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ORIGINAL PAPER

Disubstituted Dithiolethione ACDT Exerts Neuroprotective Effects Against 6-Hydroxydopamine-Induced Oxidative Stress in SH-SY5Y Cells

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Abstract
Parkinson's disease (PD) is a prevalent, progressive, neurodegenerative disorder with no known cure. Oxidative stress has been found to play a significant role in its etiology, and the search for novel neuroprotective compounds that actively prevent disease progression is currently ongoing. Dithiolethiones are a group of sulfur-containing heterocyclic compounds found in cruciferous vegetables. Using the 6-hydroxydopamine (6-OHDA) model of PD, we tested a previously identified disubstituted dithiolethione 5-amino-3-thioxo-3*H*-(1,2) dithiole-4-carboxylic acid ethyl ester (ACDT) for its neuroprotective potential. Pretreatment of SH-SY5Y cells with ACDT led to a time- and concentration-dependent induction of the antioxidant glutathione (GSH). ACDT also diminished 6-OHDA-induced cell death, lactate dehydrogenase release, elevation of caspase 3/7 activity, and increase in levels of reactive oxygen species. Inhibition of the GSH-synthesizing enzyme glutamate-cysteine ligase catalytic subunit (GCLC) led a corresponding dissipation of ACDT's neuroprotective effects, hence underlining the importance of GSH in ACDT's neuroprotective response. ACDT caused the stabilization and nuclear translocation of nuclear factor erythroid-2 related factor (Nrf2), resulting in increased protein expression of the phase II enzyme NADPH:quinone oxidoreductase 1 (NQO1), and the excitatory amino acid cysteine membrane transporter (EAAT3). Interestingly, no changes in the levels of other Nrf2-dependent molecules including GCLC were observed, indicating the possible involvement of additional alternate mechanisms behind ACDT's GSH-inducing property. Collectively, the data demonstrated ACDT to be a promising new dithiolethione for the treatment of PD, with two modifiable functional groups offering additional avenues for enhanced pharmacological application.

Keywords ACDT · Dithiolethione · Glutathione · Nrf2 · Neuroprotection · Oxidative stress

Introduction

Parkinson's disease (PD) is a neurological disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra resulting in classic motor abnormalities such as bradykinesia with tremor or rigidity [1]. Neuronal cells display high metabolic activity and are extremely prone to cellular damage induced by oxidative stress [2]. This oxidative stress can occur due to an increased generation of reactive oxygen species (ROS) or an overwhelmed cellular capacity to detoxify these reactive molecules. The presence of pro-oxidative molecules such as dopamine (DA), iron, and neuromelanin in the substantia nigra make this region more vulnerable to oxidative damage compared to other brain regions [3–5]. This is because DA can be oxidized to superoxides and DA quinones, and can react with reduced iron to form reactive hydroxyl radicals via the Fenton reaction [2]. Neuromelanin can also bind to iron and catalyze the production of ROS [6]. An overall increase in ROS levels can cause damage to cell lipid membranes, alter mitochondrial membrane potential (MMP), disrupt DNA structure, and thereby result in the disintegration of dopaminergic neurons [7–10]. Not surprisingly, increased levels of oxidized lipids and nucleic acids have been found in post mortem analyses of substantia nigra samples from PD patients [11, 12].

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To protect themselves from damage, neurons have developed the capability to synthesize cytoprotective antioxidants and enzymes upon detection of oxidative stress. Among the most inducible cytoprotective molecules are the antioxidant glutathione (GSH) and the phase II enzyme NADPH:quinone oxidoreductase 1 (NQO1) [13, 14]. GSH, by acting directly or by serving as a co-substrate in other enzymatic reactions can help detoxify ROS, thereby mitigating oxidative stress [15]. Amongst other functions, NQO1 can reduce quinones directly to hydroquinones, thereby bypassing the formation of reactive semiquinone molecules [16–18]. The substantia nigra has low levels of GSH, another factor which predisposes this brain region to damage by oxidative insult [19]. Further depleted levels of GSH [13, 20–22] and a corresponding rise in oxidative stress [23] have been hallmark features in substantia nigra samples from PD patients.

