity to PCTH (Figure 1). Examining control fibroblasts, PCIH and PCBH showed limited activity at rescuing $H_2O_2$-mediated cytotoxicity, having efficacy that was not significantly ($p > 0.05$)

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different than PIH and significantly ($p < 0.001$) less marked than PCTH (Figure 4A). In agreement with the results in Figure 2, the ability of the chelators to rescue $H_2O_2$-mediated toxicity of FA fibroblasts was less pronounced, with only PCTH leading to a marked rescue (Figure 4B). The other PCIH analogs, namely PCBBH, PCAH and PCHH (Figure 1), demonstrated no effective activity at preventing $H_2O_2$-mediated cytotoxicity in either control or FA fibroblasts, again being significantly ($p < 0.001$) less effective than PCTH (Figure 4C, D). None of the PCIH analogs when added in the absence of $H_2O_2$ showed any effect on the cellular viability of fibroblasts from control or FA patients (data not shown), in good agreement with previous studies using tumor cells (Becker and Richardson, 1999).

***The Permeable Chelators, L1 and Dipyridyl, Show High Activity at Preventing $H_2O_2$-Mediated Cytotoxicity, while Poorly Permeable Chelators Show Much Less Effect***

To understand the significance of chelator permeability in preventing $H_2O_2$-mediated toxicity in fibroblasts, the effect of PCTH was compared to two permeable chelators, namely dipyridyl (DP; Figure 1) and L1, two chelators which are known to be impermeable, DTPA and EDTA (Figure 1), and a partially permeable ligand, BPS (Kicic et al., 2001; Richardson et al., 1994; Richardson and Baker, 1994) (Figure 1).

Both L1 and DP showed activity that was slightly greater than PCTH in both control and FA fibroblasts (Figure 5A, B). For instance, control fibroblast cell viability was equal to 92%, 83% and 78% of the control in the presence of $H_2O_2$ and either L1, DP or PCTH, respectively after 24 h, while $H_2O_2$ alone reduced viability to 12% of the control (Figure 5A). Both DTPA and EDTA showed significantly ($p < 0.02$) less activity than all of the above chelators in both control and FA fibroblasts (Figure 5A, B). For example, in control fibroblasts after a 24 h incubation, cellular viability was equal to 25% and 31% of the control in the presence of $H_2O_2$ and EDTA or DTPA, respectively (Figure 5A). Notably, despite the latter two compounds

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being largely impermeable ligands (Kicic et al., 2001; Richardson and Baker, 1994), some rescuing effect against $H_2O_2$-induced toxicity was observed, suggesting that extracellular Fe chelation plays some role in preventing cytotoxicity (Figure 5). In accordance with its intermediate permeability, in control fibroblasts, BPS was slightly more effective than DTPA and EDTA at preventing the decrease in viability in the presence of $H_2O_2$ (Figure 5A). However, in FA fibroblasts, BPS showed similar activity to DTPA and EDTA, there being little rescue against $H_2O_2$-induced cytotoxicity (Figure 5B).

In summary, membrane permeability of chelators played an important role in effective rescue against the $H_2O_2$-induced decrease in cellular viability of control and FA fibroblasts.

***Iron Chelation Efficacy of the Ligands in Control and FA Fibroblasts***

To interpret the results above examining the ability of the various ligands to prevent the $H_2O_2$-induced cytotoxicity of control and FA fibroblasts, we characterized the ability of the chelators to increase $^{59}$Fe mobilization from cells. For relevant comparison, the incubation conditions were analogous to those used in the studies examining the effect of chelators and $H_2O_2$ on fibroblast viability (Figures 2-5).

Iron efflux experiments (Figure 6A) were performed by incubating control and FA fibroblasts with the physiological Fe transport protein Tf labeled with $^{59}$Fe ($^{59}$Fe-Tf; 0.75 μM). Cells were pre-labeled with $^{59}$Fe-Tf for 30 h at 37$^{o}$C and then washed and reincubated with either control medium or medium containing the chelator (50 μM) for 12 h at 37$^{o}$C. After this, $H_2O_2$ (50 μM) or media alone (control) was then added in the presence and absence of the chelator for 24 h at 37$^{o}$C. Subsequently, the overlying media containing released $^{59}$Fe was then separated from the cells. In all experiments, the addition of $H_2O_2$ did not have any effect on the ability of the chelator to induce cellular $^{59}$Fe release in comparison to chelator alone

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(Figure 6A). Thus, subsequent experiments examined the ability of control medium or chelators (50 μM) to mobilize $^{59}$Fe from the prelabeled control or FA fibroblasts without incubation with $H_2O_2$ (Figure 6B).

Normal or FA fibroblasts control medium resulted in the release of 5-6% of cellular $^{59}$Fe (Figure 6B). For all the chelators examined, there was little difference in cellular $^{59}$Fe efflux between control and FA fibroblasts. Interestingly, despite the limited effect of DFO at preventing $H_2O_2$-induced cytotoxicity in both control and FA fibroblasts, this chelator showed considerable activity at mobilizing cellular $^{59}$Fe. Indeed, DFO led to the efflux of 45 $\pm$ 1% and 39 $\pm$ 1% ($n$ = 3 determinations) of cellular $^{59}$Fe from control and FA fibroblasts, respectively (Figure 6B). Moreover, the efficacy of DFO was greater than PIH which released 24 $\pm$ 4% and 28 $\pm$ 1% of cellular $^{59}$Fe in control and FA fibroblasts (Figure 6B). These results did not appear to correlate to the ability of the chelators to prevent $H_2O_2$-induced cytotoxicity, where PIH showed significantly ($p < 0.05$) higher efficacy than DFO at preventing the reduction in viability (Figure 2C, D).

Studies using the PCIH analogs demonstrated that PCTH, PCBBH and PCBH showed similar and high activity at mobilizing cellular $^{59}$Fe in control and FA fibroblasts, resulting in the release of 33-46% of intracellular $^{59}$Fe (Figure 6B). Thus, the chelator PCTH which showed greatest efficacy of the PCIH analogs at preventing $H_2O_2$-induced cytotoxicity (Figure 4) was also very effective at mobilizing cellular $^{59}$Fe from both control and FA fibroblasts (Figure 6B). However, it is of interest that while PCBH and PCBBH showed high Fe chelation efficacy that was similar to PCTH (Figure 6B), these ligands did not markedly rescue $H_2O_2$-induced cytotoxicity (Figure 4C, D). At present, we do not understand the reason for this discrepancy in the relationship between Fe chelation efficacy and prevention of $H_2O_2$-induced

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cytotoxicity. However, we cannot rule out potential cytotoxic effects that occur between the Fe complexes of PCBH and PCBBH and $H_2O_2$.

As shown in previous studies in neoplastic cells (Becker and Richardson, 1999), the relatively hydrophilic PCIH analogs, PCHH and PCAH (Bernhardt et al., 2007), were ineffective at mobilizing $^{59}$Fe, there being no significant ($p > 0.05$) difference to the control medium alone (Figure 6B). These results indicate that both these ligands are inefficient at permeating fibroblasts and releasing $^{59}$Fe, probably due to their higher hydrophilicity than other PCIH analogs (Bernhardt et al., 2007). This low Fe chelation efficacy could explain their lack of activity at preventing the cytotoxic effects of $H_2O_2$ (Figure 4C, D).

Studies then examined the effect of EDTA, DTPA, DP, BPS and L1 at mobilizing $^{59}$Fe from control and FA fibroblasts (Figure 6B) to assess their Fe chelation efficacy relative to their ability to prevent $H_2O_2$-induced cytotoxicity (Figure 5). Control and FA fibroblasts were pre-labeled with $^{59}$Fe-Tf (0.75 μM) for 30 h at 37$^o$C, the cells washed and then reincubated with either control medium or medium containing the chelator (50 μM) for 12 h at 37$^o$C. After this, $H_2O_2$ (50 μM) or media alone (control) was then added in the presence and absence of the chelator for 24 h at 37$^o$C. Again, $H_2O_2$ had no effect on cellular $^{59}$Fe mobilization (data not shown) and the results presented are in the absence of this agent (Figure 6B).

As expected, the membrane impermeable chelators, EDTA and DTPA, showed limited ability to mobilize $^{59}$Fe in control and FA fibroblasts, having similar efficacy to that of the control (Figure 6B). This correlated to their poor ability to rescue both control and FA fibroblasts against the toxic effects of $H_2O_2$ (Figure 5) and again indicates that intracellular Fe chelation is important for preventing $H_2O_2$ cytotoxicity. The partially membrane-permeable ligand, BPS, showed moderate $^{59}$Fe mobilizing activity and was able to release 20 ± 1% and 21 ± 1%

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of intracellular $^{59}$Fe from control and FA fibroblasts, respectively (Figure 6B). This was in agreement with its intermediate ability to rescue control fibroblasts from $H_2O_2$ cytotoxicity (Figure 5A). On the other hand, the membrane permeable chelators, DP and L1, showed high $^{59}$Fe mobilizing ability in control and FA fibroblasts, mediating the release of 39-44% of $^{59}$Fe (Figure 6B). Again, this result suggested that their high Fe mobilizing efficacy played an integral role in their ability to rescue control and FA fibroblasts from the toxic effects of $H_2O_2$ (Figure 5).

