Dithiolethiones Protect Against Iron Overload-Induced Cytotoxicity and Serve as Ferroptosis Inhibitors in U-87 MG Cells

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Abstract

Iron overload-induced oxidative stress is known to trigger various cell death pathways such as apoptosis, necrosis, autophagy, and a novel iron-dependent, non-apoptotic form of cell death known as ferroptosis. Ferroptosis is driven by excessive lipid peroxidation coupled with reduced cellular antioxidant capacity and is implicated in iron overload-related neurodegenerative disorders. Dithiolethiones have emerged as promising compounds owing to their lipophilicity and cytoprotective potential against a wide range of toxicants. The two dithiolethiones 3H-1,2-dithiole-3-thione (D3T) and 5-amino-3-thioxo-3H-(1,2) dithiole-4-carboxylic acid ethyl ester (ACDT) have previously been reported to display neuroprotection by a Nrf2-mediated upregulation of the endogenous antioxidant machinery. Here, we test them against ferric ammonium citrate (FAC)-induced toxicity in U-87 MG astrocytoma cells. Exposure to 15 mM FAC for 24 hours resulted in 53% cell death. A 24-hour pretreatment with 50 µM D3T and ACDT reversed this cytotoxicity with D3T being more efficacious compared to the standard antioxidant N-acetylcysteine (NAC). Both dithiolethiones exhibited antioxidant effects by counteracting FAC-induced ROS and lipid peroxidation, reducing the need for GSH induction, and upregulating ferritin expression. Ferrostatin-1 (10 µM), a ferroptosis-specific inhibitor, inhibited FAC-induced ROS production, lipid peroxidation, and cell death, indicating for the first time that FAC induces ferroptosis in U-87 MG cells. A 24 hour pretreatment with dithiolethiones reversed erastin-induced ferroptosis by upregulating the xCT transporter and intracellular GSH levels thereby alleviating lipid peroxidation. RSL3-induced ferroptotic cell death and lipid peroxidation were also counteracted by pretreatment with D3T and ACDT. Collectively, our findings demonstrate that dithiolethiones are promising therapeutic compounds for the treatment of iron overload disorders involving ferroptotic cell death.

1. Introduction

Iron is a double-edged sword wherein both iron deficiency and iron overload conditions are deleterious to human health [1]. Although iron is essential for various biological functions, excessive levels of this redox metal lead to the generation of highly reactive oxygen species (ROS) by participation in the Fenton reaction [2]. This results in oxidative stress and causes DNA damage triggering various cell death signaling pathways such as apoptosis, necrosis, necroptosis, and autophagy [3–5].

Ferroptosis, a more recently discovered iron-dependent form of cell death [6], is characterized by uncontrolled lipid peroxidation associated with reduced intracellular antioxidant capacity [7]. It is morphologically, biochemically, and genetically distinct from all other forms of cell death. The intracellular defense mechanisms include glutathione (GSH) and glutathione peroxidase (GPX4). The former is an antioxidant capable of neutralizing free radicals while the latter is involved in detoxifying lipid peroxides [8, 9]. Inhibition of these molecules by the known ferroptosis inducers erastin and RAS-selective lethal-3 (RSL3) leads to rigorous lipid peroxidation and increased oxidative stress which then drive ferroptosis [10]. Ferroptosis is initiated by small molecules and is inhibited by iron chelators and lipophilic antioxidants [11]. Iron overload has been reported to produce ferroptosis in a previous study [12] and novel ferroptotic compounds and their underlying mechanisms continue to be identified [13]. Besides
primarily affecting the liver and heart, iron is known to accumulate in the brain [14]. Recent literature provides considerable evidence implicating iron overload and now ferroptosis in the pathophysiology of Alzheimer's disease (AD) and Parkinson's disease (PD) [15].

Chelation therapy is the mainstay of treatment for iron overload and ferroptosis, and currently, there are three FDA-approved iron chelators (deferoxamine, deferiprone, and deferasirox) on the market. Though they are highly effective in chelating iron, their use is associated with adverse effects such as agranulocytosis, neutropenia, and elevated levels of liver enzymes in patients [16]. Exploration of antioxidants to counteract iron-induced oxidative stress has thus gained interest. Several exogenous antioxidants are being explored for their direct free radical scavenging activity and indirect upregulation of the antioxidant reserves [17, 18]. Furthermore, the standard antioxidant N-acetylcysteine (NAC) in combination with an iron chelator is reported to restore brain function impaired by iron overload [19]. Nevertheless, no antioxidants have been approved by the FDA yet for the treatment of iron overload disorders.

Dithiolethiones are organosulfur compounds that possess chemotherapeutic, anti-inflammatory, and antioxidative properties [20]. They are highly lipophilic and readily cross the blood-brain barrier (BBB) [21]. They are known to induce the phase-II antioxidant system via activation of the nuclear factor erythroid 2-related factor-2-Kelch-like ECH-associated protein-1 (Nrf2-Keap1) pathway. The disulfide group in dithiolethiones reacts with molecular oxygen to form hydrogen peroxide. This generates a small amount of oxidative stress disrupting the Nrf2-Keap1 complex and causing the free Nrf2 to translocate to the nucleus and bind to an antioxidant response element (ARE) [22]. Using this mechanism, dithiolethiones upregulate various phase-II antioxidant enzymes such as NADPH: quinone oxidoreductase 1 (NQO1), glutathione-S-transferase (GST), glutamate-cysteine ligase catalytic (GCLC) subunit, and UDP-glucuronosyltransferase (UGT) in cardiovascular, hepatic, and cancer cells [23]. More importantly, activation of the Nrf2 pathway has been reported to serve as a negative regulator of ferroptosis [24].

