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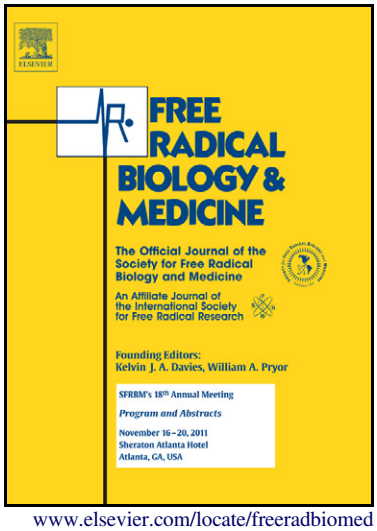

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Lipophilic adamantyl- or deferasirox-based conjugates of desferrioxamine B have enhanced neuroprotective capacity: Implications for Parkinson's disease

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Abstract

Parkinson's disease (PD) is a neurodegenerative disease characterized by death of dopaminergic neurons in the substantia nigra region of the brain. Iron content is also elevated in this region in PD, and is implicated in the pathobiology of the disease. Desferrioxamine B (DFOB) is a high affinity iron chelator and has shown efficacy in animal models of Parkinson's disease. The high water solubility of DFOB, however, attenuates its ability to enter the brain. In the current study, we have conjugated DFOB to derivatives of adamantane or the clinical iron chelator deferasirox to produce lipophilic compounds designed to increase the bioavailability of DFOB to brain cells. We found that the novel compounds are highly effective in preventing iron-mediated paraquat and hydrogen peroxide toxicity in neuronal-like BE2-M17 dopaminergic cells, primary neurons, and iron-loaded or glutathione-depleted primary astrocytes. The compounds also alleviated paraquat toxicity in BE2-M17 cells that express the PD-causing A30P mutation of α-synuclein. This protection was ~66-fold more potent than DFOB alone, and also more effective than other cell permeant metal chelators, clioquinol and phenanthroline. These results demonstrate that increasing the bioavailability of DFOB through the conjugation of lipophilic fragments greatly enhances its protective capacity. These novel compounds have potential as therapeutics for the treatment of PD and other conditions of Fe dyshomeostasis.

Keywords:

Iron, chelator, DFOB, Parkinson's disease, oxidative stress, paraquat, hydrogen peroxide

Abbreviations

BBB, blood-brain barrier; BSO, L-buthionine sulfoximine; CQ, clioquinol; DFOB, desferrioxamine B; FAC, ferric ammonium citrate; ICP-MS, inductively-coupled plasma mass spectrometry; MAO, monoamine oxidases; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PD, Parkinson's disease; PQ, paraquat; SN, substantia nigra

Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized pathologically by the loss of dopaminergic neurons in the substantia nigra (SN) region of the brain and the presence of α-synuclein-containing Lewy bodies [1]. Clinically, PD presents with resting tremor, slowness of initial movement, rigidity, and general postural instability. Additionally, PD also presents with varied non-motor deficits including sleep deficits and cognitive impairment [2].

A key pathological hallmark of PD is an accumulation of Fe in the SN, the extent of which seems to correlate with disease severity [3,4]. Iron is essential for normal cell function, and is required for many enzymes including that of dopamine synthesis, tyrosine hydroxylase [5]. However, excess redox-active Fe can catalyze the generation of highly reactive hydroxyl radicals from H_2O_2, which can damage membranes, proteins and DNA, causing oxidative stress and cell death [6]. Furthermore, Fe promotes α-synuclein aggregation [7] and has been detected in a redox-active form in Lewy bodies along with α-synuclein [8]. PD is also characterized by the loss of the antioxidant glutathione from the SN [9-11]. In addition, dopamine is degraded by monoamine oxidases (MAO) [12] and can autoxidise [13]. Both of these processes generate H_2O_2, potentially exacerbating the detrimental impact of Fe overload in the PD brain. Together, these factors render the SN highly susceptible to oxidative stress, which is implicated in the loss of SN dopaminergic neurons and the pathogenesis of PD [14,15].

Exposure to the common pesticide paraquat (PQ), may be a causative event in the pathogenesis of PD. Peripheral/systemic administration of PQ to rodents causes parkinsonism, kills SN dopaminergic neurons and increases brain ROS levels [16,17]. In humans, recent epidemiological studies indicate a strong association between long-term PQ exposure and PD [18,19]. PQ is a redox cycling agent which in the presence of reducing equivalents and oxygen, generates superoxide radicals, which in turn generate H_2O_2 [20]. This may be particularly relevant in regions of elevated Fe, such as the SN in PD.

In light of the central role of excess Fe in promoting dopaminergic neuron death, a potential therapeutic avenue for PD is to limit excess Fe by application of chelating

compounds. One such compound is desferrioxamine B (DFOB), a hexadentate, high affinity Fe^{3+} chelator ($\log\beta_{110}$ = 30.5) [21]. DFOB is protective in vivo in several pharmacological animal models of PD [22-26]. However, in all of these models, DFOB was administered directly into the brain, or peripherally in weanling mice without a fully functional blood-brain barrier (BBB). DFOB is highly water soluble and there is only limited evidence it can cross the BBB or enter cells. It is used clinically for conditions of Fe overload such as β-thalassemia and sickle cell aenemia [27-29]. DFOB has also been evaluated in a single clinical trial for the treatment of Alzheimer's disease and elicited a beneficial outcome [30]. Although this effect was ascribed to chelation of aluminium, the potential for effective chelation of brain Fe by DFOB remains a possibility [31]. Novel chelators have been developed with greater capacity to enter cells, and these have shown efficacy in animal models of PD [32-36]. Hence, Fe chelation therapy remains a potential avenue for the treatment of PD.

In the current study, we aimed to increase the bioavailability of DFOB through conjugation to actual or analogues of orally-available, lipophilic compounds in clinical use. We recently reported that relative to DFOB, selected candidates from this DFOB conjugate library were 2-3-fold more efficient at mobilizing intracellular Fe from SK-N-MC-neuroepithelioma cells, and that this activity was closely correlated with lipophilicity [37]. In the current study, we investigated the protective capacity of a subset of the novel DFOB conjugates in PD-relevant in vitro models of oxidative stress. We have directly compared the effectiveness of the novel compounds with DFOB, and to other relevant metal chelating compounds. We also assessed the ability of the compounds to mobilize cell-associated Fe. We found that the novel compounds were more effective at attenuating oxidative stress than other chelating compounds, and substantially more effective than DFOB. These results are likely to be due to the increased cell permeability of the novel compounds over DFOB.

Materials and methods

Materials

Acetonitrile (biotech grade), adamantane-1-acetic acid (98%), L-Buthionine sulfoximine (BSO), 5-chloro-7-iodo-8-quinolinol (clioquinol), desferrioxamine B·mesylate (DFOB, 95%), 3,5-dimethyladamantane-1-carboxylic acid (97%), *N*-(3-

dimethylaminopropyl)-*N*′-ethylcarbodiimide·HCl (EDC, protein sequence grade), dimethylformamide (DMF, biotech grade), ferric ammonium citrate (FAC), methyl viologen dichloride (PQ) and 1,10-phenanthroline were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). H_2O_2 was from ChemSupply (Gillman, SA, Australia). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Amresco. *N*-Hydroxybenzotriazole (HOBt) was obtained from Auspep (Parkville, VIC, Australia) and 4-[3,5-bis(2-hydroxyphenyl)-1,2-4-triazol-1-yl]benzoic acid (deferasirox, 98%) was obtained from AmplaChem (Carmel, IN) or Ontario Chemical Inc (Guelph, ON, Canada). *N*,*N*-Diisopropylethylamine (DIPEA, 99%) was purchased from Lancaster Synthesis, Inc. (Pelham, NH). All culture reagents were purchased from Gibco (Invitrogen; Mount Waverley, Victoria, Australia). All chemicals and solvents were used as received. Culture plates were obtained from Nunc (ThermoFisher; Scoresby, VIC, Australia) or Corning (InVitro Technologies; Noble Park, VIC, Australia).