Both DFO and PCTH markedly increased $^{59}$Fe mobilization from fibroblasts (Figure 6B), but under the same conditions, DFO did not rescue $H_2O_2$-induced cytotoxicity, while PCTH did (Figure 2). This led to the hypothesis that it could be the rate at which these chelators penetrate cells that could be important in terms of their protective effect. To assess this, FA fibroblasts were prelabeled with $^{59}$Fe-Tf (0.75 μM) for 30 h at 37$^o$C, washed and then reincubated for 30 min to 24 h at 37$^o$C with control medium or the chelators, PCTH, DFO, PCIH, PCBH or PCBBH at 50 μM. Importantly, PCTH rapidly penetrated cells to induce $^{59}$Fe efflux, while DFO was significantly ($p < 0.05$) slower (Figure 6C). The remaining chelators, PCBH, PCBBH and PCIH also demonstrated an initial slow rate of $^{59}$Fe efflux. After 30 min, PCTH was rapidly able to mediate the release of 25 ± 2% of cellular $^{59}$Fe, while DFO, PCBH, PCBBH and PCIH were significantly ($p < 0.001$) less effective, leading to the efflux of 4-11% of cellular $^{59}$Fe (Figure 6C). Only after 6 h did PCTH and DFO start to show similar $^{59}$Fe mobilizing efficacy. In addition, PCIH, PCBBH and PCBH showed reduced levels of cellular $^{59}$Fe release in comparison to DFO and especially PCTH (Figure 6C). These results suggest that the increased ability of PCTH in preventing $H_2O_2$-induced cytotoxicity in comparison to these other ligands could be explained by its rapid rate of penetrating cells to induce efficient $^{59}$Fe mobilization from $H_2O_2$-sensitive pools. Hence, the ability of the ligand to permeate the cell membrane, and particularly the rate at which it does so, were crucial factors in preventing

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$H_2O_2$-induced cytotoxicity. This would be particularly the case when the preincubation time with PCTH is short (*e.g.*, 2 h), relative to much longer preincubation periods (*e.g.*, 12 h).

***Combination of DFO and PCTH Increases the Efficacy of Rescue Against $H_2O_2$-Induced Cytotoxicity in Control and FA Fibroblasts***

Previous studies using several ligands, including L1 and PIH, have demonstrated that combination of a poorly permeable chelator (*i.e.*, DFO) and a permeable ligand (*i.e.*, L1 or PIH) can markedly increase Fe chelation efficacy (Link et al., 2001; Link et al., 2003). This is probably due to the shuttle principle, whereby a permeable chelator enters cells, binds Fe and then delivers it to a largely extracellular ligand (Link et al., 2003). This chelator outside the cell removes Fe from the complex allowing the permeable ligand to enter the cell to chelate more Fe. Considering this, we assessed the combination of the highly permeable PCTH ligand with DFO. In these experiments the concentration of DFO was kept constant at 50 μM, while the concentration of PCTH was increased from 5-50 μM (Figure 7A, B).

As shown previously (see Figure 2), DFO had no significant effect on rescuing cells from $H_2O_2$-induced toxicity. However, upon the addition of increasing concentrations of PCTH to DFO, cellular viability in the presence of $H_2O_2$ markedly increased (Figure 7A, B). In fact, the combination of the highest PCTH concentration (50 μM) with DFO resulted in greater ability to prevent $H_2O_2$-induced cytotoxicity in control (Figure 7A) and FA fibroblasts (Figure 7B) than 50 μM PCTH alone. However, only for FA fibroblasts was the activity of the combination of DFO with PCTH significantly ($p < 0.006$) more effective than PCTH alone (Figure 7B). This may indicate a shuttle effect between PCTH and DFO that led to greater activity than PCTH alone. Further studies then examined if the combination of increasing concentrations of PIH (5-50 μM) with DFO (50 μM) also led to some improved ability of the former to prevent the $H_2O_2$-mediated decrease in fibroblast viability (Figure 7C, D). However,

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this combination did not lead to any significant alteration in cellular viability in comparison to PIH alone (Figure 7C, D). This difference in the protective effect of the combination of DFO with PIH or PCTH is intriguing. However, it could be due to the fact that in contrast to PCTH that binds Fe(II) alone, PIH is a Fe(III) chelator that may less readily donate its Fe to DFO (Bernhardt et al., 2007). The pM values that allow comparison of the relative ability of different ligands to bind a metal ion under comparable physiological conditions have been reported for PCTH and PIH (Bernhardt et al., 2007) and support this concept.

***PCTH is More Effective at Preventing the $H_2O_2$-Mediated Decrease in Viability of Fibroblasts than Free Radical Scavengers***

The efficacy of PCTH at preventing the $H_2O_2$-induced decrease in viability of control and FA fibroblasts was compared to a variety of well known radical scavengers alone or in combination (Figure 8A, B). These scavengers were used at concentrations that have been shown to be effective in previous investigations (Chaston et al., 2004; Konorev et al., 1999; Kwok and Richardson, 2002) and included SOD (1000 U/mL) which eliminates superoxide, Cat (1000 U/mL) which degrades $H_2O_2$ and the permeable glutathione peroxidase mimetic, MnTBAP (200 μM), that also decreases $H_2O_2$ levels at the expense of oxidizing glutathione. All scavengers either alone or in combination were found to effectively prevent the $H_2O_2$-mediated decrease in viability of control and FA fibroblasts. However, PCTH showed slightly greater activity than all of the above, there being a significant ($p < 0.015$) difference between all of the scavengers and PCTH after 24 h when examining control fibroblasts (Figure 8A). Examining FA fibroblasts, a significant ($p < 0.05$) difference in the efficacy of preventing $H_2O_2$-induced cytotoxicity was only found between PCTH and SOD. None of the radical scavengers alone or in combination had any effect on cellular viability when added in the absence of $H_2O_2$ (data not shown), all being well-tolerated.

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Considering the efficacy of the radical scavengers at preventing the decreased viability observed in the presence of $H_2O_2$, studies were designed to assess the effect of combining these agents with PCTH (Figure 9A, B). The combination of Cat and SOD markedly prevented the decreased viability of control and FA fibroblasts in the presence of $H_2O_2$ (Figure 9A, B). Interestingly, the combination of Cat and SOD with PCTH did not significantly improve cellular viability over that observed with PCTH alone (Figure 9A, B). A similar effect was observed with L1, where the combination of Cat and SOD with L1 again did not significantly improve cellular viability over that seen with L1 alone (Figure 9A, B).

***Idebenone is Less Effective than PCTH at Rescuing the $H_2O_2$-Induced Decrease in Fibroblast Viability***

Idebenone has shown some beneficial effects in the treatment of FA patients in clinical trials due to its anti-oxidant properties (Di Prospero et al., 2007; Rustin et al., 2002). Hence, it was an appropriate positive control to compare the effects of PCTH in the current model. In contrast to 50 μM PCTH which did not reduce fibroblast viability when it was incubated with cells in the absence of $H_2O_2$ (Figure 2A-D), idebenone alone markedly reduced cellular viability at 25 and 50 μM (Figure 10A, B). In the presence of $H_2O_2$, there was increased fibroblast viability from both control and FA patients in comparison to $H_2O_2$ alone as the idebenone concentration increased to 25 μM (Figure 10C, D). However, at an idebenone concentration of 50 μM, the efficacy at preventing $H_2O_2$-induced cytotoxicity was similar to that found at 5 μM in control and FA fibroblasts. This decreased efficacy at the highest idebenone concentration in the presence of $H_2O_2$ (Figure 10C, D) is probably due to toxicity observed with idebenone alone (Figure 10A, B). Collectively, these studies demonstrate that PCTH was better tolerated and more effective under the current conditions at preventing the $H_2O_2$-induced cytotoxicity of fibroblasts from control and FA patients.

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**Discussion**

The mitochondrial Fe-loading and oxidative stress in FA may play a role in this disease (Pandolfo, 2006). Thus, we investigated if our novel PCIH Fe chelators, in comparison to other ligands, could prevent $H_2O_2$-mediated cytotoxicity. We showed that one of the most biologically-active ligands of the PCIH class, PCTH (Becker and Richardson, 1999; Wong et al., 2004), markedly prevented $H_2O_2$-mediated cytotoxicity in normal and FA fibroblasts.

In this investigation, a range of Fe chelators with varying properties were assessed for their ability to prevent the decreased viability induced by $H_2O_2$. This was important considering that several chelators have shown protective effects in control and FA fibroblasts (Sturm et al., 2005; Wong et al., 1999). In our investigation, DFO had no significant effect on rescuing $H_2O_2$-mediated cytotoxicity. This could be explained by its slow permeation rate due to its relatively high hydrophilicity (Richardson et al., 1994; Richardson et al., 2001). However, under the same experimental conditions, $^{59}$Fe efflux studies demonstrated that DFO effectively permeated the cell and resulted in pronounced Fe efflux from control and FA fibroblasts (Figure 6A, B). Additionally, the effect of DFO at inducing $^{59}$Fe mobilization was more marked than the lipophilic ligand, PIH. This could be reconciled considering the relatively long 36 h incubation period with the chelators which would facilitate the slow access of DFO to $^{59}$Fe pools (Figure 6A, B).