3H-1,2-Dithiole-3-thione (D3T) and 5-amino-3-thioxo-3H-(1,2) dithiole-4-carboxylic acid ethyl ester (ACDT) are dithiolethiones that have previously shown neuroprotective effects both in vitro and in vivo. D3T is the most potent, unsubstituted parent compound in this series and has displayed neuroprotective activity against AD [25], neuroinflammation [26], stroke [27], and amyloid beta-induced toxicity [28]. ACDT, a disubstituted dithiolethione with pharmacokinetically favorable functional groups has exhibited protection against neuroinflammation [29], a 6-hydroxydopamine (6-OHDA) model of PD [30], and manganese-induced neurotoxicity [31]. Overlap in the toxicity profiles of manganese and iron as well as the strong association of iron with neurodegenerative diseases like PD and AD [32, 33] led us to explore the protective role of these dithiolethiones against iron-induced toxicity in U-87 MG cells. Additionally, since activation of the Nrf2 pathway protected against ferroptosis [24], we also evaluated whether dithiolethiones protect U-87 MG cells against ferroptosis induced by erastin and RSL3.

2. Materials And Methods
2.1. Reagents and supplies

The dithiolethiones D3T and ACDT were generously donated by Dr. Dennis Brown (Manchester University College of Pharmacy, Natural & Health Sciences, Fort Wayne, IN). U-87 MG human glioblastoma cells (ATCC® HTB-14) and Eagle's Minimum Essential Medium (EMEM, 30-2003) were procured from American Type Culture Collection (ATCC, Manassas, VA). Fetal bovine serum (FBS, 26140079), trypsin-ethylenediaminetetraacetic acid (25200056), penicillin-streptomycin antibiotics (15070063), FluoroBrite DMEM medium (A1896701), bicinchoninic acid assay kit (BCA, 23225), mammalian protein extraction reagent (MPER, 78501), and Hank's balanced salt solution (HBSS, no calcium, no magnesium, no phenol red, 14170161) were purchased from ThermoFisher Scientific (Waltham, MA). Ferric ammonium citrate (FAC, reagent grade, powder, ammonium iron (III) citrate, F5879), NAC (cell-culture tested, A9165), and 2',7'-dichlorofluorescin diacetate dye (DCFDA, D6883) were ordered from Sigma Aldrich (St. Louis, MO). CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS, G3580), Caspase-Glo 3/7 luminescence assay kit, and the luminescence-based GSH-Glo glutathione assay kit (V6911) were purchased from Promega (Madison, WI). Erastin (17754), RSL3 (19288), ferrostatin-1 (17729), and TBARS Assay Kit® (10009055) were procured from Cayman Chemical (Ann Arbor, MI).

2.2. Cell culture

Human glioblastoma (U-87 MG) cells were cultured in EMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO\textsubscript{2} at 37°C. Stock solutions of D3T (50 mM), ACDT (50 mM), erastin (20 mM), RSL3 (10 mM), and ferrostatin-1 (10 mM) were prepared in dimethyl sulfoxide (DMSO). The stock solutions were diluted in EMEM media before being added to the cells (final DMSO concentrations ≤ 0.1% v/v).

2.3. Cell viability assay

U-87 MG cells at a density of 2 x 10\textsuperscript{4} cells/well were seeded in 96-well microplates and incubated overnight. For a baseline toxicity evaluation, the cells were treated with FAC (0-20 mM) for 24 hours. To examine the protective ability of dithiolethiones against iron toxicity, the cells were pretreated with 50 µM D3T, ACDT, or NAC for 24 hours before the media being replaced with that containing 15 mM of FAC for an additional 24 hours. To determine whether FAC induces ferroptotic cell death, the cells were pretreated with 10 µM ferrostatin-1, a ferroptosis inhibitor, for 24 hours followed by exposure to 15 mM FAC for another 24 hours. Finally, to evaluate if D3T and ACDT act as ferroptosis inhibitors, cells were pretreated with 50 µM of either D3T, ACDT, or 10 µM ferrostatin-1 for 24 hours, followed by subsequent exposure to the ferroptosis inducers, erastin (20 µM) or RSL3 (10 µM). The MTS assay was used to determine cell viability as previously described [34] and absorbance was measured at a wavelength of 490 nm using the Synergy HT multimode microplate reader (Biotek instruments, Winooski, VT).

2.4. Microscopy

U-87 MG cells were seeded at a density of 1x10\textsuperscript{6} cells in 25 cm\textsuperscript{2} flasks (for the effects of D3T, ACDT, NAC against FAC) and 1x10\textsuperscript{5} cells/well in 6-well plates (for ferroptosis experiment) for each group and
incubated for 24 hours. The cells were treated with compounds of interest either alone or in combination with FAC. After a wash with PBS, phenol red-free media was added, and cell morphology changes were captured at a phase contrast mode using the Keyence All-in-One Fluorescence Microscope (BZ-X800, Woburn, MA).