Given the importance of oxidative stress in the underlying pathophysiology of PD, strategies using antioxidants such as exogenous GSH, GSH precursor N-acetylcysteine (NAC), vitamin C, neuromelanin, and silymarin have been tested for their neuroprotective effects against PD [24–29]. Unfortunately, limited bioavailability, poor permeability through the blood brain barrier, and related adverse effects have raised questions regarding the efficacy of these approaches [30–32]. In recent years, a novel strategy designed to induce the synthesis of endogenous antioxidants in neuronal cells by using chemical inducers such as dithiolethiones has gained interest. Dithiolethiones are naturally occurring sulfur-containing compounds found in cruciferous vegetables. The simplest member of this class, namely 3H-1,2-dithiole-3-thione (D3T), increases GSH synthesis in hepatic and cardiovascular cells [33, 34]. It has been found to be neuroprotective against standard oxidative stressors such as dopamine, 6-hydroxy dopamine (6-OHDA), hydrogen peroxide (H_2O_2), 4-hydroxy-2-nonenal (HNE), and acrolein [35, 36]. D3T has also been found to be an effective neuroprotectant in animal models of autoimmune encephalomyelitis [37, 38], ischemic stroke [39], and Alzheimer's disease [40, 41], thus demonstrating high bioavailability and appropriate blood brain barrier permeability.

In our search for a dithiolethione which would resemble the pharmacodynamic profile of D3T but offer the additional advantage of modifiable functional groups allowing for physicochemical and pharmacokinetic enhancements, we pursued compound 6a [42]. In this previously published structure–activity relationship study, the disubstituted dithiolethione 5-amino-3-thioxo-3*H*-(1,2) dithiole-4-carboxylic acid ethyl ester called compound 6a (henceforth referred to as ACDT) was identified to be the most effective at inducing GSH in neuronal cells. This was attributed to the presence of an electron-withdrawing ester at position 4 and an electron-donating amino group at position 5 of this compound (Fig. 1). The current study was designed to establish a detailed pharmacological profile of this relatively understudied dithiolethione using an in vitro model of PD. We assessed the pre-treatment effects of ACDT in SH-SY5Y cells exposed to 6-OHDA. A significant increase in total GSH levels and overall cell viability, and a corresponding decrease in markers of oxidative stress established this disubstituted dithiolethione to have strong neuroprotective properties. Further investigation for an underlying mechanism of action indicated numerous similarities and a few noteworthy differences from D3T, leaving some unanswered questions open for future exploration.

Materials and Methods

Chemicals and Materials

The dithiolethione ACDT was purchased from Oakwood Chemical (West Columbia, SC). A stock solution (50 mM) was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich Corporation, Natick, MA), and further diluted in media to obtain test concentrations (final DMSO concentration 0.1% v/v). Dulbecco's modified Eagle medium:nutrient mixture (DMEM:F12), fetal bovine serum (FBS), trypsin–EDTA solution, Dulbecco's phosphate buffered saline (DPBS), and penicillin–streptomycin solution were purchased from GE Healthcare Life Sciences (Uppsala, Sweden).

Cell Culture

The human neuroblastoma SH-SY5Y cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in DMEM:F12 media (1:1), supplemented with FBS (10%), penicillin (100 U/mL), and streptomycin (100 µg/mL) in 150 cm^2 tissue culture flasks in a humidified atmosphere at 37 °C and 5% CO_2. The media was replaced every 3–4 days, and cells were subcultured using a trypsin–EDTA solution once they reached 70–80% confluence.

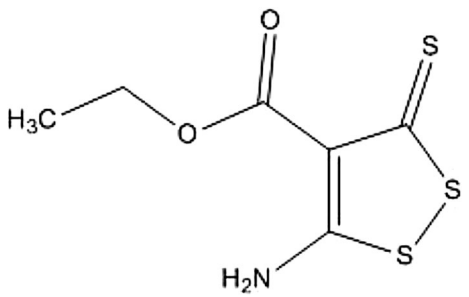

Fig. 1 Chemical structure of ACDT (5-amino-3-thioxo-3*H*-(1,2) dithiole-4-carboxylic acid ethyl ester)