While PIH was significantly more active than DFO at preventing $H_2O_2$-mediated cytotoxicity, it was less efficient than other closely related aroylhydrazones, such as PCTH (Figure 1). Indeed, of all the PCIH analogues, PCTH was the most effective, markedly preventing the effect of $H_2O_2$ at inducing decreased fibroblast viability. Previous studies showed PCTH to be highly efficient at mobilizing Fe from neoplastic cells, being particularly effective at low concentrations (Becker and Richardson, 1999). Further, PCTH was also efficient at chelating

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Fe from Fe-loaded mitochondria of reticulocytes (Richardson et al., 2001). From the work comparing the ability of PIH and DFO to prevent $H_2O_2$-mediated cytotoxicity, it could be suggested the ability of chelators to penetrate membranes and bind Fe pools is important to prevent the effects of $H_2O_2$. Significantly, this was clearly demonstrated by kinetic studies examining the ability of the chelators to enter cells, bind intracellular Fe pools and induce Fe efflux as a function of time. These experiments showed that PCTH could rapidly mobilize intracellular $^{59}$Fe within only 30 min of incubation and was the most effective chelator tested (Figure 6C). In marked contrast, DFO only showed similar $^{59}$Fe mobilizing efficacy to PCTH after 6 h. Furthermore, PCBH, PCIH and PCBBH were shown to induce cellular Fe-depletion at a slower rate than DFO and particularly PCTH. These results suggest the rapid rate of penetration by PCTH into fibroblasts in comparison to DFO, PCIH, PCBH and PCBBH and its ability to efficiently mobilize $^{59}$Fe may be crucial in preventing $H_2O_2$-induced cytotoxicity. Hence, these data indicate that it is the rate at which the ligand permeates the cell which dictates its ability to prevent $H_2O_2$-mediated cytotoxicity. In view of this, it is notable that $H_2O_2$ can, within seconds, rapidly partition across cell membranes (Antunes and Cadenas, 2000) and thus its cytotoxic effects *via* interaction with Fe occur very quickly. While the ability of chelators to permeate cells has been shown to be important for chelation of intracellular Fe (Lipinski et al., 1997; Porter et al., 1988; Ma et al., 2006), this study demonstrates that the rapid access to intracellular Fe is vital for protection against $H_2O_2$-mediated cytotoxicity.

The low activity of the hydrophilic PCHH and PCAH ligands at preventing $H_2O_2$-induced cytotoxicity is probably explained by their inability to act as efficient Fe chelators to mobilize $^{59}$Fe from fibroblasts (Figure 6B), as observed in Fe chelation studies in tumor cells (Becker and Richardson, 1999). Hence, lipophilicity plays an important role in the effect observed and it is notable that PCTH and its Fe complex display high hydrophobicity (Bernhardt et al.,

2007). However, PCBBH and its Fe complex are also very lipophilic (Bernhardt et al., 2007), but far less active than PCTH in preventing $H_2O_2$-induced toxicity, indicating other factors are also important.

Considering other beneficial properties of these compounds, in contrast to PIH, we showed that in the presence of $H_2O_2$ and Fe(II), PCIH analogs did not induce hydroxyl radical generation (Chaston and Richardson, 2003). In fact, the PCIH analogs form Fe(II) complexes with a high potential Fe(III/II) redox couple (>500 mV *vs.* NHE), and thus, the generation of reactive oxygen species would not be facile (Bernhardt et al., 2007). Hence, the lack of redox activity of the formed PCTH-Fe complex may explain, in part, its low toxicity and high efficacy in preventing the $H_2O_2$-mediated decrease in fibroblast viability (Figure 2). Another potentially important chemical property of the PCIH ligands, relative to PIH which has strong affinity for Fe(III) (Richardson, 1998), is that the PCIH chelators are well-characterized Fe(II) ligands (Bernhardt et al., 2007). The chelation of Fe(II) pools that are known to exist within cells (St. Pierre et al., 1992) are probably crucial, as these interact with $H_2O_2$ leading to toxic radicals. It is also of interest to note that due to dynamic equilibria, an Fe(III) chelator also has indirect access to chelatable Fe(II) and thus can prevent $H_2O_2$ toxicity.

While the latter factors are significant, further evidence of the importance of membrane permeability and Fe chelation to the protective effects of a compound was demonstrated using a range of ligands with different membrane permeability characteristics that ranged from highly permeable (ie, DP, L1) to limited permeability (BPS), to not permeable (EDTA, DTPA) (Kicic et al., 2001; Richardson and Baker, 1994). The ability of DP to effectively prevent $H_2O_2$-mediated cellular toxicity in control and FA fibroblasts agreed with previous studies (Sturm et al., 2005). The orally-effective chelator, L1 (Richardson, 2001), also showed very high activity, probably due to its efficacy to permeate cells and chelate Fe pools

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(Fredenburg et al., 1996), as illustrated by our Fe mobilization experiments (Figure 6B). Of interest, in a small, poorly controlled clinical trial, L1 given in combination with idebenone improved neuropathy and ataxic gait in FA patients (Boddaert et al., 2007). However, since idebenone has beneficial effects on neurological symptoms of FA patients (Di Prospero et al., 2007), further studies are essential to confirm these preliminary findings with L1.

Both DTPA and EDTA showed little activity to prevent $H_2O_2$-mediated cytotoxicity, probably due to their extracellular location and inability to permeate cells (Kicic et al., 2001; Richardson and Baker, 1994). The Fe(II) ligand, BPS, showed activity that was slightly greater than DTPA and EDTA and much less than DP, L1 or PCTH. The structure of BPS includes two sulphonate groups (Figure 1) that are negatively charged at physiological pH and the molecule has been suggested to be impermeable. However, our studies using fibroblasts (Figure 6B) and previous investigations implementing neoplastic cells (Richardson and Baker, 1994), indicate that BPS is capable of limited cellular Fe mobilization, being more effective than DTPA and EDTA, but less active than DP.

The ability of PCTH to prevent $H_2O_2$-mediated cytotoxicity of fibroblasts was also compared with classical anti-oxidants. Interestingly, PCTH was similarly or more effective than SOD, Cat, MnTBAP or idenbenone as single agents or when used in combination. The anti-oxidant, idebenone, is of relevance as it is beneficial for treating the cardiomyopathy (Rustin et al., 2002) and neurological deficits (Di Prospero et al., 2007) of FA patients. Surprisingly, in this study, this agent alone was shown to be cytotoxic at 50 μM. At lower idebenone concentrations its activity at preventing $H_2O_2$-mediated cytotoxicity was far less than PCTH.

An interesting observation was that while control and FA fibroblasts could be rescued by chelators, the FA fibroblasts were more sensitive to $H_2O_2$. Chelators and anti-oxidants were

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less capable of preventing the decreased viability in FA fibroblasts. The higher sensitivity of FA fibroblasts to $H_2O_2$ was previously reported (Sturm et al., 2005), but their decreased response to Fe chelators and anti-oxidants was not described. The reason for their higher sensitivity could relate to the known alterations in mitochondrial Fe metabolism in FA patients (Napier et al., 2005; Pandolfo, 2006). In fact, mitochondrial Fe deposits are known in FA fibroblasts (Delatycki et al., 1999), which could react with $H_2O_2$. However, both control and FA fibroblasts were rescued from the $H_2O_2$-mediated toxicity by Fe chelation, suggesting common physiologically-relevant Fe pools were targeted by this oxidant. There is oxidative stress in FA (Pandolfo, 2006; Wong et al., 1999) and the sensitivity of FA fibroblasts to $H_2O_2$ could be due to alterations in mitochondrial Fe metabolism and/or processes important in redox management.

In summary, the novel orally-effective chelator, PCTH, shows high activity at preventing the cytotoxic effects of $H_2O_2$ in fibroblasts being as, or more effective, than classical anti-oxidants. Our results suggest that it is the rate at which permeable chelators penetrate the cell that dictates their effectiveness at rescuing $H_2O_2$-mediated toxicity. These data support the use of PCTH as an agent suitable to treat Fe-loading conditions.



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## Legends for Figures

**Figure 1. The chemical structures of iron chelators described in this study.** Desferrioxamine (DFO), deferiprone (L1), ICL670A (deferasirox), pyridoxal isonicotinoyl hydrazone (PIH), dipyridyl (DP), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), bathophenanthroline disulphonate (BPS), 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH), 2-pyridylcarboxaldehyde 2-thiophene hydrazone (PCTH), 2-pyridylcarboxaldehyde benzoyl hydrazone (PCBH), 2-pyridylcarboxaldehyde *m*-bromobenzoyl hydrazone (PCBBH), 2-pyridylcarboxaldehyde *p*-aminobenzoyl hydrazone (PCAH) and 2-pyridylcarboxaldehyde *p*-hydroxybenzoyl hydrazone (PCHH).

**Figure 2. PCTH, but not DFO or PIH, effectively rescues control and Friedreich's ataxia (FA) fibroblasts from $H_2O_2$-mediated cytotoxicity.** Fibroblasts were preincubated with control medium or medium containing the chelator (50 μM) for 0.5 h or 12 h at 37°C. Then $H_2O_2$ (50 μM) or media alone was added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37°C. The results are a typical experiment from 3 performed.

**Figure 3. PCTH, but not DFO or PIH, effectively rescues control and Friedreich's ataxia (FA) lymphoblasts from $H_2O_2$-mediated cytotoxicity.** Control and FA lymphoblasts were preincubated with control medium or medium containing the chelator (50 μM) for 12 h at 37°C. $H_2O_2$ (50 μM) or media alone was then added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37°C. The results are a typical experiment from 3 performed.

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**Figure 4. PCTH, but not its closely related analogs PCIH, PCBH, PCBBH, PCAH or PCHH, effectively rescues control and Friedreich's ataxia (FA) fibroblasts from $H_2O_2$-mediated cytotoxicity.** Fibroblasts were preincubated with control medium or medium containing the chelator (50 μM) for 12 h at 37°C. $H_2O_2$ (50 μM) or media alone was then added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37°C. The results are a typical experiment from 3 performed.