2.5. Measurement of intracellular ROS

The ROS levels were determined using the DCFDA dye according to the manufacturer’s protocol. Briefly, 5x10^4 cells/well were seeded in 96-well, black microplates (3916, Corning®) and incubated overnight. Culture media was aspirated and the cells were incubated with 10 µM DCFDA fluorescent probe in phenol red-free media for 45 minutes. After removal of the dye and a wash with HBSS, cells were pretreated with D3T, ACDT, or NAC for 24 hours followed by another 4 hour exposure to 15 mM FAC. For ferroptosis experiments, cells were pretreated with ferrostatin-1 for 24 hours followed by a 4 hour exposure to FAC. The fluorescence signals directly proportional to the levels of intracellular ROS levels were measured at excitation/emission wavelengths of 495/529 nm. ROS fold-change was calculated relative to vehicle control.

2.6. Measurement of intracellular GSH levels

Cells were seeded in 96-well, clear bottom, white microplates at a density of 2x10^4 cells/well and incubated overnight. They were pretreated with D3T, ACDT, or NAC for 24 hours in phenol red-free media followed by exposure to 15 mM FAC for an additional 24 hours. For ferroptosis experiments, cells were pretreated with D3T, ACDT, or ferrostatin-1 for 24 hours in phenol red-free media followed by exposure to 20 µM erastin for an additional 24 hours. The GSH-Glo glutathione assay kit was used to evaluate GSH levels as per the manufacturer’s protocol. The Relative Luminescent Units (RLU) were recorded using a microplate reader and the total GSH levels were calculated relative to vehicle control.

2.7. Lipid peroxidation assay

U-87 MG cells were seeded in 75 cm^2 flasks at a density of 1x10^7 cells/flask and incubated overnight. The cells were pretreated with D3T, ACDT, or NAC for 24 hours followed by another 24 hour exposure to FAC. For ferroptosis experiments, cells were pretreated with D3T, ACDT, or ferrostatin-1 for 24 hours before being exposed to FAC for an additional 24 hours. Cell lysates were prepared, and the thiobarbituric acid reactive substances (TBARS) assay was performed according to the manufacturer’s instructions. The absorbance values were measured at 540 nm and malondialdehyde (MDA) levels were normalized to a standard curve.

2.8. Western blot analysis

Cells were seeded at a density of 1x10^6 cells in 25 cm^2 flasks for each treatment group. After overnight incubation, they were treated with D3T, ACDT, NAC, or ferrostatin-1 for 24 hours. Media was replaced with serum-free media containing either FAC, erastin, or RSL3 for another 24 hours. The cells were lysed, protein extraction was carried out using protease inhibitors and MPER lysis buffer, and total protein concentration was evaluated using the BCA assay. The samples were reduced using sodium dodecyl
sulfate (SDS) buffer for 5 minutes at 95°C. A total of 40 µg of protein from each sample was subject to gel electrophoresis at 125 V for 50 minutes using 4-20% Miniprotein gradient TGX precast gels (BioRad, Hercules, CA). The separated proteins were transferred onto nitrocellulose membranes and blocked using 5% non-fat dried milk in tris-buffered saline plus 0.1% tween 20 detergent (TBS-T) for 1 hour at room temperature (RT). The membranes were then incubated with primary antibodies overnight at 4°C. The primary antibodies used were recombinant anti-ferritin monoclonal antibody (ab75973, 1:1000, Abcam®), anti-ferroportin polyclonal antibody (PAB15509, 1:1000, Abnova, Taipei, Taiwan), anti-GPX4 monoclonal antibody (ab75973, 1:500, Abcam®) and the anti-xCT monoclonal antibody (ab175186, 1:1000, Abcam®). After three washes, the membranes were incubated with HRP goat anti-rabbit secondary antibody H&L (ab97051, 1:10000, Abcam®) for 1 hour. The anti-beta actin antibody (HRP) (ab49900, 1:20000, Abcam®) was used to detect the loading control. The membranes were washed three additional times and the bands were obtained after incubating with 1:1 of Super Signal West Pico chemiluminescent substrate and enhancer solution (Catalog. no. 34580, Thermo Fisher scientific) for 5 minutes. The bands were visualized using a C-Digit Scanner (Li-COR biosciences, Lincoln, NE), and the Image Studio Lite® (Li-COR biosciences, Lincoln, NE) program was used for densitometric analysis. The protein levels were calculated relative to the vehicle control.

2.9. Measurement of caspase 3/7 activity to detect apoptosis

Cells were seeded in 96-well, clear bottom, white microplates at a density of 1.5x10⁴ cells/well and incubated overnight. They were pretreated with D3T, ACDT, or NAC for 24 hours in phenol red-free media followed by an additional 24 hour exposure to FAC. The Caspase-Glo 3/7 luminescence assay kit was used to evaluate the caspase activity as previously described (Zhang et al., 2018). The RLU were recorded using a microplate reader and the caspase activity in the treatment groups was calculated relative to vehicle control.