Cell culture

BE2-M17 neuroblastoma cells stably transfected with empty vector (M17), human wild-type α-synuclein (WT-α-syn) or α-synuclein containing the PD-causing A30P point mutation (A30P-α-syn; from Prof. M. Farrer, Mayo Clinic, FL, USA) [38] were maintained in Optimem media supplemented with pyruvate, non-essential amino acids, penicillin/streptomycin and 10% fetal bovine serum (FBS) and selected with 5% G418 geneticin. Cells were passaged every 3-4 days and used between passage numbers 40-80. Cultures were used when cells had reached ~80-90% confluency.

Primary cortical neurons were harvested from C57BL6/J E14 mouse embryos and maintained in neurobasal media supplemented with glutamine, gentamycin, and 10% B27 as previously described [39]. Cells were used at 6-7 days in vitro, 24 h after renewal of media in which B27 was substituted for B27 without antioxidants.

Primary astrocyte cultures were harvested from the brains of newborn C57BL6/J mouse pups and maintained in DMEM supplemented with penicillin/streptomycin and 10% FBS as previously described [40]. Media was renewed every 7 days and cultures were used between 15-20 days in vitro, 24 h after media renewal.

Cells were plated in 48-well culture plates for toxicity analyses or 6-well plates or 10-cm culture dishes for ICP-MS metal analyses. Cultures were plated at the following densities: $2x10^4$ cells cm^{-2} (M17); $3x10^4$ cells cm^{-2} (WT-α-syn); $1.6x10^4$ cells cm^{-2} (A30P-α-syn); $16x10^4$ cells cm^{-2} (primary neurons); $16x10^4$ cells cm^{-2} (primary astrocytes).

Synthesis of novel DFOB conjugates

DFOB conjugates (compounds 1, 2 and 3; Fig. 1) were synthesized as previously described using an HOBt-based, EDC-activated coupling reaction [37]. The compounds were purified to >95% using preparative reverse-phase HPLC and were characterized by ^1H NMR spectroscopy and by electrospray ionisation mass spectrometry.

Experimental incubations

For toxicity studies, cultures were incubated with 300 μL treatment media (growth media without FBS (M17 cells, astrocytes) or B27 substituted for B27 without antioxidants (neurons)) containing the indicated concentrations of chelators prepared from 1000-fold stock solutions in DMSO. After 2 h, the chelator-containing media was aspirated and replaced with treatment media containing the indicated stressor (H_2O_2 or PQ) for a further 24 h, upon which time media was sampled for lactate dehydrogenase (LDH) activity and cell viability assessed via MTT assay.

In a series of experiments, astrocytes were pre-incubated for 24 h in 300 μL treatment media containing 1 mM BSO or for 2 h in 300 μL treatment media containing 100 μM FAC, before removal of this media and incubation with chelators as above.

For ICP-MS analyses, cultures were treated for 2 h with 100 μM FAC before the media was aspirated and replaced with media containing the indicated concentrations of chelators for a further 2 h. Cells were washed 3 times with ice-cold PBS before being collected and centrifuged at 720 g for 3 min and re-suspended in 1 mL PBS. The suspension was sampled for protein content via the bicinchoninic acid (BCA) protein assay (Pierce), with the remainder centrifuged at 15,000 g for 3 min and supernatant discarded. The pellet was used for ICP-MS analysis of metal content.

Inductively-coupled plasma mass spectrometry (ICP-MS) analysis of metal content

Cell pellets were dissolved in 30-50 µL 14.4 M nitric acid (Suprapur, Merck) overnight before heating at 90°C for 20 min, followed by the addition of 0.8-1 mL of 1% nitric acid. Media samples were diluted to 1 mL with 1% nitric acid. Samples were measured on an Agilent 7700 series ICP-MS instrument, as previously described [41].

Viability assays

Extracellular LDH activity was determined using the LDH Cytotoxicity Detection Kit (Roche; Castle Hill, NSW, Australia) according to the manufacturer's instructions. Values were standardized to cells lysed in treatment media containing 1% triton X-100. Activity of intracellular reductases was assessed by MTT reduction. Briefly, 20 µL of 12 mM MTT was added to the existing media and cells were incubated for 1-2 h before removal of the media and lysing the cells in DMSO. The increase in absorbance at 540 nm was standardized to identically-treated cells incubated in the absence of chelators or stressors.

Western blot analysis of protein expression

Cell lysates prepared in Phosphosafe Extraction Reagent (Merck; Kilsyth, Victoria, Australia) were mixed with electrophoresis SDS sample buffer and separated on 12% SDS-PAGE Tris-glycine gels. Proteins were transferred to PVDF membranes and blocked with 4% skim milk solution in PBST before immunoblotting for α-synuclein (Ab 97/8 raised to the C-terminal region amino acids 116-131; 1:1000) [42]. Secondary antiserum was rabbit-HRP (Cell Signaling Technology; 1:5,000). Blots were developed using GE Healthcare ECL Advance Chemiluminescence and imaged on a Fujifilm LAS3000 imager (Berthold, Bundoora, Australia). Expression of β-actin (Cell Signaling Technology; 1:3,000) was used as a protein loading control.

Data presentation and statistical analyses

Experiments were performed twice for cell lines and on 3 independently-prepared primary cultures with at least triplicate wells for each data point. Data are presented as means ± standard deviations of representative experiments (cell lines) or average of each experimental mean (primary cultures) with n=3 for all conditions.

Results

Novel DFOB conjugates as bioavailable iron chelators

The novel compounds were generated by conjugating DFOB with 3,5-dimethyladamantane-1-carboxylic acid (compound 1) or adamantane-1-acetic acid (compound 2) or deferasirox (compound 3; Fig 1 [37]). Several adamantyl-based compounds are used to treat Parkinson's disease (amantadine), Alzheimer's disease (memantine), and influenza A (amantadine, rimantadine; [43-46]). These compounds are orally active and are generally well tolerated by patients. Deferasirox is a tridentate Fe chelator developed as an alternative to DFOB for clinical use in conditions of Fe overload [47,48]. Orally available deferasirox is generally well tolerated by most patients and circumvents the arduous slow subcutaneous infusion administration required for DFOB [27].

These novel compounds were tested in addition to parallel tests with the isolated fragments, DFOB and deferasirox; and also to other known metal chelating agents, 1,10-phenanthroline and clioquinol (Fig 1). Phenanthroline and clioquinol (CQ) are bidentate chelators with broader metal ion specificity (Fe(II), Zn(II), Cu(II)) [49] than DFOB (Fe(III)). CQ is an 8-hydroxylquinoline derivative that has shown efficacy in clinical trials for Alzheimer's disease [50], and protects SN neurons from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity in mice by Fe chelation [33].

Novel chelators protect M17 cells from oxidative stress

Both H_2O_2 and PQ are known to promote Fe-mediated oxidative stress. Concentrations of both H_2O_2 and PQ sufficient to elicit a substantial loss of cell viability to M17 neuroblastoma cells were established as 1.5 mM and 4 mM, respectively (Supp. Fig 1). The protective capacity of the compounds was tested at a range of concentrations. The lowest concentrations to elicit substantial protection are shown in all figures. In the case of phenanthroline or CQ, the most protective concentrations tested are shown, even though these concentrations sometimes induced mild toxicity in the absence of stressors. Higher concentrations of phenanthroline or CQ caused substantial toxicity. Deferasirox was tested at equivalent concentrations to compound 3.