Assay for Cell Viability and Neuroprotection

SH-SY5Y cells (5×10^4 cells/well) were seeded in 96-well plates. After an overnight attachment period, the media was replaced with fresh media containing ACDT (6.25–100 μM) or DMSO (0.1% v/v) for 24 h. The CellTiter-Glo® Luminescent Cell Viability Assay from Promega (Madison, WI) was used to measure cellular viability and establish the cytotoxicity profile for ACDT. To measure the neuroprotective potential of ACDT against 6-OHDA (Millipore Sigma, St. Louis, MO), an additional 24 h co-incubation with 40 μM 6-OH prepared in DMEM:F12 (1:1) media was allowed before performing the MTT cell viability assay (Alfa Aesar, Ward Hill, MA). To assess the impact of glutamate-cysteine ligase catalytic subunit (GCLC) inhibition on this neuroprotection, cells were co-incubated with ACDT (50 μM) and 1.25–20 μM buthionine sulfoximine (BSO, Millipore Sigma) for 24 h before the addition of 6-OHDA (40 μM) for an additional 24 h. Cell viability was expressed as a percentage of control.

Assay for LDH Cytotoxicity

SH-SY5Y cells (5×10^4 cells/well) in 96-well plates were exposed to ACDT (0–50 μM) for 24 h, followed by the addition of 6-OHDA (40 μM) for another 24 h. The LDH release was measured using the Pierce™ LDH Cytotoxicity Assay Kit (ThermoFisher Scientific) using the manufacturer's instructions, and values were expressed as fold-change of control.

Assay for Total GSH Content

Total glutathione levels were measured using the GSH/GSSG-Glo™ Assay kit (Promega). SH-SY5Y cells were seeded in 96-well plates (2×10^4 cells/well) and allowed to attach overnight. Media was removed and replaced with fresh media containing ACDT (6.25–100 μM) for 24 h, or 50 μM ACDT for a range of time points. Control cells received 0.1% v/v DMSO. To assess GSH induction in the presence of 6-OHDA, cells were incubated with ACDT for 24 h, followed by co-incubation with 6-OHDA (40 μM) for an additional 24 h prior to performing the assay. The impact of GCLC inhibition was measured by co-incubating the cells with 50 μM ACDT and a range of BSO concentrations (1.25–20 μM) for 24 h, followed by the measurement of total GSH levels. Values were expressed as a percentage of control.

Measurement of ROS Levels

SH-SY5Y cells (5×10^4 cells/well) were seeded into poly-D-lysine coated black opaque clear bottom 96-well plates and allowed to attach overnight. The media was replaced with that containing 50 or 100 μM ACDT for 24 h. Cells were then washed with FluoroBrite-DMEM and stained with FluoroBrite-DMEM containing 20 μM CM-H_2DCFDA dye (ThermoFisher Scientific) for 30 min. Next, the cells were rinsed and incubated with FluoroBrite-DMEM for an additional 30 min to allow for cell recovery. The cells were then co-incubated with ACDT and the ROS inducers H_2O_2 (300 μM) or 6-OHDA (100 μM) for 2 h. The fluorescence intensities were acquired by the Synergy HT micro plate reader (Biotek, Winooski, VT) at 485/20 nm and 528/20 nm, for excitation and emission, respectively.

Assay for Caspase 3/7 Activity

SH-SY5Y cells (5×10^4 cells/well) were plated in 96-well plates and were exposed to media containing ACDT (50 μM) for 24 h, followed by co-incubation with 40 μM 6-OHDA for additional 7 h. The caspase 3/7 activity was measured using the Caspase-Glo® 3/7 Assay Systems kit from Promega following the manufacturer's instructions. Values were expressed as fold-change of control.