2.10. Cytotoxicity assay for necrosis

A cytotoxicity assay was performed to evaluate necrotic cell death using the ethidium homodimer-1 fluorescent dye (E1169, Thermo Fischer Scientific). Cells were seeded in 24-well plates at a density of 2x10⁵ cells/well and incubated for overnight attachment. They were pretreated with D3T, ACDT, or NAC for 24 hours followed by exposure to media containing FAC for an additional 24 hours. After the removal of media, 200 µL of ethidium homodimer in sodium buffer (pH 7.4) was added to each well followed by a 45 minute incubation in the dark. The fluorescence was then measured at excitation/emission wavelengths of 528/20 nm and 590/35 nm using a microplate reader. Next, 20 µL of 10% digitonin was added to each well and incubated for 2 hours in the dark followed by measurement of resulting fluorescence. The percent cytotoxicity for each treatment group was calculated upon normalization to digitonin-induced cell death.

2.11. Statistical analysis
Data are reported as mean ± SEM. Comparisons between multiple groups were performed using a one-way analysis of variance (ANOVA) followed by an appropriate post hoc test. Results with a p-value ≤ 0.05 were considered to be statistically significant. Each experiment was performed as three independent replicates. GraphPad Prism version 8.0 for Windows (La Jolla, CA) was utilized for statistical analysis and graphical representation of the data.

3. Results

3.1. Impact of dithiolethiones on FAC-induced cell death

A 24 hour exposure of U-87 MG cells to FAC (0-20 mM) resulted in a concentration-dependent cytotoxicity at concentrations of 5 mM and higher (Fig. 1a). As the cell viability decreased to 53.3% (F_{9,20} = 31.62; p < 0.0001) with 15 mM FAC, this concentration was selected for consecutive experiments. Pretreatment with 50 µM of D3T, ACDT, or NAC significantly increased the cell viability to 94.3%, 89.6%, and 75.3%, respectively in comparison to the FAC alone-treated group (Fig. 1b) (F_{4,10} = 24.41; p < 0.0001). These results demonstrate that pretreatment with D3T or ACDT protects the U-87 MG cells against FAC-induced cytotoxicity with D3T being significantly more efficacious than NAC, a standard comparator (Fig. 1b). The dithiolethiones and NAC alone had no impact on cell viability. Cells treated with 15 mM FAC alone were visually sparse in number, appeared stressed and rounded in comparison to those in the control group, while cells pretreated with the dithiolethiones and NAC appeared morphologically healthier (Fig. 1c).

3.2. Impact of dithiolethiones on FAC-induced oxidative stress

The exposure of cells to 15 mM FAC significantly increased intracellular ROS levels beginning as early as 1 hour (1.4-fold) with a peak at 4 hours (2-fold) (F_{7,16} = 102.3; p < 0.0001) (Fig. 2a). Pretreatment with dithiolethiones and NAC counteracted this effect at 4 hours (F_{7,16} = 48.32; p < 0.0001) (Fig. 2b). In addition, the TBARS assay showed a significant increase (38.3 to 53.5 µM) in MDA levels with a 24 hour exposure to FAC in comparison to control. This was successfully counteracted by the pretreatment with D3T, ACDT, and NAC from 53.5 to 40.3, 40.7, and 40.6, respectively (F_{7,16} = 18.03; p < 0.0001, Fig. 2c). Furthermore, exposure to FAC led to a 3-fold increase in intracellular GSH level which was alleviated by pretreatment with D3T, ACDT, and NAC to 1.41, 1.43, 1.60-fold, respectively (F_{7,16} = 72.52; p < 0.0001, Fig. 2d). These data indicate that D3T and ACDT rescue U-87 MG cells from FAC-induced oxidative stress as effectively as NAC. While exposure to FAC alone significantly upregulated (2.4-fold) the expression of the iron storage protein ferritin, D3T and ACDT increased this protein level even further (1.6-fold and 1.5-fold, respectively compared to FAC alone group) (F_{7,16} = 20.24; p < 0.0001, Fig. 2e). Treatment of cells with 50 µM NAC did not alter the levels of ferritin (Fig. 2e). No significant changes were observed in the protein expression of iron export transporter ferroportin with any of the compounds tested (Fig. 2f).
3.3. Impact of dithiolethiones on various cell death pathways triggered by FAC

In evaluating the effects of dithiolethiones on FAC-induced apoptosis, we observed no significant changes in the caspase 3/7 activity with any of the treatment groups (Supplementary Fig. 1). We also performed ethidium homodimer necrosis assay which revealed a significant increase (94.3%) in cell death upon exposure to FAC ($F_{7,16} = 35.31; p < 0.0001$) which was successfully counteracted by pretreatment with D3T (80.6%), ACDT (57.6%), and NAC (78.6%) (Supplementary Fig. 2). This shows that the dithiolethiones inhibit FAC-induced necrosis in U-87 MG cells. Finally, we evaluated whether FAC induces ferroptosis, a newly discovered iron-dependent form of cell death in these cells. We observed rounded, detached cells with loss of cell structural integrity and reduced cell number with FAC exposure and these morphological changes were similar to those seen in the cells treated with ferroptosis inducers erastin and RSL3 (Fig. 3a). A 24 hour exposure to FAC alone reduced cell viability to 51.3% ($F_{3,8} = 554.0, p < 0.0001$) and pretreatment with the specific ferroptosis inhibitor ferrostatin-1 increased it to 78.3% (Fig. 3b). Ferrostatin-1 also counteracted FAC-induced ROS production (1.7-fold to 0.8-fold) ($F_{3,8} = 73.16, p < 0.0001$, Fig. 3c) and MDA levels (41.1 µM to 9.4 µM) ($F_{3,8} = 20.84, p = 0.0004$, Fig. 3d). Therefore, inhibition of FAC-induced cytotoxicity by ferrostatin-1 collectively indicates that FAC causes ferroptosis in U-87 MG cells.