**Figure 5. PCTH, L1 and dipyridyl, but not BPS, DTPA, or EDTA, effectively rescues control and Friedreich's ataxia (FA) fibroblasts from $H_2O_2$-mediated cytotoxicity.** Fibroblasts were preincubated with control medium or medium containing the chelator (50 μM) for 12 h at 37°C. $H_2O_2$ (50 μM) or media alone was then added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37°C. The results are a typical experiment from 3 performed.

**Figure 6. Effect of chelators at mediating $^{59}$Fe mobilization from prelabeled control and Friedreich's ataxia (FA) fibroblasts. (A) The effect of PIH, DFO, PCBBH and PCTH in the presence and absence of $H_2O_2$.** Fibroblasts were prelabeled with $^{59}$Fe-Tf (0.75 μM) for 30 h at 37°C and washed. The cells were then incubated with either control medium or medium containing the chelator (50 μM) for 12 h at 37°C. $H_2O_2$ (50 μM) or media alone was then added to each of these conditions and incubated for 24 h at 37°C. **(B) The effect of DFO, PIH and the PCIH analogs on cellular $^{59}$Fe mobilization.** Fibroblasts were prelabeled with $^{59}$Fe-Tf (0.75 μM) for 30 h at 37°C and washed. The cells were then incubated with either control medium or medium containing the chelator (50 μM) for 36 h at 37°C. **(C) The effect of control medium, PCTH, DFO, PCIH, PCBH and PCBBH on $^{59}$Fe mobilization from fibroblasts as a function of time.** Fibroblasts were prelabeled with $^{59}$Fe-Tf (0.75 μM) for 30 h at 37°C, washed, and then reincubated with control medium, PCTH, DFO, PCIH, PCBH or

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PCBBH at 50 μM for up to 24 h at 37°C. The results are a typical experiment from 3 performed.

**Figure 7. Combination of (A, B) DFO with PCTH or (C, D) DFO with PIH increases the efficacy of PCTH alone, but not PIH alone, at rescuing control and Friedreich's ataxia (FA) fibroblasts from $H_2O_2$-mediated cytotoxicity.** Control and FA fibroblasts were preincubated with control medium or medium containing DFO (50 μM) in the presence of a range of concentrations of PCTH (5-50 μM) in **(A)** control or **(B)** FA fibroblasts; or PIH (5-50 μM) in **(C)** control and **(D)** FA fibroblasts for 12 h at 37°C. Then $H_2O_2$ (50 μM) or media alone was added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37°C. The results are a typical experiment from 3 performed.

**Figure 8. PCTH is similar to, or more effective, than free radical scavengers at rescuing (A) control and (B) Friedreich's ataxia (FA) fibroblasts from $H_2O_2$-mediated cytotoxicity.** Fibroblasts were preincubated with control medium or medium containing either: PCTH (50 μM), MnTBAP (200 μM), catalase (Cat; 1000 U/mL), SOD (1000 U/mL), SOD (1000 U/mL) + Cat (1000 U/mL), or MnTBAP (200 μM) + SOD (1000 U/mL) + Cat (1000 U/mL) for 12 h at 37°C. Then $H_2O_2$ (50 μM) or media alone was added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37°C. The results are a typical experiment from 3 performed.

**Figure 9. The combination of PCTH with free radical scavengers does not lead to increased efficacy at rescuing (A) control or (B) Friedreich's ataxia (FA) fibroblasts from $H_2O_2$-mediated cytotoxicity.** Fibroblasts were preincubated with control medium or medium containing either: PCTH (50 μM), L1 (50 μM), catalase (Cat; 1000 U/mL) + SOD (1000 U/mL), PCTH (50 μM) + Cat (1000 U/mL) + SOD (1000 U/mL) or L1 (50 μM) + Cat (1000 U/mL) + SOD (1000 U/mL) for 12 h at 37°C. $H_2O_2$ (50 μM) or media alone was then

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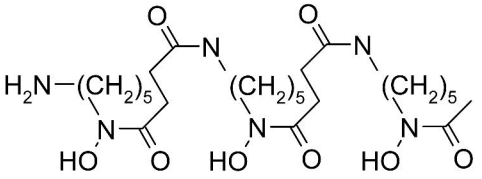
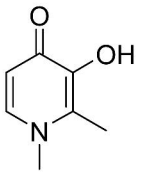
added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37$^{o}$C. The results are a typical experiment from 3 performed.

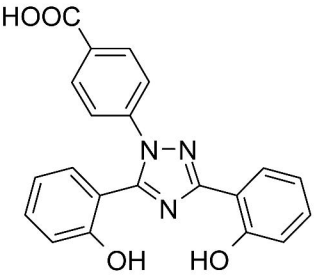
**Figure 10. Idebenone in contrast to PCTH decreases the cell number of: (A) control and (B) Friedreich's ataxia (FA) fibroblasts as the concentration is increased to 25 and 50 μM. Idebenone is not as effective as PCTH at rescuing (C) control or (D) FA fibroblasts from $H_2O_2$-mediated cytotoxicity.** Control or FA fibroblasts were preincubated with control medium or medium containing either PCTH (50 μM) or idebenone (1-50 μM). Then either: **(A, B)** medium alone or **(C, D)** $H_2O_2$ (50 μM) was added to each of these conditions and the incubation continued for 1 min, 2 h, 12 h or 24 h at 37$^{o}$C. The results are a typical experiment from 3 performed.



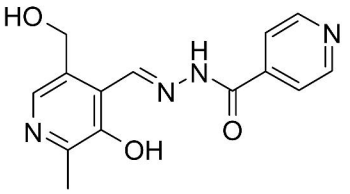
DFO



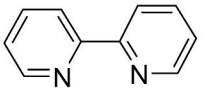
Deferiprone (L1)



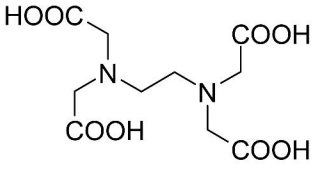
Deferasirox



PIH



DP



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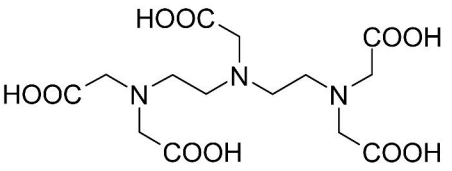
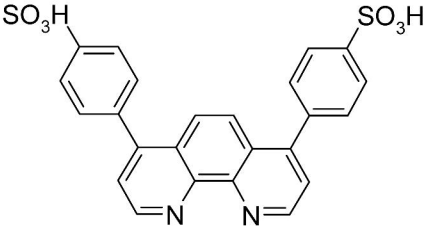
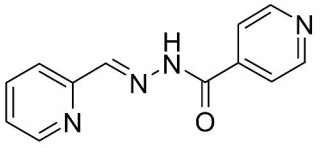