In the absence of stressors, 100 μM phenanthroline and 20 μM CQ were mildly toxic to M17 neuronal-like cells (Fig 2A). The novel compounds at 100 μM (2) or 50 μM (1, 3); and DFOB at 100 μM were well tolerated, while 3,300 μM DFOB slightly decreased MTT reduction (Fig 2A). In the absence of chelators, 1.5 mM H_2O_2 was highly toxic, with 70% of total LDH activity found extracellularly and reduction of MTT almost completely inhibited (Fig 2B). When treated with H_2O_2, 100 μM DFOB had no effect, while equivalent concentrations of compounds 1-3 and a much higher concentration of DFOB (3,300 μM) almost completely abrogated the toxicity of H_2O_2. In contrast, phenanthroline, CQ and isolated deferasirox had no effect on H_2O_2-induced toxicity (Fig 2B). The lack of protection given by deferasirox alone was in stark contrast to the best-performing DFOB-deferasirox conjugate (compound 3). This demonstrates that the properties of deferasirox have been significantly altered through conjugation to DFOB.

In the absence of chelators, PQ induced a small increase in LDH release, but substantially decreased MTT reduction (Fig 2C). While 100 μM DFOB had no effect on PQ-induced toxicity, all other compounds significantly alleviated PQ-induced toxicity (Fig 2C). However, phenanthroline and CQ were not as effective as the novel compounds. Lower concentrations of phenanthroline and CQ averted their toxicity in the absence of PQ, but were not protective in the presence of PQ (data not shown).

Novel chelators protect M17 cells transfected with A30P-α-synuclein from paraquat

To assess the protective capacity of the novel compounds in a PD-relevant model, M17 cells stably transfected with the PD-associated A30P mutant form of human α-synuclein (A30P-α-syn) were used. This form of α-synuclein causes familial PD and is implicated in the pathogenesis of the disease [51]. When compared to M17 cells transfected with the empty vector or wild-type human α-synuclein (WT-α-syn), A30P-α-syn cells were rendered highly vulnerable to PQ toxicity. PQ caused substantial toxicity in A30P-α-syn cells at concentrations which resulted in only mild toxicity in vector control cells (Supp. Fig 2). This enhanced susceptibility was not due to overexpression of α-synuclein alone, as M17 cells transfected with wild-type α-syn were as sensitive as control cells with endogenous levels of α-synuclein (Supp. Fig 2), even though both WT-α-syn and A30P-α-syn cells had similar levels of α-synuclein,

and much higher expression than control cells (Supp. Fig 3). This indicates that the A30P mutant form of α-synuclein specifically enhances sensitivity to PQ.

In the absence of stressors, phenanthroline and CQ induced mild toxicity in A30P-α-syn cells, while all other compounds were well tolerated (Fig 3A), similar to M17 cells with normal levels of α-syn (Fig. 2A). To confirm the increased sensitivity of A30P-α-syn cells to PQ, these cells were exposed to PQ at the same dose as applied to normal M17 cells (4 mM). At this dose, PQ killed most A30P-α-syn cells, and no compounds were able to substantially abrogate PQ toxicity (Fig 3C). However, PQ applied at a 2.5-fold lower concentration (1.6 mM; Fig 3B) induced a similar level of toxicity in A30P-α-syn cells as did 4 mM PQ in normal M17 cells. At this dose of PQ (1.6 mM), compounds 1-3, deferasirox and phenanthroline significantly improved viability, while an equivalent concentration of DFOB had no effect. Compound 3 almost completely abolished PQ toxicity and was substantially more effective than the isolated fragment deferasirox, phenanthroline, and compounds 1 and 2. DFOB gave a similar result when applied at a 66-fold higher dose than compound 3, while CQ exacerbated PQ toxicity in A30P-α-syn cells (Fig 3B).

Primary neurons are protected by novel chelators

To verify that the novel compounds were not only protective in cell lines, primary mouse cortical neurons were prepared. In the absence of stressors, phenanthroline and CQ were highly toxic when applied at 100 μM and 20 μM, respectively (data not shown). Accordingly, lower concentrations were applied to cortical neurons. Phenanthroline still exhibited limited toxicity at 50 μM, but all other compounds were well tolerated (Fig 4A). Primary neurons were far more vulnerable to oxidative stress than M17 cells, as only 100 μM H_2O_2 induced ~90% loss of MTT reduction and 60% increase in release of cellular LDH (Fig 4B). The novel compounds strongly abrogated H_2O_2-induced toxicity, as did a considerably higher concentration of DFOB (3,300 μM). However deferasirox, phenanthroline and CQ were completely ineffective in preventing H_2O_2-induced toxicity in primary neurons (Fig 4B).

Novel chelators alleviate oxidative stress in iron-loaded cells

A key feature of PD is an increased burden of Fe in the SN [4]. To recapitulate these conditions, the protective capacity of the novel compounds was next investigated in

Fe-loaded cells. When incubated for 2 h with 100 μM FAC, M17 cells did not strongly accumulate Fe from the media (Supp. Fig 4). In contrast, primary mouse astrocytes readily accumulate Fe in the form of FAC [52]. Accordingly, the cellular Fe content of primary mouse astrocytes treated for 2 h with 100 μM FAC increased ~20-fold (Supp. Fig 4), indicating strong accumulation of Fe from the media. Furthermore, evidence suggests that the increase in SN Fe content may be localized to astrocytes [3,53]. Hence, the protective capacity of the compounds under conditions of excess Fe was examined in Fe-loaded primary mouse astrocytes. The accumulation of Fe observed in the current study was not in itself toxic (Fig 5A). However, it greatly exacerbated the toxicity of H_2O_2 and PQ, such that 1 mM H_2O_2 or 2 mM PQ was significantly more toxic in Fe-loaded cells than in cells with normal Fe content (Supp. Fig 5). That exposure to 1 mM H_2O_2 or 2 mM PQ was not substantially toxic in the absence of excess cellular Fe indicates that the resultant cell death is contingent upon the increased cellular Fe content.

In Fe-loaded astrocytes treated in the absence of additional stressors, CQ was mildly toxic, while all other compounds were well tolerated (Fig 5A). When exposed to 2 mM PQ following FAC treatment, MTT reduction was decreased by 80% while LDH release increased to 40% (Fig 5B). 100 μM DFOB was ineffective in rescuing cells, while equivalent concentrations of compounds 1-3 significantly improved astrocyte survival. DFOB applied at 3,300 μM was also effective, as was 100 μM phenanthroline. In contrast, deferasirox and CQ did not improve cell survival; and as similarly observed in A30P-α-syn cells, CQ exacerbated PQ toxicity.

Exposure to 1 mM H_2O_2 following FAC treatment was highly toxic, with MTT reduction almost completely inhibited, and almost 80% of cellular LDH released into the medium (Fig 5C). 100 μM DFOB did not alter H_2O_2-induced toxicity. In contrast, a lower concentration (50 μM) of compounds 1 and 3 significantly rescued astrocytes, as did compound 2 at a slightly elevated concentration (200 μM). DFOB exhibited a similar protective capacity at a 66-fold greater concentration (3,300 μM). In contrast, deferasirox, phenanthroline and CQ were ineffective at preventing H_2O_2-induced toxicity in Fe-loaded cells.