3.4. D3T and ACDT protected against erastin-induced lipid peroxidation and ferroptosis

We attempted to delineate whether the dithiolethiones serve as inhibitors of erastin-induced ferroptosis. Erastin significantly reduced cell viability (75.3%) which was successfully counteracted by pretreatment with D3T (101.6%), ACDT (91.3%), and the standard ferroptosis inhibitor ferrostatin-1 (90.6%) ($F_{7,16} = 27.38, p < 0.0001$, Fig. 4a). An erastin-induced drop in xCT protein expression (0.37-fold) was also reverted by both dithiolethiones and ferrostatin-1 ($F_{4,10} = 13.40, p = 0.0005$, Fig. 4b). Moreover, while erastin significantly depleted the intracellular GSH levels to 0.2-fold ($F_{4,10} = 77.30, p < 0.0001$), pretreatment with D3T, ACDT, and ferrostatin-1 increased these levels to 0.5-fold, 0.43-fold, and 0.46-fold, respectively (Fig. 4c). D3T, ACDT, and ferrostatin-1 also reduced the erastin-induced increase in MDA levels from 17.7 µM down to 8.4 µM, 7.6 µM, and 6.5 µM, respectively ($F_{7,16} = 12.77, p < 0.0001$, Fig. 4d). These results show that the dithiolethiones act as inhibitors of erastin-induced ferroptosis comparable to ferrostatin-1.

3.5. D3T and ACDT protected against RSL3-induced lipid peroxidation and cell death

RSL3 produced a significant reduction in cell viability (59.6%) and pretreatment with D3T, ACDT, and ferrostatin-1 increased it to 100.6%, 92.6%, 94.6%, respectively ($F_{7,16} = 31, p < 0.0001$) (Fig. 5a).
Furthermore, the steep rise in MDA levels caused by RSL3 was successfully counteracted by D3T, ACDT, and ferrostatin-1 ($F_{7,16} = 18.03, p < 0.0001$) (Fig. 5b). No significant changes were observed in GPX4 levels with any of the treatment groups (Fig. 5c). These observations demonstrate that dithiolethiones inhibit RSL3-induced ferroptosis by inhibiting lipid peroxidation and these effects are comparable to the standard ferroptosis inhibitor ferrostatin-1.

4. Discussion

Iron overload and accumulation are linked to various diseases like PD, AD, and neurodegeneration with brain iron accumulation (NBIA) [35]. Given the wide range of adverse effects associated with current iron chelators, exogenous antioxidants such as NAC, flavonoids, carotenoids, and diterpenes have been tested against iron overload [19, 36]. Taking into account the promising antioxidant activity and favorable pharmacokinetic and adverse effect profiles of dithiolethiones [37], we present their effects against iron-induced cytotoxicity and ferroptosis in this study.

Astrocytes have gained a spotlight as models of iron overload-induced oxidative stress [38]. These cells serve as iron stores and gatekeepers for the transport of iron and other toxic substances in the brain [39]. Therefore, any toxic damage to astrocytes is associated with neurodegeneration [40]. In our study, we used U-87 MG cells which are astrocytoma cells. The FAC salt was used to induce iron toxicity owing to its readily water-soluble property and widespread use in past iron-related studies [5].

While D3T and ACDT have previously shown cytoprotection against various toxicants, to our knowledge, this is the first study depicting their effects against iron-induced toxicity and associated cell death pathways. A 50% cytotoxicity with 10 mM FAC at 24 hours [41] and 5 mM FAC at 48 hours [42] were previously reported in SH-SY5Y neuroblastoma cells. The requirement of higher concentration of FAC (15 mM) in our study to produce similar of cell death is likely due to the cell line itself, as astrocytes can store large amounts of iron and are more resistant to toxicity compared to neurons [43]. Here, we chose the 50 µM concentration for both our test dithiolethiones based on the results from previous reports. ACDT at 50 µM concentration exerted significant neuroprotection against 6-OHDA [30] and Mn-induced cytotoxicity [31] in SH-SY5Y cells. Similarly, Li et al., showed that 50 µM D3T protected against ultra-violet radiation-induced toxicity in retinal cells [44]. We included NAC as a concentration-matched standard comparator. This dual antioxidant directly neutralizes free radicals and indirectly acts a precursor to GSH [45]. Importantly, all three compounds demonstrated an excellent counteractivity against iron overload-induced cell death (Fig. 1b and Fig. 1c). A structural comparison of D3T and ACDT with NAC reveals that dithiolethiones are at an advantage wherein they possess three sulfur groups while NAC has only one. This may help partly explain the stronger antioxidant effect observed with these compounds in some of our reported experiments.