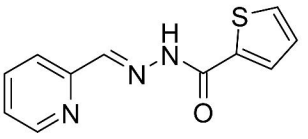
DTPA



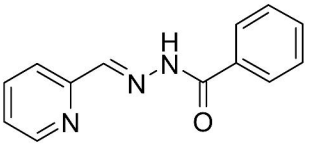
BPS



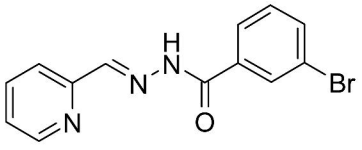
PCIH



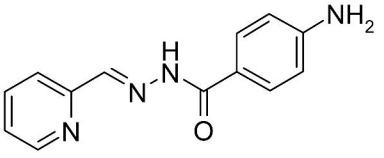
PCTH



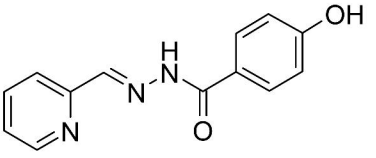
PCBH



PCBBH



PCAH



PCHH

**Figure 1**

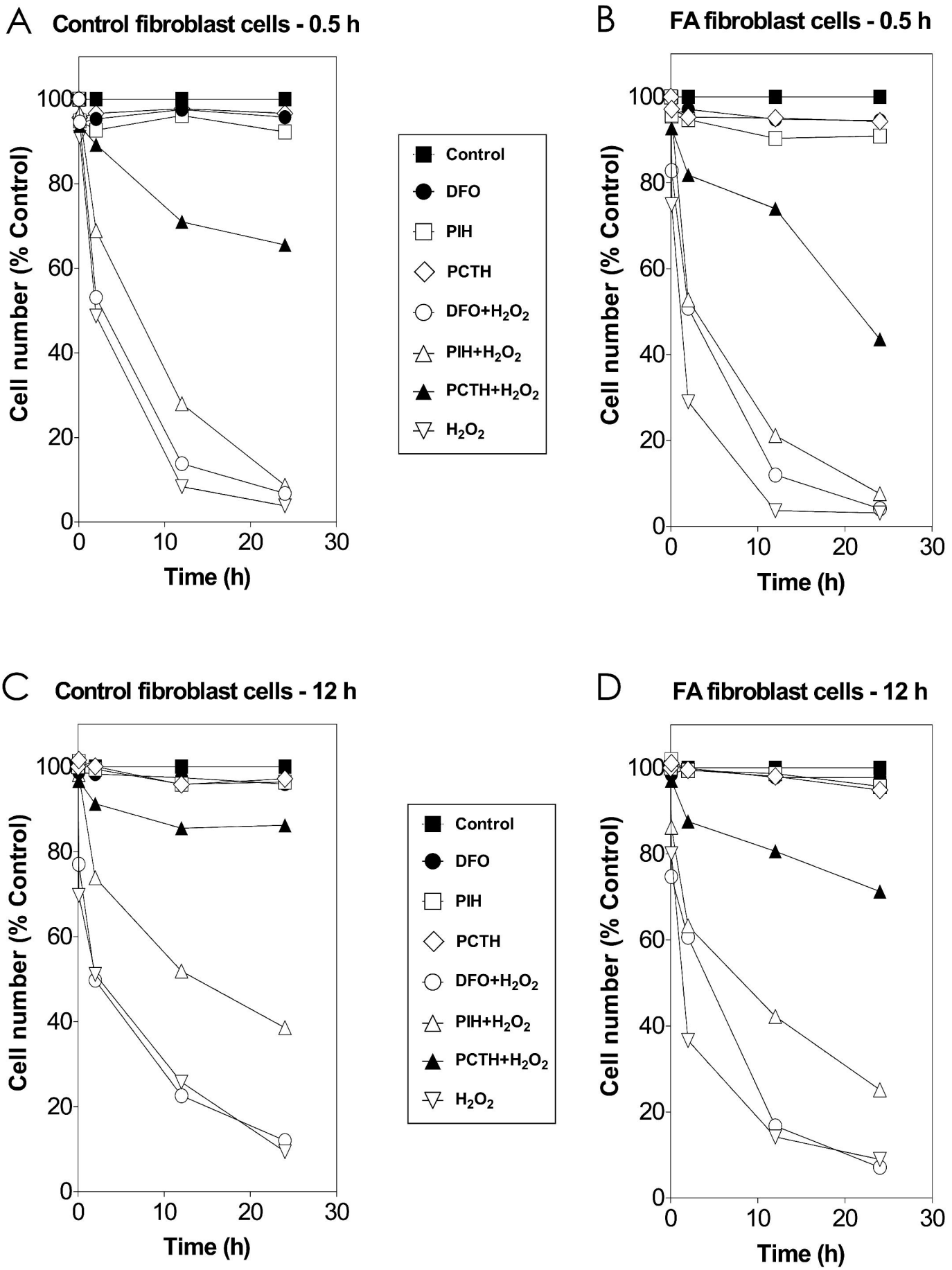



Figure 2

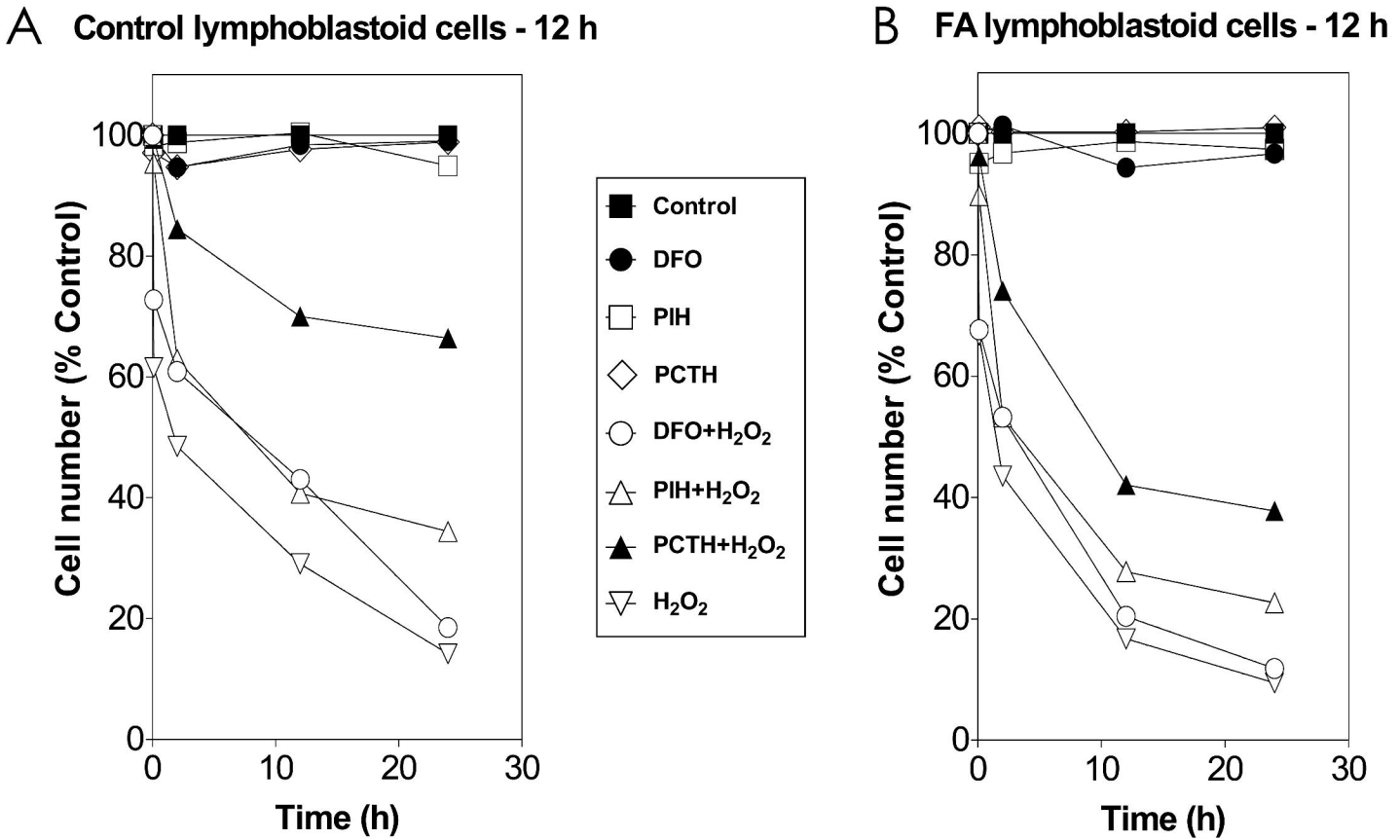
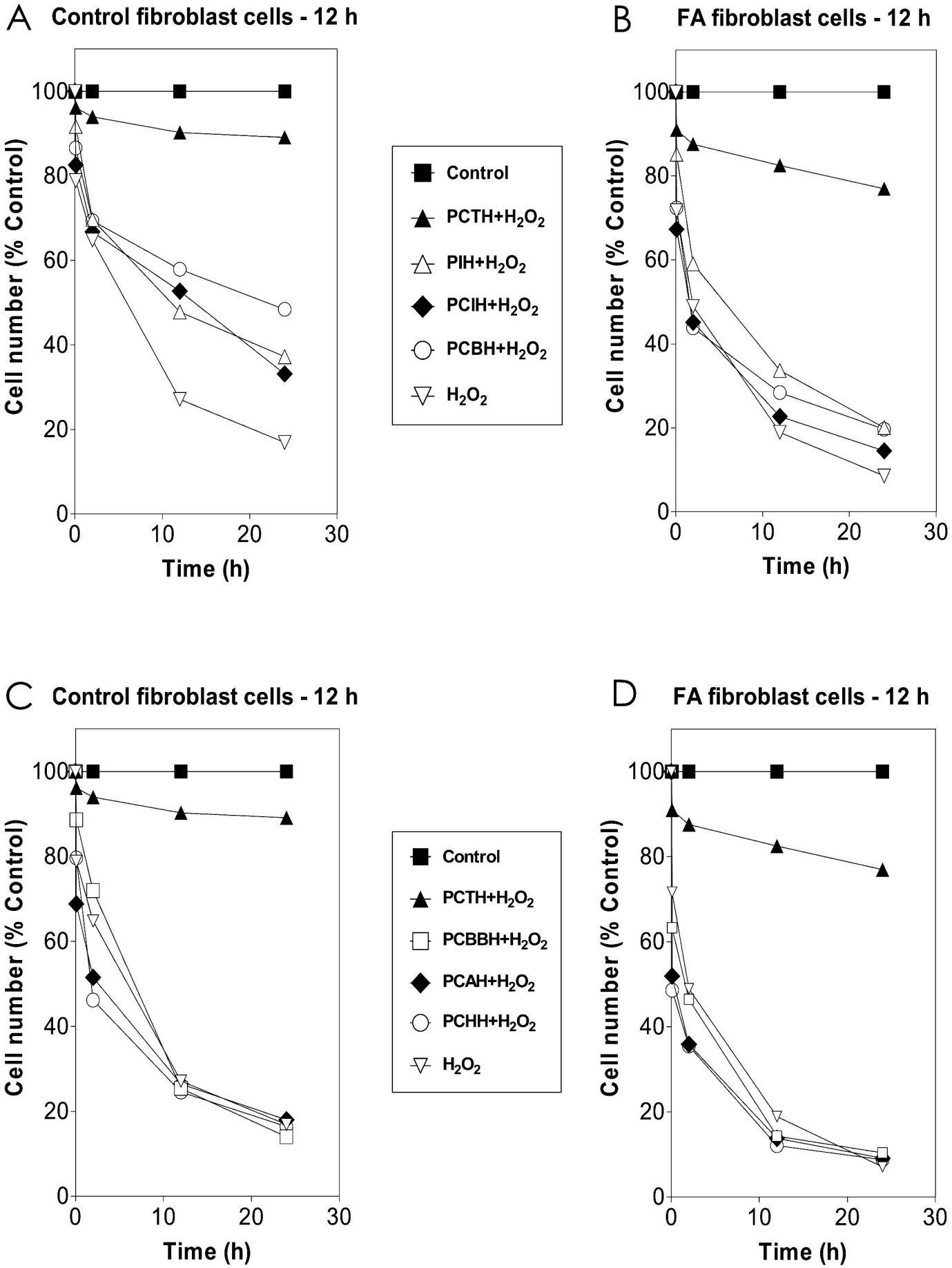