Novel chelators protect cells depleted of glutathione from oxidative stress

A pathological hallmark of PD is the loss of glutathione from the SN, and evidence suggests that glutathione may be selectively lost from astrocytes [9,54]. Along with increased Fe content, this loss of glutathione is implicated in the pathogenesis of PD [14,15]. Cultured astrocytes depleted of glutathione are highly sensitive to elevated Fe content (unpublished results), hence glutathione is required to prevent Fe-mediated toxicity. To assess the protective capacity of the compounds in astrocytes depleted of glutathione, astrocytes were treated with the glutathione synthesis inhibitor BSO. After 24 h, 1 mM BSO decreased cellular glutathione content by 90% [55]. Such depletion of glutathione alone is not toxic, but greatly potentiates the toxicity of H_2O_2 [55]. A normally non-toxic dose of H_2O_2 (200 μM; Supp. Fig 5) induced severe toxicity in astrocytes depleted of glutathione (Fig 5D). As opposed to the models described above, 100 μM DFOB partially rescued astrocytes from this toxicity, while concentrations of compounds 1-3 as used above (50-200 μM) almost completely prevented H_2O_2-induced toxicity (data not shown). Accordingly, lower concentrations of the compounds were tested, and compounds 1-3 were found to be highly effective at 5, 50, and 5 μM, respectively (Fig 5D). DFOB at 330 μM matched this protection. Although more effective than in the previous models, this concentration of DFOB remains 66-fold higher than compounds 1 and 3. Deferasirox at a similar concentration (10 μM) to the deferasirox-DFOB conjugate compound 3 had no effect, nor did CQ. Phenanthroline was effective at an elevated concentration (200 μM).

Mobilization of cell-associated iron by novel chelators

The protective capacity of the chelators is likely due to sequestering cellular Fe, thus preventing Fe-mediated radical generation. To examine this, the Fe content of FAC-treated astrocytes was investigated. FAC treatment increased cell-associated Fe content by ~20-fold (Fig 6). A further 2 h incubation in the absence of extracellular Fe did not significantly alter Fe content, indicating that the Fe from FAC remained associated with astrocytes for at least 2 h. In the presence of any chelator, the amount of cell-associated Fe significantly decreased. 100 μM DFOB substantially decreased the amount of cell-associated Fe, to a similar extent to compounds 2 and 3, deferasirox and phenanthroline. Compound 1 and a higher dose of DFOB decreased cell-associated Fe levels to a slightly greater degree than the other treatments.

Discussion

DFOB is a high affinity Fe(III) chelator [21], and is effective at decreasing Fe-mediated oxidative stress, exhibiting efficacy in in vivo models of PD [22-26]. However, DFOB was only effective in these studies when applied directly into the brain, and is much less effective when administered peripherally, highlighting the poor propensity of DFOB to cross the BBB and enter the brain. This is likely due to its short plasma half-life (~15 min) [56], high water solubility and poor capacity of DFOB to pass through membranes [57]. The present study attempted to increase the bioavailability of DFOB by conjugating it to lipophilic, orally available compounds in clinical use. The protective capacity of these novel DFOB conjugates was assessed in multiple PD-relevant models of oxidative stress. The novel compounds were consistently found to be substantially more effective than DFOB, and also outperformed other PD-relevant metal-chelating compounds.

In the present study, we found that the novel chelators were more effective than DFOB, phenanthroline and CQ at preventing PQ toxicity in neuronal-like M17 cells, M17 cells transfected with mutated α-synuclein and Fe-loaded astrocytes. These results suggest that the toxicity of PQ observed in vitro in the current study is mediated by redox-active Fe. As PQ generates superoxide radicals, which in turn form H_2O_2 [20], the observed toxicity of PQ is consistent with Fe catalyzing the generation of hydroxyl radicals from PQ-produced H_2O_2. Given that there is increasing evidence that PQ exposure may be a causative event in the pathogenesis of PD [18,19], protecting cells from PQ toxicity demonstrates that the novel compounds have potential as useful therapeutics.

H_2O_2 itself was investigated as it is involved in multiple PD-relevant stress pathways. H_2O_2-derived radicals are implicated in the cell death caused by many PD-related neurotoxins, including MPTP and rotenone [58,59]. Additionally, it is a primary downstream product of PQ cycling in cells. Furthermore, H_2O_2 can be generated by MAO during dopamine turnover [12] and by the autoxidation of dopamine [13]. Therefore, a key therapeutic aim for PD treatment is to prevent H_2O_2-induced toxicity. Chelation of Fe by DFOB effectively prevents H_2O_2 toxicity in vitro [55,60,61]. In the current study, the novel DFOB conjugates prevented H_2O_2 toxicity more effectively than DFOB, phenathroline, CQ and deferasirox in multiple cell

models. As determined from the MTT assay, compound 3 (50 μM) demonstrated a close-to-complete rescue of M17 neuronal-like cells under 1.5 mM H_2O_2 insult. This was significantly greater than the activity of the isolated components (DFOB or deferasirox) at comparable concentrations (Fig. 2B), which demonstrates that the properties of the conjugate do not derive merely from a sum of its parts. Although highly effective in the M17 neuroblastoma cell line, it was important to confirm this protection in primary neurons, which are exquisitely sensitive to H_2O_2 toxicity. That protection was as effective in primary neurons as in M17 cells demonstrates that the compounds can protect authentic neurons. Furthermore, PD brain has an increased burden of Fe in the SN [4]. Hence, chelators will only have therapeutic potential if they can effectively alleviate toxicity in cells that recapitulate this core feature of PD. The novel DFOB conjugates effectively alleviated toxicity in Fe-loaded astrocytes, indicating that the compounds can effectively sequester sufficient cellular Fe to prevent the toxicity of H_2O_2 and PQ.

The A30P mutation of α-synuclein causes familial PD [62]. In culture, cells expressing mutant α-synuclein, including the A30P mutant, are more vulnerable to PD-related stressors including H_2O_2, Fe, and dopamine [63-68]. In line with these findings, we found cells transfected with A30P α-synuclein were substantially more vulnerable to PQ toxicity. This sensitization was specific for the mutant form of α-synuclein, as cells overexpressing wild-type α-synuclein were no more vulnerable to PQ than cells with normal α-synuclein expression levels. The novel chelators (particularly compound 3) were more effective than all other chelators tested. These results demonstrate that mutated α-synuclein increases the susceptibility of neuronal-like cells to the PD-related neurotoxin PQ, likely to involve α-synuclein-mediated mitochondrial dysfunction [69]. These results suggest that the toxicity of PQ in the presence of mutated α-synuclein is mediated by redox-active Fe, and can be alleviated by Fe chelation.

Another pathological feature of PD is the loss of glutathione from the SN [14,15], particularly from astrocytes [9,54]. Depletion of cellular glutathione greatly sensitized astrocytes to H_2O_2 toxicity. The novel compounds were extremely effective in protecting cells depleted of glutathione from H_2O_2 toxicity, with significant alleviation of toxicity evident at much lower concentrations than in any other model

examined in the current study. This suggests that the compounds may have enhanced efficacy in tissues with impaired antioxidative capacity, such as the SN of PD patients. Although the reasons for this remain unclear, under conditions of diminished antioxidative capacity (i.e. depleted glutathione content), the Fe(III)/(II) speciation, compartmentalization and equilibria, may have been modulated in a fashion that required less Fe(III)-specific chelator for rescue.

Although phenanthroline and CQ are cell permeant, they provided less effective protection than the novel DFOB-based compounds. This may be due to the different binding characteristics of these compounds. DFOB is highly specific towards Fe(III) ($\log K_a$ 31) and the $[Fe(DFOB(3-))]^{+/0}$ redox potential ($E_{½}$ -0.48 V vs NHE at pH 7.5) [70,71] indicates that the one-electron reduction of $[Fe(DFOB(\tilde{3})]^{+}$ to corresponding Fenton-reactive Fe(II) species will not occur within the biological redox window. This property confers significant value upon DFOB-based Fe chelation. Bidentate 1,10-phenanthroline is an Fe(II) specific chelator ($\log\beta_3$ 16.5). The molecular skeleton of bidentate CQ, 8-hydroxyquinoline, is more specific towards Fe(III) ($\log\beta_3$ 38) than Fe(II) ($\log\beta_3$ 22.1) and the redox potential of $[Fe(8\text{-hydroxyquinoline})_3]^{3+/2+}$ (E_f -0.25 V) [72] indicates the ability to move between Fe(III) and Fe(II) species. Compared to the DFOB conjugates, the lower protection towards oxidative insult offered by phenanthroline and CQ is likely due at least in part to the incomplete sequestration of redox-active Fe(II). Further, incomplete binding of Fe by CQ or phenanthroline may increase the solubility of Fe and increase the likelihood of damaging reactions [73]. Such reactions may explain the increased toxicity observed for CQ in the presence of PQ. Finally, both CQ and phenanthroline are potent Cu chaperones, shuttling extracellular Cu into cells [74]. Although this effect is associated with positive amyloid β-altering effects [74], increased cellular Cu in the current models may be exacerbating the toxicity of the stressors examined.