Western Blotting for Protein Levels

SH-SY5Y cells were seeded in 6-well plates (2×10^6 cells/well), and allowed to attach overnight. The media was replaced with that containing ACDT (range of concentrations or different incubation times). The control cells received 0.1% v/v DMSO. Following the appropriate incubation period, the drug-containing media was aspirated, and the cells were lysed with RIPA buffer containing the Pierce™ Protease Inhibitor (ThermoFisher Scientific, Cambridge, MA). The samples were then centrifuged at 6000 rpm for 5 min at 4 °C. The supernatants were collected and assessed for protein levels using the Pierce BCA Assay Kit (ThermoFisher Scientific). Equal amounts of protein were loaded and resolved using 12% Mini-Protean® TGX™ Precast Protein Gels (BioRad, Hercules, CA) and transferred to an Immobilon-FL PVDF membrane (Millipore Sigma). After membrane blocking at 25 °C for 1 h, the levels of different proteins were measured using the following dilutions of primary polyclonal rabbit antibodies (Proteintech Group, Rosemont, IL)—GCLC, glutamate-cysteine ligase modifier subunit (GCLM), and NQO1 (1:1000); beta actin and GAPDH (1:2000); nuclear factor erythroid-2 related factor (Nrf2) (1:500); and Kelch-like ECH-associated protein 1 (Keap1) (1:5000). Cell membrane fractions were isolated using MEM-Per Plus (ThermoFisher Scientific) according to manufacturer's protocol, and the blotted PVDF membranes were incubated with excitatory amino acid transporter 3 (EAAT3) (1:1000) primary antibodies (Abcam, Cambridge, MA). After overnight incubation at 4 °C and subsequent

washing, the membranes were incubated with a fluorophore-linked secondary IRDye® 680RD goat anti-rabbit antibody (1:5000, 1 h at 25 °C) from LI-COR (Lincoln, NE). The membranes were then scanned and analyzed using the LI-COR Odyssey® Fc Imaging System.

Quantitative PCR for mRNA Levels

SH-SY5Y cells were seeded in 6-well plates (1×10^6 cells/well) and allowed to adhere overnight. Cells were then treated with either DMSO or different concentrations of ACDT for multiple time points. Culture media was then removed, and total RNA was isolated, purified, and quantified using the TRIzol™ reagent (ThermoFisher Scientific). cDNA was formed from purified RNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific), and qPCR was then performed using TaqMan chemistry (ThermoFisher Scientific).

Immunocytochemistry for Nrf2 Nuclear Translocation

The procedure was performed as previously described [43]. Briefly, SH-SY5Y cells were cultured in 8-well Lab-TekTM II Chamber Slides (Nunc™) and allowed to grow for 2 days. Cells were then exposed to 50 µM ACDT for 2 or 4 h. Cells were rinsed and fixed with PBS and 4% paraformaldehyde in PBS, respectively. Next, the slides were permeabilized with 0.1% Triton-X (Sigma-Aldrich) and blocked with 4% BSA, 10% goat-serum and 0.3 M glycine in PBS-Tween 20 (0.1%). Subsequently, the slides were exposed to anti-Nrf2 antibody (1:100) overnight at 4 °C. The slides were washed and then incubated with Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:400, Abcam). Following immunostaining, f-actin was labelled with Actin Red-555 reagent (ThermoFisher Scientific) for 20 min and the slides were then mounted with diamond mounting medium containing DAPI (Thermo Fisher). The slides were finally visualized using the epifluorescence microscope Nikon Eclipse E600 and the pictures were edited using the platform Fiji-ImageJ [44].

Nrf2 Gene Silencing

SH-SY5Y cells were plated in 6-well plates (2×10^6 cells/well) using antibiotic-free media and allowed to reach 30–50% confluence. Transfection was carried out using small interfering RNA (siRNA) for Nrf2 and GAPDH based on the manufacturer's instructions using Lipofectamine® 2000. OPTI-MEM®I Reduced Serum Medium was used to make all transfection solutions. Control cells received only transfection reagents (no siRNA). After a 24 h incubation with siRNA followed by a 24 h incubation with fresh antibiotic-free media, half the cells from each group received ACDT (50 µM). Total GSH was measured 24 h later using the GSH/GSSG-Glo™ assay kit (Promega) and values were expressed as percentage of control. All reagents for transfection were purchased from ThermoFisher Scientific.

Statistical Analysis

Data has been expressed as mean ± S.E.M (n = 3 to 6). Analysis of differences between mean values of multiple groups was performed using one-way ANOVA followed by the post hoc Dunnett's multiple comparison test. Data were considered to be statistically different at $p < 0.05$.

Results

Impact of ACDT on Cell Viability

To assess if ACDT alone had any impact on cell viability, SH-SY5Y cells were treated with various concentrations (6.25, 12.5, 25, 50, and 100 µM) of this compound for 24 h. One-way ANOVA depicted no significant cytotoxicity for concentrations up to 100 µM (data not shown).