A
Control lymphoblastoid cells - 12 h
B
FA lymphoblastoid cells - 12 h
Cell number (% Control)
Time (h)
0
20
40
60
80
100
0
10
20
30
Control
DFO
PIH
PCTH
DFO+H2O2
PIH+H2O2
PCTH+H2O2
H2O2

**Figure 3**

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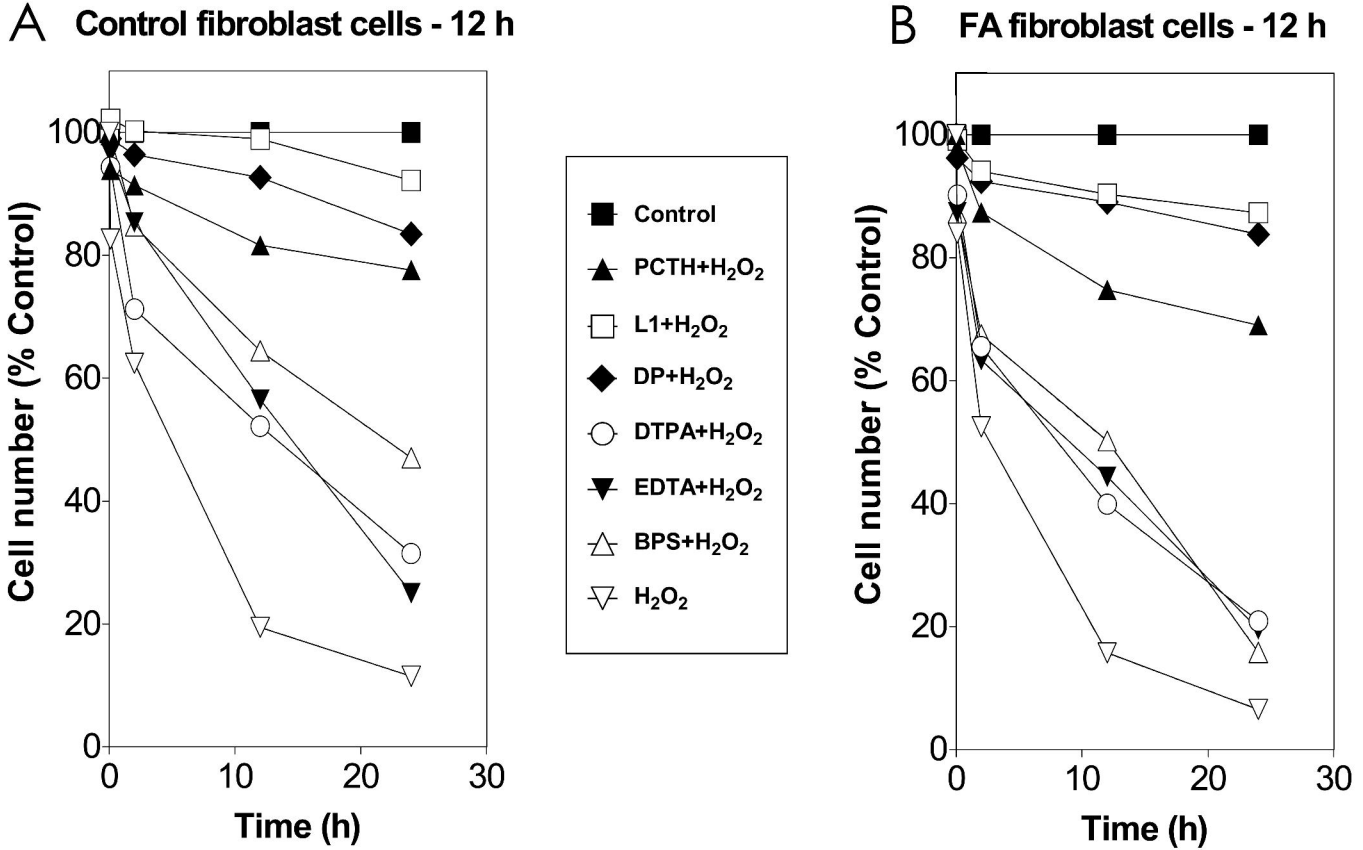
A
Control fibroblast cells - 12 h
Cell number (% Control)
Time (h)
0
20
40
60
80
100
0
10
20
30
Control
PCTH+H2O2
L1+H2O2
DP+H2O2
DTPA+H2O2
EDTA+H2O2
BPS+H2O2
H2O2
B
FA fibroblast cells - 12 h
Cell number (% Control)
Time (h)

Figure 5

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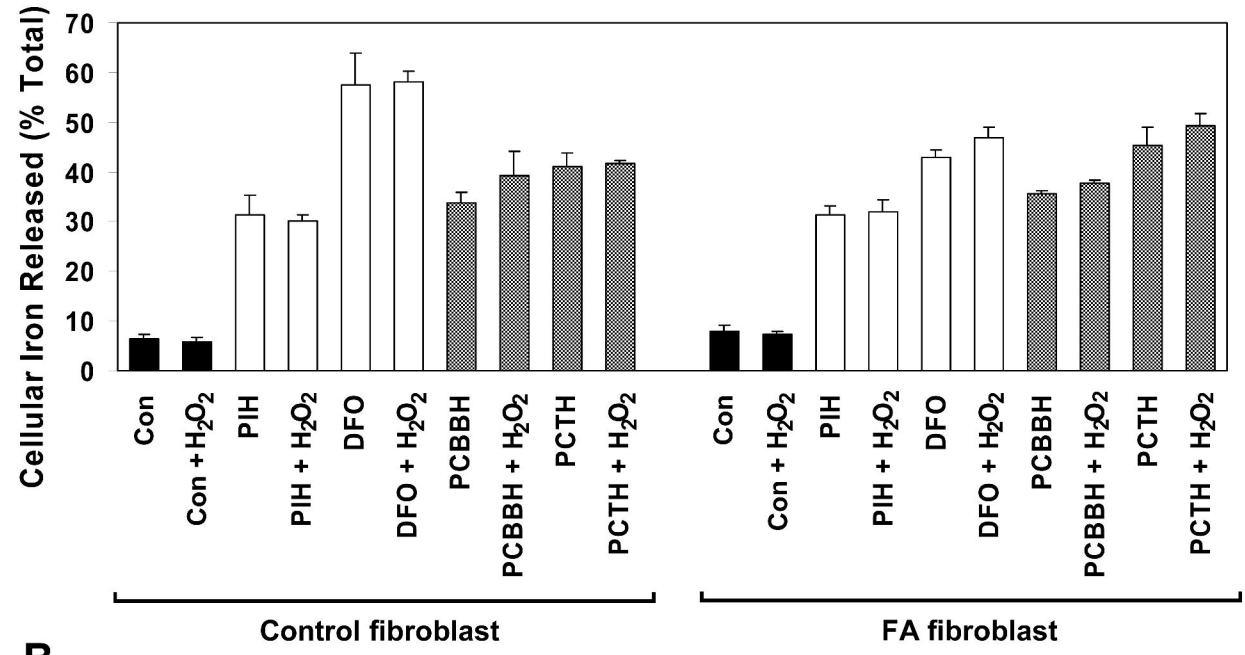