FAC-induced oxidative stress (ROS generation and lipid peroxidation) is the principal mechanism of iron toxicity [46]. The successful mitigation of ROS production (Fig. 2b) and lipid peroxidation (Fig. 2c) by D3T and ACDT are key findings. These effects are explained by the established mechanism of a Nrf2-
driven upregulation of the cellular antioxidant machinery by dithiolethiones [47]. A 4 hour FAC exposure for the ROS assay was chosen because ROS levels peaked significantly at this time point (Fig. 2a) and this is in line with the previous FAC-related studies [48]. The significant increase in intracellular GSH observed with exposure to FAC (Fig. 2d) was consistent with a past study [49] and likely due to the cellular response to FAC-induced oxidative insult. Exposure to D3T alone for 24 hours has previously been reported to increase GSH levels in SH-SY5Y cells [23] and cardiomyocytes [50]. Likewise, Betharia et al. showed that a 24 hour treatment with ACDT increases intracellular GSH in SH-SY5Y cells [30]. In our study, the FAC-induced increase in GSH levels was successfully reduced by D3T and ACDT (Fig. 2d). We hypothesize that pretreatment with dithiolethiones may have preemptively bolstered the GSH stores via Nrf2 activation thereby preventing FAC-induced oxidative stress and reducing the need for further rise in GSH production. Interestingly, this effect of dithiolethiones was similar to that observed with the well-established antioxidant NAC which increases GSH levels by acting as a source of cysteine, an amino acid required for GSH biosynthesis.

The labile iron pool is strictly regulated by various mechanisms including the Nrf2-mediated transcriptional upregulation of ferritin and ferroportin (FPN1) expression [51]. Both these proteins reduce cellular iron wherein ferritin is an intracellular iron storage protein that stores excess iron in a redox inactive form [52], while FPN1 is the iron exporter that exports iron from the cell into the plasma [53]. Incubation with ferric iron has been reported to increase ferritin expression in Caco-2 cells [54] and astrocytes [55] as a potential cellular defense mechanism against iron overload which is in line with our results (Fig. 2e). Remarkably, pretreatment with D3T and ACDT but not NAC, further increased ferritin expression against FAC. In explanation, dithiolethione-mediated activation of the Nrf2-ferritin heavy chain (Fth1)-ARE pathway can induce ferritin expression beyond normal in response to oxidative stress [56]. In our study, in contrast to the results from Tangudu and group [57], FAC did not alter FPN1 levels (Fig. 2f). However, hepcidin levels were unchanged in response to FAC in astrocytes [58]. Since hepcidin regulates FPN1 expression, the absence of FPN1 upregulation in our study is justified.

Iron overload-induced oxidative stress can cause DNA damage and trigger various cell death pathways such as apoptosis, necrosis, autophagy, and the more recently identified ferroptosis [59]. Activation of caspases and the pro-apoptotic protein Bax are reported in past studies in response to ferroptosis [60]. Since we did not observe activation of caspases (supplementary Fig. 1), we evaluated the possibility of a lesser explored necrotic cell death by FAC. Similar to studies in rat hepatocytes [61] and in bone marrow cells [62], our results indicate FAC-induced necrosis for the first time in U-87 MG cells (supplementary Fig. 2). The successful counteraction of this form of cell death by dithiolethiones can be attributed to the alleviation of ROS and MDA-induced oxidative stress by the Nrf2-mediated upregulation of GSH and ferritin levels. It is important to point out that while the effects of dithiolethiones matched those of NAC in ROS, GSH, and MDA experiments, their effects on ferritin upregulation and protection against cytotoxicity were significantly higher.

While reports on iron overload-induced necrosis are sparse, recent research has expanded to other forms of iron-induced cell death such as ferroptosis. First coined in 2012, ferroptosis is an iron-dependent, non-
apoptotic cell death characterized by increased lipid peroxidation [63]. The specific ferroptosis inhibitor ferrostatin-1 is a lipophilic antioxidant which specifically inhibits ferroptosis by preventing lipid peroxidation and lacks an effect on other forms of cell death [64]. FAC-induced cell death (Fig. 3b), ROS production (Fig. 3c), and lipid peroxidation (Fig. 3d) were counteracted by ferrostatin-1, thus collectively providing the first evidence that FAC induces ferroptosis in U-87 MG cells. This is in line with a previous study where 10 µM ferrostatin-1 counteracted the lipid peroxidation and ferroptosis against FAC in primary hepatocytes [12]. Additionally, FAC-induced changes in the morphology of U-87 MG cells (Fig. 3a) were similar to those observed in three different colorectal cancer cells exposed to the ferroptosis inducer RSL3 [65].