Concentration- and Time-Dependent Effects of ACDT on GSH Levels

ACDT was found to increase the levels of total GSH in a concentration-dependent manner ($F_{5,18} = 74.81$, $p < 0.0001$), and this increase was statistically significant in cells exposed to 50 µM (2-fold) and 100 µM (2.9-fold) ACDT for 24 h (Fig. 2a). Based on this data, the lower 50 µM concentration was selected for further exploration. SH-SY5Y cells exposed to 50 µM ACDT also led to a time-dependent increase in total GSH levels ($F_{5,18} = 3.885$, $p = 0.0145$), with effects being significant with 6, 8, and 24 h incubation periods (Fig. 2b). The 24 h incubation period was selected for consequent experiments to be consistent with other in vitro studies on dithiolethiones [35, 36].

Concentration-Dependent Effects of ACDT on 6-OHDA-Induced Lactate Dehydrogenase (LDH) Release

Neuronal cell exposure to the oxidative stressor 6-OHDA is an established in vitro model of PD. As expected, exposure to 6-OHDA led to a 23-fold increase LDH release, which is considered a marker for cell cytotoxicity. The effect was significantly mitigated by a 24 pre-treatment with 25 or 50 µM ACDT (from 23-fold to 13- and ninefold respectively; $F_{4,10} = 7.154$, $p = 0.0055$), which was indicative of significant neuroprotection (Fig. 3a).

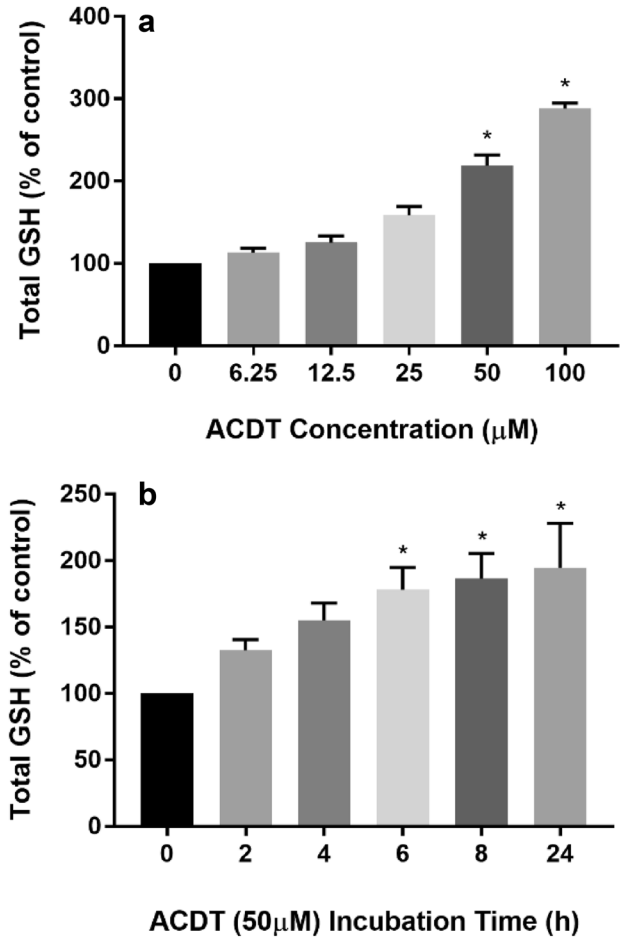

Fig. 2 Concentration- and time-dependent induction of GSH by ACDT in SH-SY5Y cells. Cells were treated with increasing concentrations of ACDT for 24 h (**a**), or 50 μM ACDT for increasing periods of time (**b**), and levels of total GSH were measured. Data are represented as percent change from DMSO control, and values are expressed as mean ± S.E.M. *p < 0.05 versus DMSO control

Concentration-Dependent Effects of ACDT on 6-OHDA-Induced Decrease in Cell Viability

Incubation with 6-OHDA (40 μM for 24 h) led to an expected decrease in cell viability (to 19% of control) as determined by the MTT assay. However, a 24 h pre-treatment with 50 μM ACDT was able to significantly protect the cells from these toxic effects (Fig. 3b), increasing the cell viability to 46% of control ($F_{4,10} = 40.99$, $p < 0.0001$).