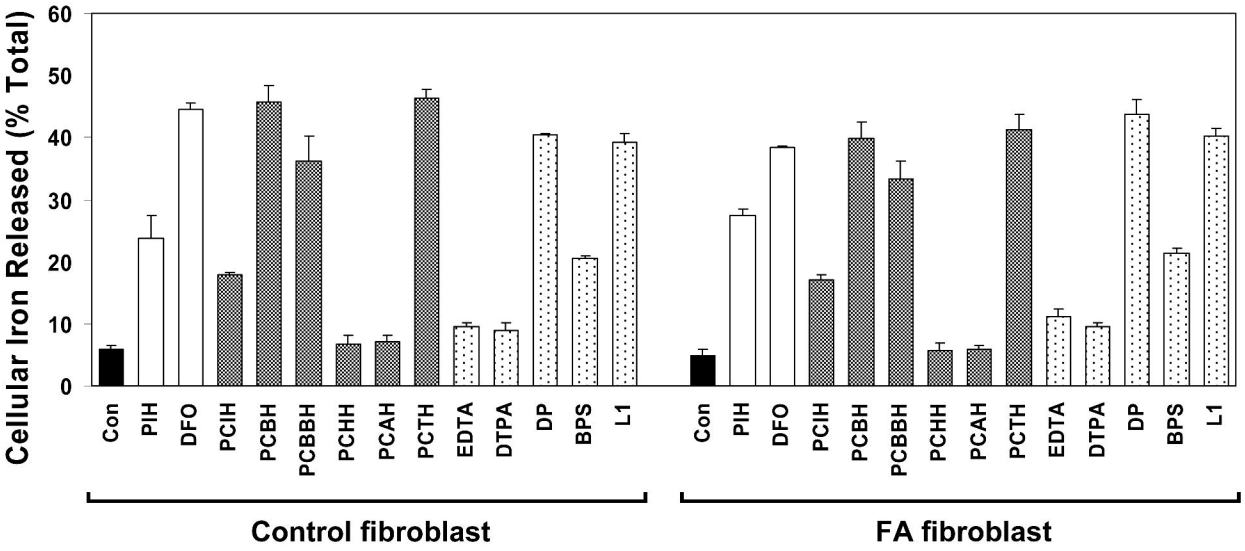


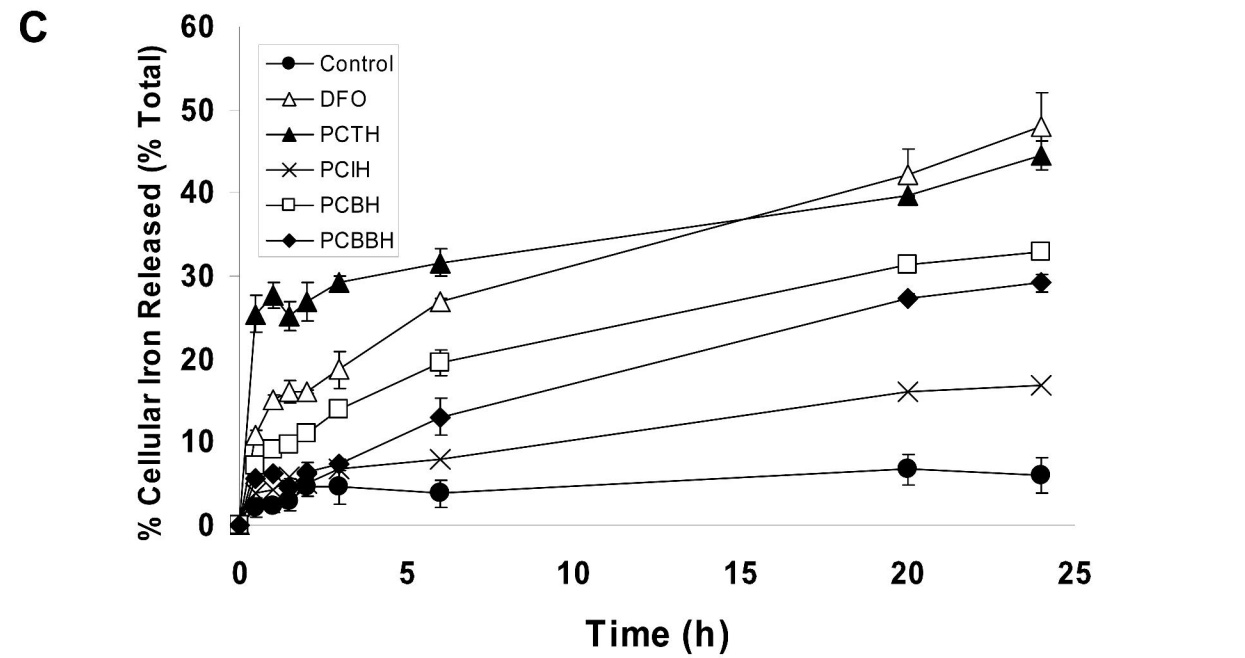


Figure 6

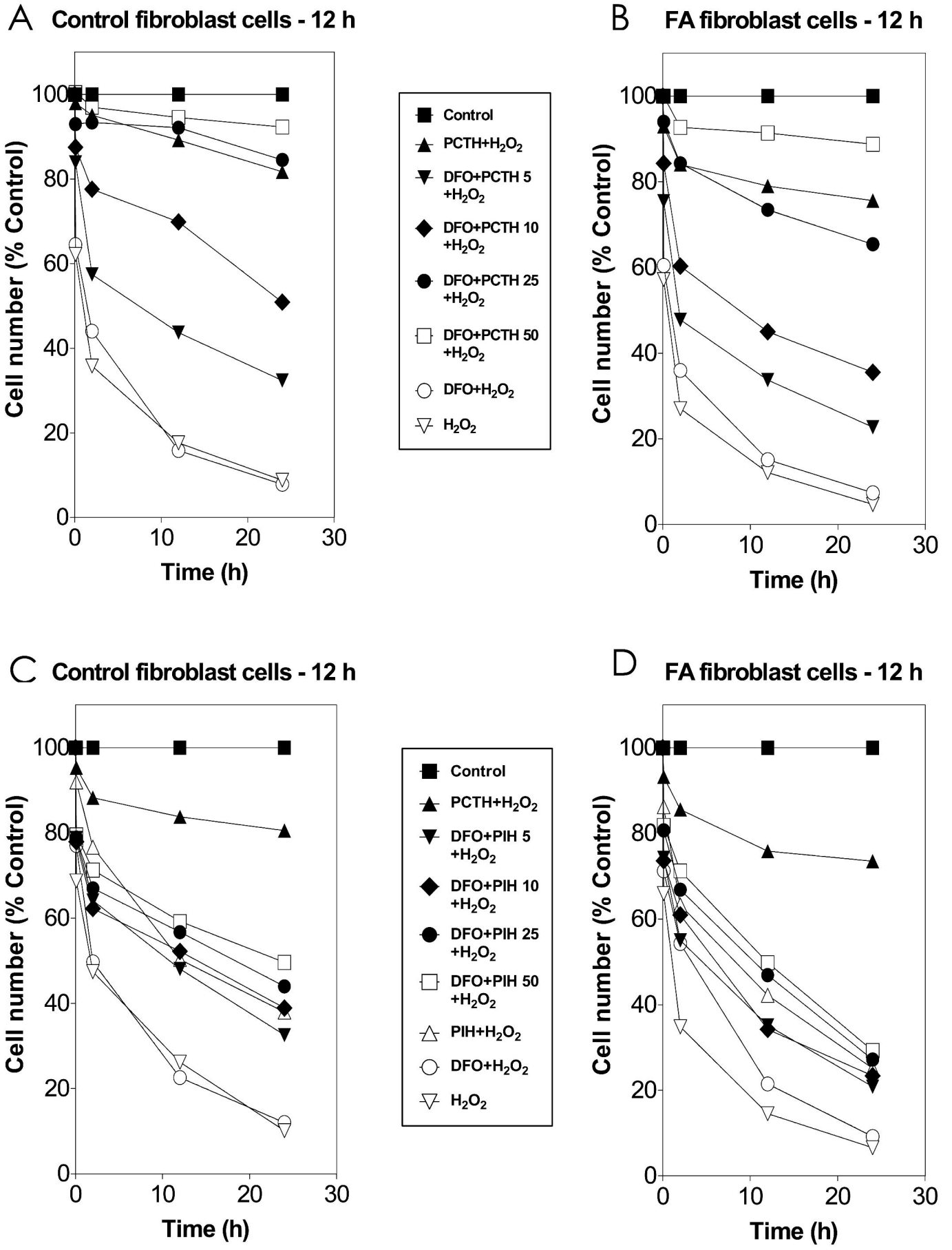


**Figure 7**

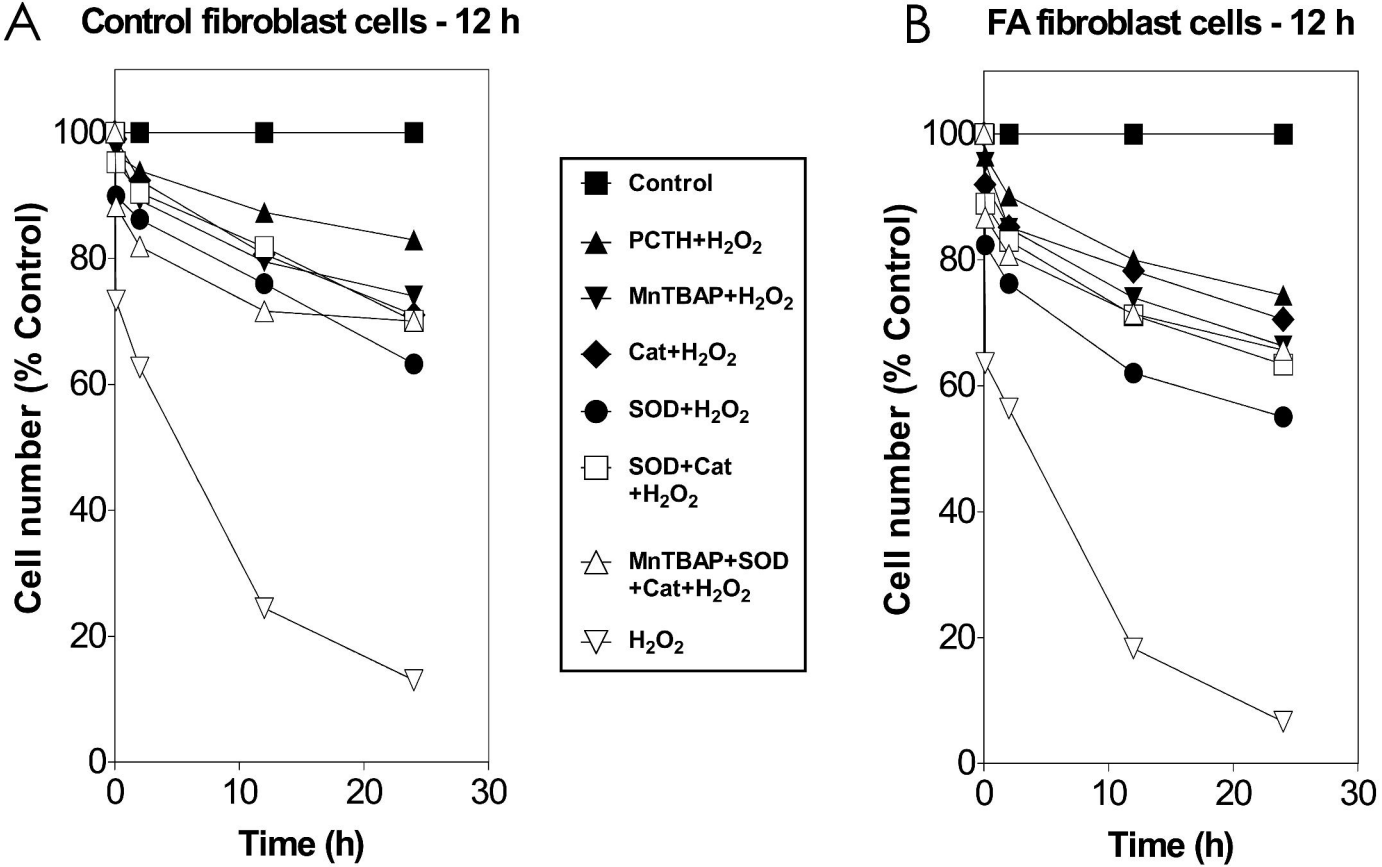
A
Control fibroblast cells - 12 h
B
FA fibroblast cells - 12 h
Cell number (% Control)
Time (h)
0
20
40
60
80
100
0
10
20
30
Control
PCTH+H2O2
MnTBAP+H2O2
Cat+H2O2
SOD+H2O2
SOD+Cat +H2O2
MnTBAP+SOD +Cat+H2O2
H2O2