In the current study, following loading of cells with Fe, all chelating compounds decreased the amount of cell-associated Fe to a similar extent, which appeared not to correlate with the differential protective capacity of the compounds. The protective capacity is likely due to effects beyond simply coordinating Fe from the bulk sample, and would be expected to be tuned by compound-specific characteristics, including the ability to enter and exit cells, the nature of accessible Fe pools and Fe:compound

binding ratios. Additionally, glial cells are known to readily release accumulated Fe [75], and it may be that a significant proportion of the Fe bound by the chelators may be adventitiously bound in the extracellular compartment. The exact nature of Fe-binding by the chelators is beyond the scope of the currrent study and would require detailed analysis to fully elucidate the Fe pools responsible for mediating toxicity and the differential access of the compounds to these pools.

The novel complexes are likely to attenuate Fe-mediated free radial generation through Fe chelation. The superiority of the novel compounds over DFOB is most likely a result of the increased partition coefficients, which manifest as an improved carriage across the cell membrane. In addition, evidence suggests Fe chelation can also stabilize hypoxia-inducible factor-1α (HIF-1α), leading to the upregulation of several downstream neuroprotective signaling pathways [35,76,77], an effect which prevents the toxicity of MPTP [78]. DFOB and the novel chelators may be acting via these mechanisms. Other antioxidant properties of DFOB that are independent of its ability to sequester Fe(III) include its acting as a substrate for peroxidase; and as a radical scavenger, to generate relatively non-toxic hydroxamate-derived nitroxide radicals [79,80]. Furthermore, compounds 1 and 2 may have actions attributable to the adamantane domain, which is structurally similar to clinically approved compounds amantadine, memantine and rimantadine. Amantadine is in clinical use to treat PD and is thought to antagonize glutamatergic neurotransmission [44]. The possibility of such additional actions cannot be excluded in the current study.

Of the conjugates between DFOB and adamantyl-based fragments, compound 1 was more effective than 2 in all models tested. These results likely reflect the greater capacity of compound 1 to enter cells. The log*P* value of compound 1 (2.36 ± 0.50) is ideal for passing through membranes, while compound 2 is more water soluble (log*P* 1.65 ± 0.49; [37]). Compound 3 (log*P* 3.12 ± 1.00) is a conjugate of DFOB and the clinically approved Fe chelator deferasirox. This novel compound was more effective in abrogating oxidative stress than deferasirox or DFOB alone, and was more effective than compound 2. Its superiority above compound 2 may be due to the extra Fe-binding domain provided by the deferasirox moiety. While the molecular weight of compound 3 when fully Fe(III) saturated (M_r 1989.6 g mol^{-1}) would likely impede

its exit from the cell, *in vitro* studies have indicated the presence of at least two sub-stoichiometric Fe(III)-loaded forms of this compound, which would have variable distribution properties. It remains possible that Fe bound to the deferasirox motif of compound 3 in a coordinatively unsaturated fashion could result in increased redox-active Fe, potentially increasing the generation of radicals.

In conclusion, while DFOB is a potent Fe chelator, its high water-solubility renders it unable to easily cross cell membranes and it remains poorly bioavailable. We have increased the bioavailability of DFOB by conjugating it to analogues of orally available, clinically approved adamantyl-based compounds or deferasirox. The top selling 25 CNS-active drugs have a mean calculated log*P* value, clog*P* = 2.8, with a preferred range of clog*P* = 2-4 [81]. The experimental log*P* values of compound 1 and 3 lie within this boundary, while for compound 2, the value lies just outside the lower limit. While the ability to cross the BBB is derived from many factors, log*P* remains a simple measure used in drug design to predict for this property. These novel compounds protected cultured cells from a variety of PD-relevant, Fe-mediated insults. The novel DFOB conjugates are significantly more effective than DFOB, and other cell-permeant metal chelators phenanthroline and CQ. These compounds protect neuronal-like cell lines, cells overexpressing the PD-related A30P mutant form of α-synuclein, primary neurons, and primary astrocytes loaded with Fe or depleted of cellular glutathione. Together these results indicate the potential of these DFOB conjugates as therapeutics for PD and related conditions involving Fe overload or Fe-mediated stress.

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Figure Legends

Figure 1. Structure of chelator compounds studied in this work. Desferrioxamine B (DFOB) and deferasirox (4-[3,5-bis(2-hydroxyphenyl)-1,2-4-triazol-1-yl]benzoic acid) are in clinical use for treating transfusion-dependent secondary iron overload. Standard amide coupling procedures were used to prepare DFOB-*N*-(3,5-dimethyladamant-1-yl)carboxamide (1), DFOB-*N*-(adamantane-1-acetyl)carboxamide (2) or DFOB-*N*-[4-(3,5-bis(2-hydroxyphenyl)-1,2-4-triazol-1-yl)benzyl]carboxamide (3). Common chelators 1,10-phenanthroline and clioquinol (CQ) were also examined.

Figure 2. Chelators protect M17 neuronal-like cells from oxidative stress. Confluent M17 cells were incubated for 2 h with the indicated concentrations of chelators (in μM) before being incubated for 24 h in the absence (A) or presence of either H_2O_2 (1.5 mM; B) or paraquat (4 mM; C). Cells were assayed for viability and toxicity via MTT (top panels) and LDH assays (bottom panels), respectively. * $P<0.05$, ** $P<0.01$ as compared to relevant control (No chelator for DFOB, deferasirox, phenanthroline and clioquinol; DFOB (100 μM) for compounds 1-3).

Figure 3. Chelators protect A30P-α-synuclein transfected M17 cells from oxidative stress. Confluent M17 cells stably transfected with the mutant A30P form of α-synuclein were incubated for 2 h with the indicated concentrations of chelators (in μM) before being incubated for 24 h in the absence (A) or presence of 1.6mM

paraquat (B) or 4 mM paraquat (C). Cells were assayed for viability and toxicity via MTT (top panels) and LDH assays (bottom panels), respectively. * P<0.05, ** P<0.01 as compared to relevant control (No chelator for DFOB, deferasirox, phenanthroline and clioquinol; DFOB (100 μM) for compounds 1-3).

Figure 4. Chelators protect primary cultured neurons from oxidative stress. Primary cortical neurons were incubated for 2 h with the indicated concentrations of chelators (in μM) before being incubated for 24 h in the absence (A) or presence of H_2O_2 (100 μM; B). Cells were assayed for viability and toxicity via MTT (top panels) and LDH assays (bottom panels), respectively. ** P<0.01 as compared to control (No chelator).

Figure 5. Chelators protect compromised primary astrocytes from oxidative stress. Primary cultured astrocytes were incubated for 2 h with 100 μM FAC or for 24 h with 1 mM BSO before the media was completely aspirated and replaced with media containing the indicated concentrations of chelators (in μM) for a further 2 h. FAC-treated astrocytes were then incubated for 24 h in the absence (A) or presence of paraquat (2 mM; B) or H_2O_2 (1 mM; C), while BSO-treated astrocytes were incubated for a further 24 h with 200 μM H_2O_2 (D). Cells were assayed for viability and toxicity via MTT (top panels) and LDH assays (bottom panels), respectively. * P<0.05, ** P<0.01 as compared to relevant control (No chelator for DFOB, deferasirox, phenanthroline and clioquinol; DFOB (100 μM) for compounds 1-3). †† P<0.01 as compared to No chelator.