The Nrf2 transcription factor is involved in inhibiting ferroptosis by regulating the expression of various ferroptosis-related proteins [66]. As dithiolethiones are activators of the Nrf2 pathway, we tested their potential role in inhibiting ferroptosis against the canonical ferroptosis inducers erastin and RSL3 using ferrostatin-1 as a standard comparator. Notably, ours is the first study to test the effects of erastin and RSL3 in U-87 MG cells. Erastin causes ferroptosis by inhibiting the transmembrane cystine/glutamate antiporter (xCT). This transporter normally allows the uptake of cystine into the cells which is then reduced to cysteine, a molecule indispensable in GSH synthesis. Therefore, inhibition of xCT leads to a decrease in cysteine levels and consequent intracellular GSH depletion, increased lipid peroxidation, and resulting ferroptosis [67]. Our results conform with previous reports of erastin-induced downregulation of xCT expression and increased lipid peroxidation in primary cortical neurons [68], and GSH depletion in human cervical adenocarcinoma cells [67] and HT-1080 human fibrosarcoma cells [69]. Moreover, increase in the xCT expression by D3T and ACDT against erastin (Fig. 4b) is one of our valuable findings. This effect is likely via activation of the Nrf2 pathway which is reported to control the expression of xCT antiporter [70]. Notably, the dithiolethiones also inhibited erastin-induced cell death (Fig. 4a), GSH depletion (Fig. 4c), and lipid peroxidation (Fig. 4d) providing the first evidence that they serve as ferroptosis inhibitors similar to ferrostatin-1. As Nrf2 target antioxidants are involved in upregulation of intracellular antioxidants and prevention of lipid peroxidation, the protective role of D3T and ACDT against ferroptosis is justified.

RSL3 is reported to directly inhibit the GPX4 enzyme by covalently binding to the catalytic selenocysteine moiety of GPX4 [71]. This creates an imbalance between lipid peroxidation and intracellular antioxidant capacity thus leading to ferroptosis. A study in cortical neurons showed that the exposure to RSL3 decreased GPX4 protein expression [72]. In line with a previous study [73], we found RSL3-induced lipid peroxidation (Fig. 5b) and cytotoxicity (Fig. 5a) to be successfully counteracted by dithiolethiones likely via their impact on the Nrf2 pathway. Furthermore, since the GPX4 enzyme is a Nrf2 target antioxidant, dithiolethiones have been reported to upregulate GPX4 expression [74]. Surprisingly, we saw no change in GPX4 levels with D3T, ACDT, or even ferrostatin-1 in our study (Fig. 5c). A possible explanation could be the difference in duration of exposure. A study in human primary cardiomyocytes has reported that a 48 hour exposure to 50 µM of D3T induced GPX expression [75]. Additional studies are needed to reconcile this discrepancy. A schematic representation of the effects of these dithiolethiones against iron-induced toxicity and ferroptosis is shown below (Fig. 6).
5. Conclusions

This study is the first to report that dithiolethiones D3T and ACDT exert significant cytoprotection against iron overload-induced cytotoxicity in U-87 MG cells. Dithiolethiones counteracted cell death by upregulating ferritin and reducing ROS production, lipid peroxidation, and intracellular GSH demand. The differential effects on ferritin upregulation and resulting cytoprotection are striking advantages of dithiolethiones over the standard antioxidant NAC. Ferrostatin-1, a specific ferroptosis inhibitor, successfully counteracted FAC-induced ROS and lipid peroxidation indicating that FAC causes ferroptosis in this cell line. Dithiolethiones D3T and ACDT successfully inhibited erastin- and RSL3-induced ferroptosis by increasing xCT expression and GSH levels, alleviating lipid peroxidation, and thereby protecting against cell death in a manner similar to that of the standard comparator ferrostatin-1. Hence for the first time, we introduce D3T and ACDT as ferroptosis inhibitors. Finally, our assessment provides compelling evidence that these dithiolethiones are strong candidates for the treatment of iron overload conditions, ferroptosis, and associated brain disorders.

Abbreviations

6-OHDA – 6-hydroxydopamine

ACDT – 5-Amino-3-thioxo-3H-(1,2) dithiole-4-carboxylic acid ethyl ester

AD – Alzheimer’s disease

ANOVA – Analysis of variance

ARE – Antioxidant response element

BBB – Blood-brain barrier

D3T – 3H-1,2-dithiole 3-thione

DCFDA – 2’,7’-Dichlorofluorescin diacetate

DMSO – Dimethyl sulfoxide

EMEM – Eagle’s Minimum Essential Medium

EAE – Experimental autoimmune encephalomyelitis

FAC – Ferric ammonium citrate

FBS – Fetal bovine serum

FPN1 – Ferroportin
GCLC – Glutamate-cysteine ligase catalytic
GSH – Glutathione
HBSS – Hank’s Balanced Salt Solution
Keap1 – Kelch-like ECH-associated protein-1
Mn – Manganese
MDA – Malondialdehyde
MTS – 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAC – N-acetyl cysteine
NBIA – Neurodegeneration with brain iron accumulation
Nrf2 – Nuclear factor erythroid 2-related factor 2
PD – Parkinson’s disease
ROS – Reactive oxygen Species
RSL3 – RAS-selective lethal-3
TBARS – Thiobarbituric acid reactive substances

Declarations

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Declaration of conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Neha Kulkarni and Swati Betharia contributed to the conception, validation, and design of the study. Material preparation, data collection and analysis were performed by Neha Kulkarni and Rajitha Gadde. The first draft of the manuscript was written by Neha Kulkarni and Rajitha Gadde, and Swati Betharia critically reviewed and commented on previous versions of the manuscript. All authors read and approved the final manuscript. Funding acquisition and project supervision was carried out by Swati Betharia.

Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval**

Not applicable

**Consent to participate**

Not applicable

**Consent for publication**

Not applicable

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Figures
Impact of dithiolethiones on FAC-induced cell death. The MTS assay was used to determine the effect of (a) 24 hour exposure to FAC (0-20 mM) and (b) a 24 hour pretreatment with 50 µM D3T, ACDT, or NAC prior to a 24 hour exposure to 15 mM FAC on cell viability. Data are represented as mean ± SEM (n=3) analyzed by one-way ANOVA followed by either Dunnett’s or Tukey’s post hoc test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001 vs. control. ##p ≤ 0.01 and ####p ≤ 0.0001 vs. FAC alone group; $p ≤ 0.05 vs. NAC+FAC treated group. (c) Phase contrast images of U-87 MG cells indicating changes in cell morphology (scale 100 µM, magnification 10X). (d) Chemical structures of the tested dithiolethiones and NAC, a standard antioxidant.
Figure 2

Impact of dithiolethiones on FAC-induced oxidative stress. DCFDA dye was used to determine the ROS levels (a) upon exposure to FAC for 0-24 hours and (b) upon a 24 hour pretreatment with 50 µM dithiolethiones or NAC prior to a 4 hour exposure to FAC. A similar pretreatment prior to a 24 hour exposure to FAC was used for analysing (c) MDA levels (TBARS assay), (d) intracellular GSH levels (GSH-Glo assay), and protein expression of (e) ferritin and (f) ferroportin (western blotting). Data are represented as mean ± SEM (n=3) analyzed by one-way ANOVA followed by either Dunnett’s or Tukey’s post hoc test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 vs. control and #p ≤ 0.05, ####p ≤ 0.001 vs. FAC alone group; §§ p ≤ 0.01 vs. NAC+FAC group.
Figure 3

Impact of FAC on ferroptotic cell death. (a) The phase contrast image of U-87 MG cells treated with FAC (15 mM) for 24 hours was compared with those of cells treated with ferroptosis inducers erastin (20 µM) and RSL3 (10 µM) (scale 100 µM, magnification 10X). The effect of a 24 hour pretreatment with 10 µM ferrostatin-1 before exposure to 15 mM FAC for (b) 24 hours on cell viability using MTS assay, (c) 4 hours on ROS using DCFDA dye, and (d) 24 hours on MDA levels using TBARS assay was determined. Data are represented as mean ± SEM (n=3), analyzed by one-way ANOVA followed by Tukey's post hoc test. **p ≤ 0.01, ****p ≤ 0.0001 vs. control; ###p ≤ 0.001, ####p ≤ 0.0001 vs. FAC alone treated group.
Figure 4

Impact of dithiolethiones on erastin-induced ferroptosis. The effect of a 24 hour pretreatment with 50 µM D3T, ACDT, or 10 µM ferrostatin-1 before a 24 hour exposure to 20 µM erastin on (a) cell viability, b) xCT protein expression, (c) total GSH levels, and (d) MDA levels was determined. Data are represented as mean ± SEM (n=3), calculated by one-way ANOVA using Tukey's post hoc test. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 vs. control group; #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001, ####p ≤ 0.0001 vs erastin alone group; and $p ≤ 0.05 vs. D3T+erastin treated group.

Figure 5
Impact of dithiolethiones on RSL3-induced ferroptosis. The effect of a 24 hour pretreatment with 50 µM D3T, ACDT, or 10 µM ferrostatin-1 before a 24 hour exposure to 10 µM RSL3 on (a) cell viability, (b) lipid peroxidation, and (c) GPX4 protein expression was determined. Data are represented as mean ± SEM (n=3) analyzed by one-way ANOVA followed by Tukey's post hoc test. ****p ≤ 0.0001 vs. control; ###p ≤ 0.001 and ####p ≤ 0.0001 vs. RSL3 alone group.

Figure 6

Dithiolethiones protect against iron-induced cytotoxicity and serve as ferroptosis inhibitors against erastin and RSL3. **On the left**: Iron in the form of Fe^{2+} enters the cells via DMT1 transporters where it is utilized for cellular processes or is bound to the iron storage protein ferritin. Excess unbound iron mediates the generation of ROS by participating in the Fenton reaction thereby resulting in uncontrolled lipid peroxidation and cell death. Dithiolethiones (D3T and ACDT) and NAC (a standard comparator) are capable of protecting the cells against iron overload-induced cell death by blocking this pathway. Unlike NAC, these dithiolethiones also upregulate the expression of ferritin. The iron-induced increase in ROS and MDA levels is successfully inhibited by the ferroptosis inhibitor ferrostatin-1 (F-1) indicating that iron induces ferroptosis in these cells. **On the right**: Erastin inhibits the xCT antiporter and RSL3 directly inactivates the GPX4 enzyme, both leading to increased lipid peroxidation and consequent ferroptotic cell death. Dithiolethiones D3T and ACDT increase the expression of xCT transporters and intracellular GSH, and decrease lipid peroxidation against erastin. These effects are comparable to those of F-1. D3T and ACDT also inhibit RSL3-induced lipid peroxidation and cell death, collectively demonstrating their anti-ferroptotic effects in U-87 MG cells.
Supplementary Files

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