**Figure 8**

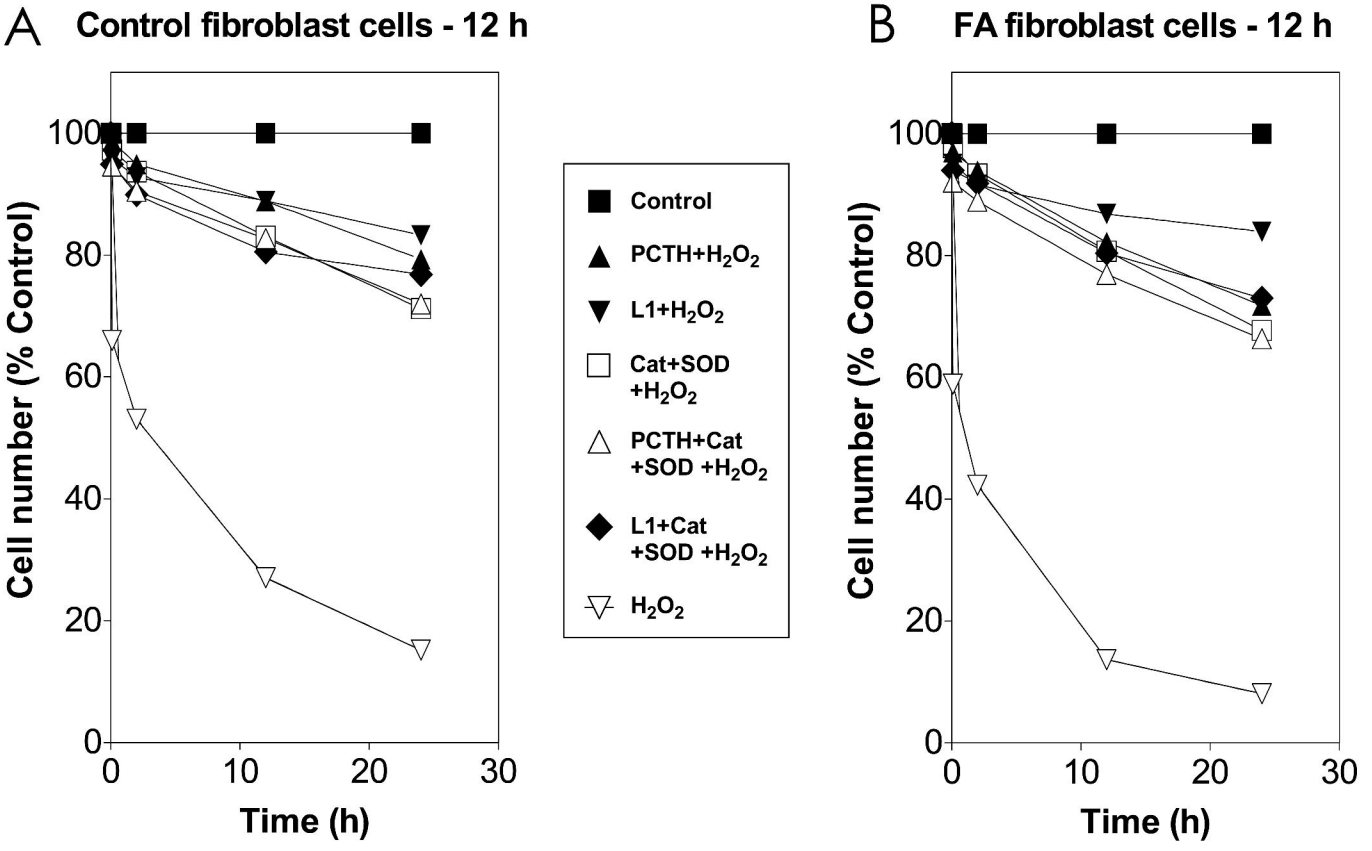
A
Control fibroblast cells - 12 h
B
FA fibroblast cells - 12 h
Cell number (% Control)
Time (h)
Control
PCTH+H2O2
L1+H2O2
Cat+SOD +H2O2
PCTH+Cat +SOD +H2O2
L1+Cat +SOD +H2O2
H2O2

Figure 9

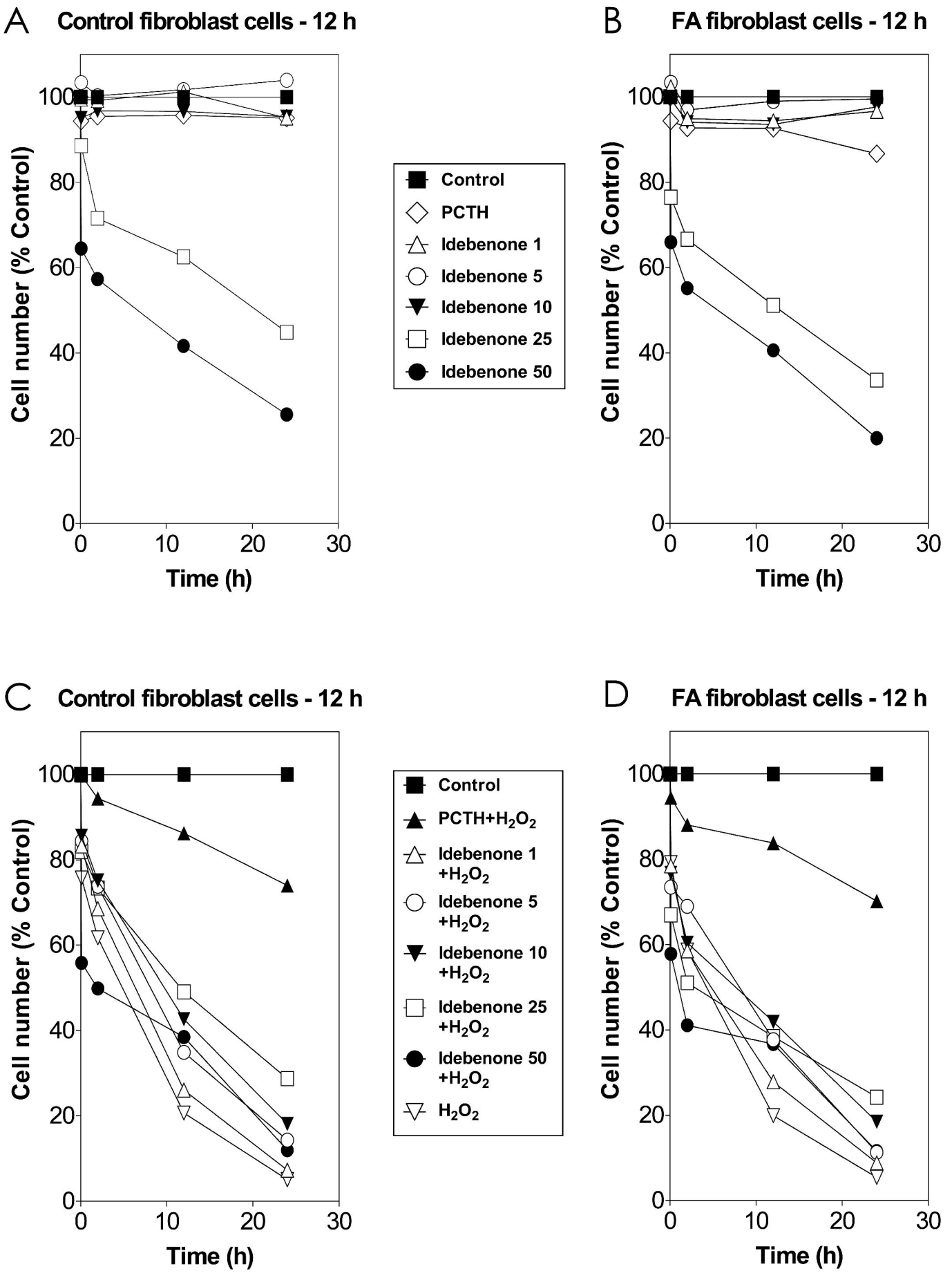


**Figure 10**