Figure 6. Mobilization of cell-associated iron by chelators. Primary astrocytes were pre-loaded with 100 μM FAC for 2 h (Fe loading) before the media was completely aspirated and replaced with media containing the indicated concentrations of chelators (in μM) for a further 2 h. Cells were subsequently collected for metal analysis by ICP-MS. * P<0.05, ** P<0.01 as compared to cells treated in the absence of chelators (No chelator).

Highlights

1. Lipophilic conjugates greatly enhance the neuroprotective capacity of DFOB
2. More protective against iron-mediated paraquat and H_2O_2 toxicity in neuronal cells
3. More protective in iron-loaded, GSH-depleted or A30P-α-synuclein expressing cells

Graphical Abstract (for review)

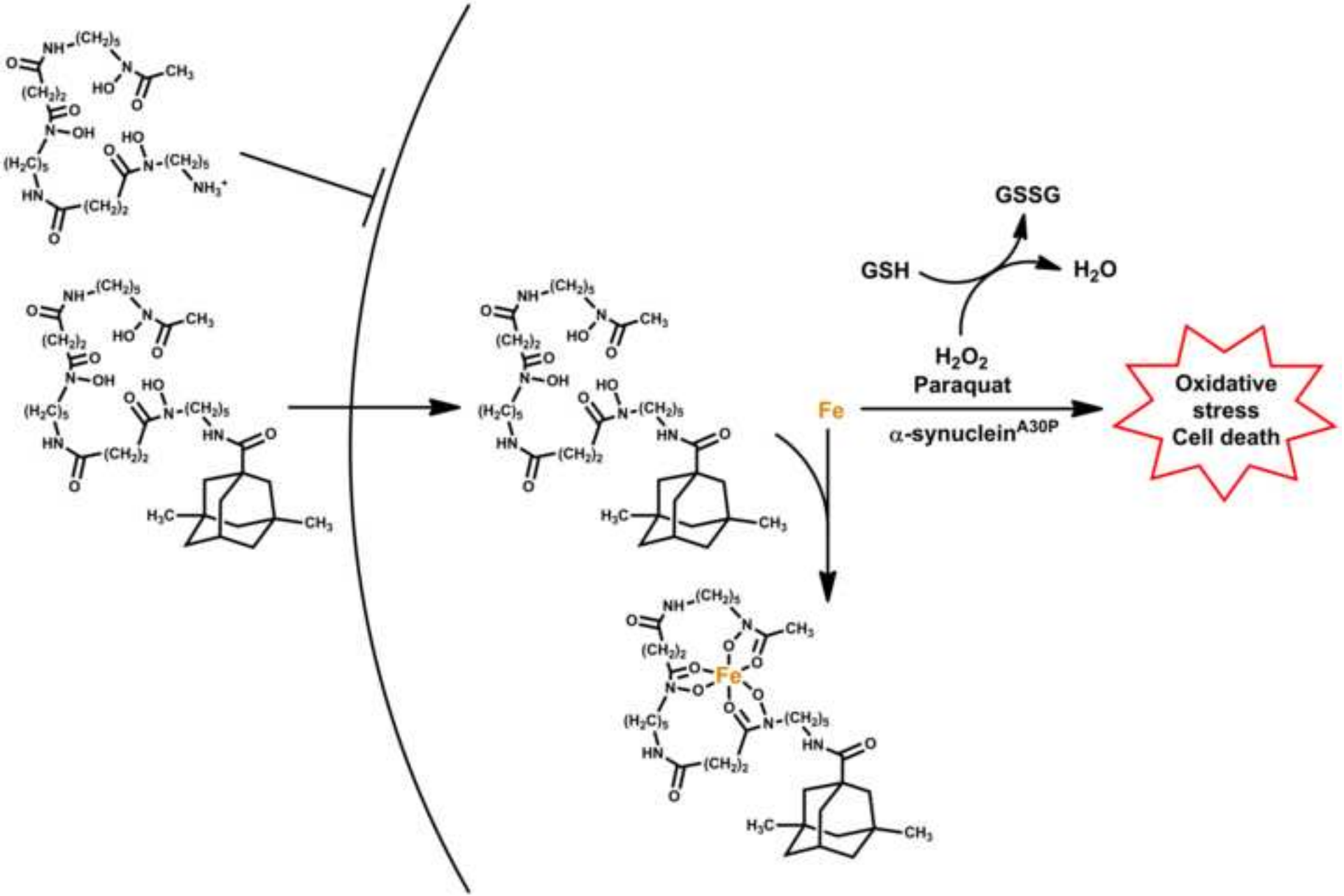
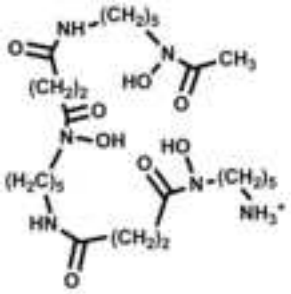
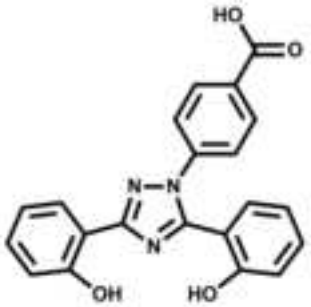


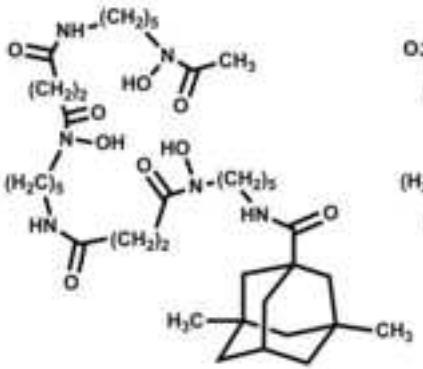
Figure 1



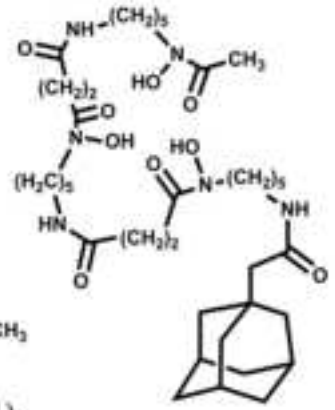
DFOB



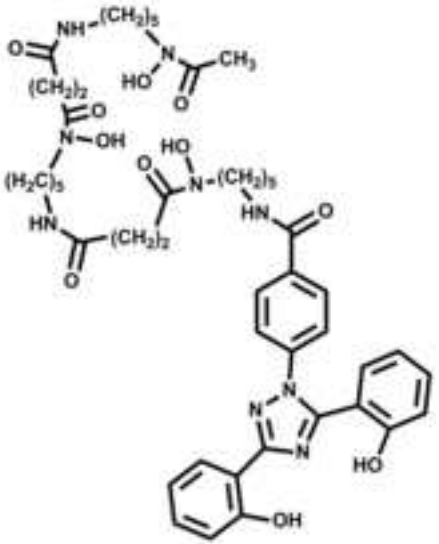
deferasirox



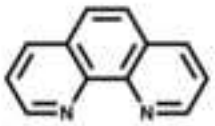
DFOB-AdA$_{dMe}$ (**1**)



DFOB-AdAc (**2**)



DFOB-L$_{DX}$ (**3**)



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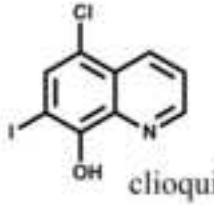

clioquinol (CQ)

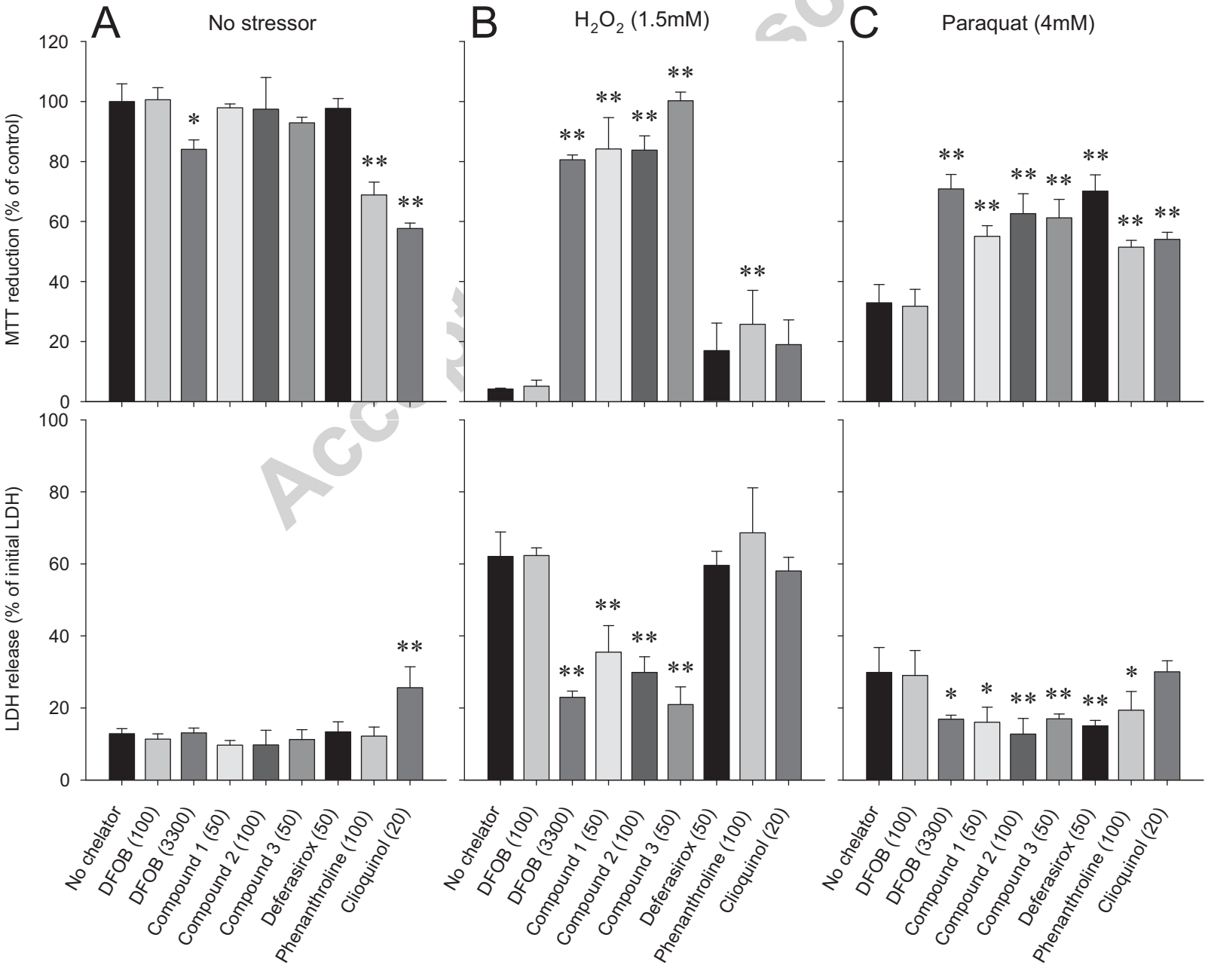

Figure 3

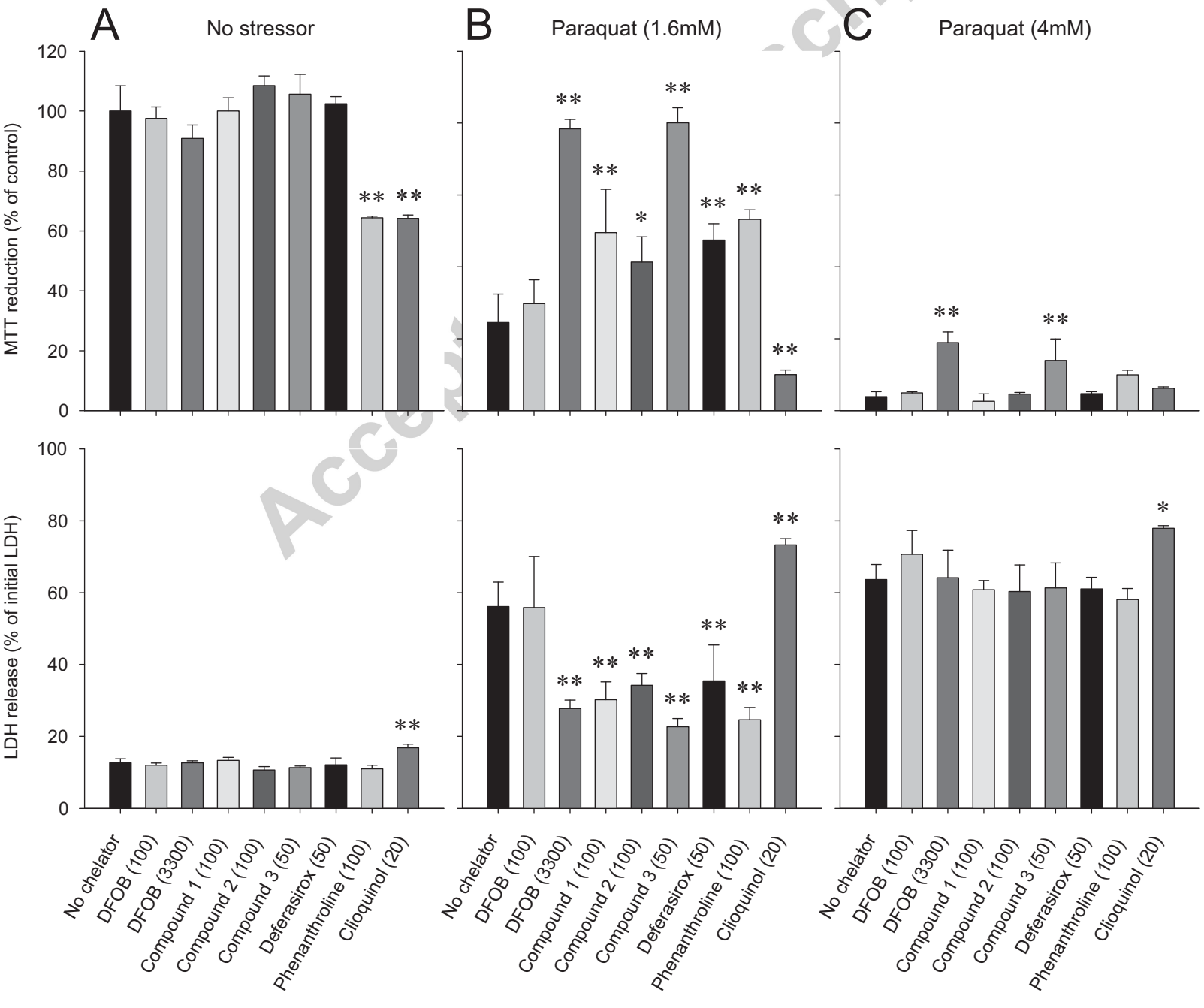

Figure 4

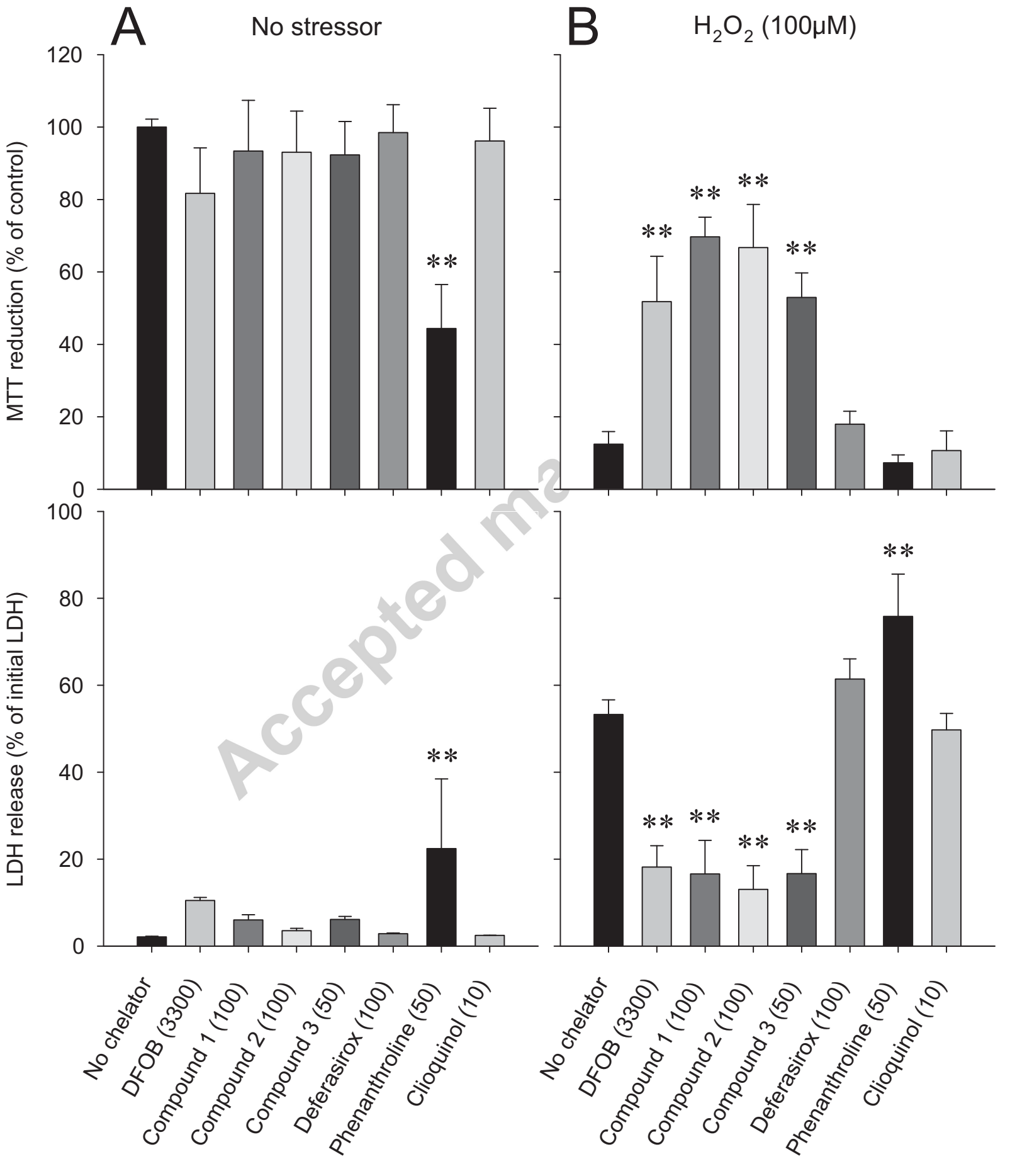

Figure 5

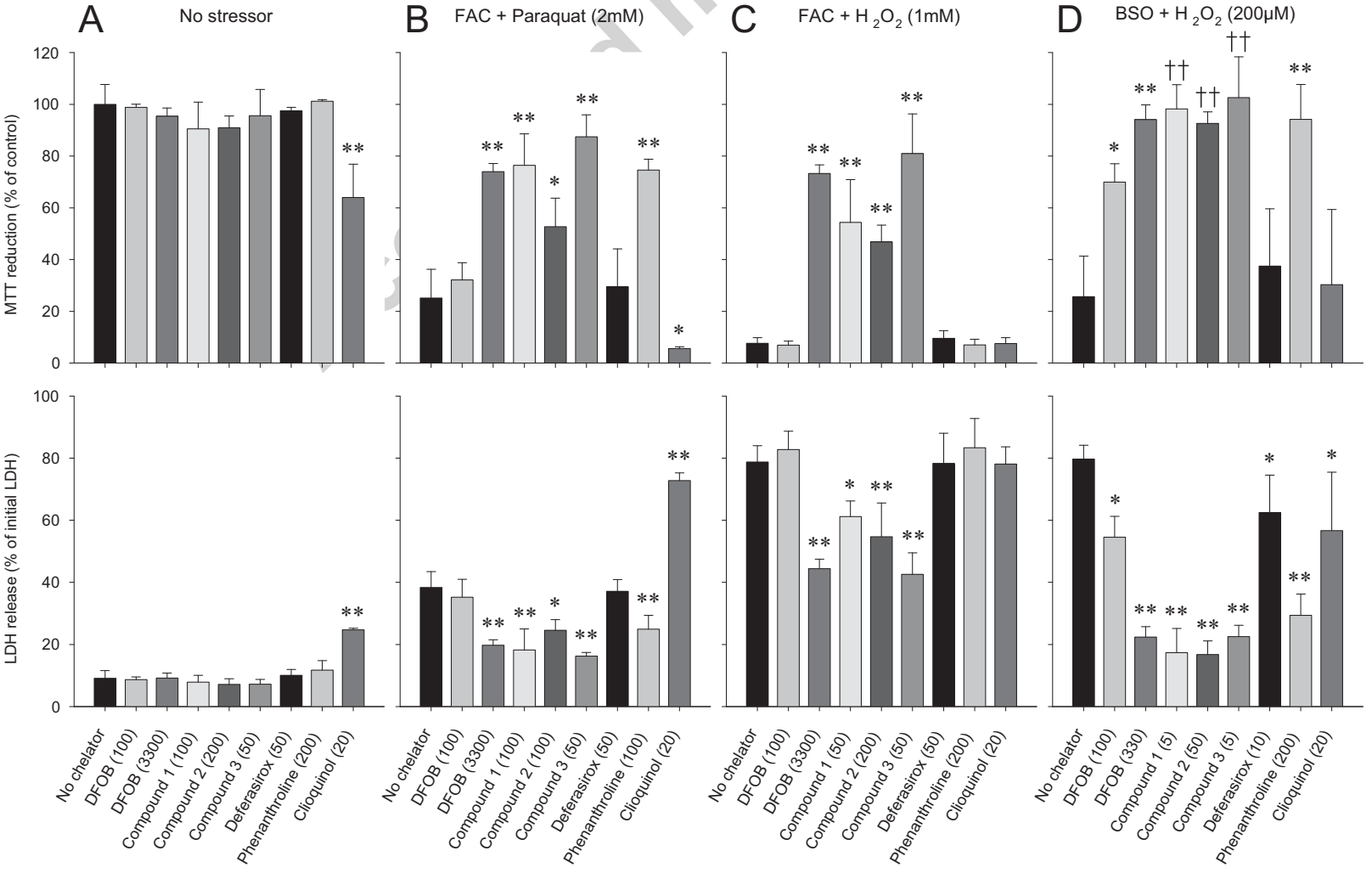

Figure 6