Concentration-Dependent Effects of ACDT on 6-OHDA-Induced Increase in Total GSH Levels

In cells pretreated with increasing concentrations of ACDT for 24 h followed by the addition of 6-OHDA (40 μM) for another 24 h, levels of total GSH normalized to cell viability

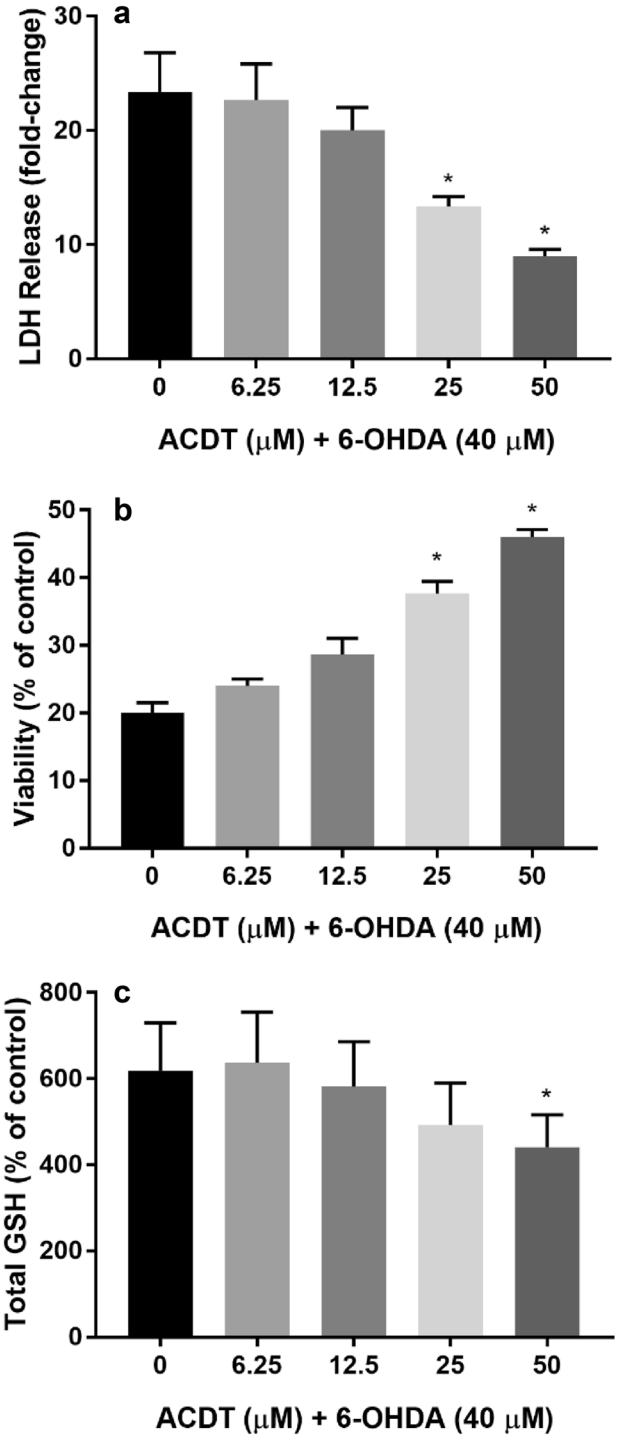

Fig. 3 Neuroprotective effects of ACDT against oxidative stress. SH-SY5Y cells were pre-treated with 50 μM ACDT for 24 h, followed by the addition of 40 μM 6-OHDA for another 24 h. Fold-change in LDH enzyme release (**a**) percent cell viability (**b**) and total GSH levels normalized to cell viability (**c**) were measured. Values are expressed as mean ± S.E.M. *p < 0.05 versus 6-OHDA alone

were found to decrease in a concentration-dependent manner (Fig. 3c; $F_{4,10} = 64.98$, $p < 0.0001$).

Concentration-Dependent Effects of ACDT on 6-OHDA or H_2O_2-Induced Increase in ROS Levels

The effects of ACDT pre-treatment on ROS generation by standard oxidative stressors were measured next. Both 6-OHDA and H_2O_2 are known inducers of ROS [45, 46], and a 2 h incubation of SH-SY5Y cells with 100 and 300 µM concentrations of these stressors respectively led to a significant increase in ROS levels (Fig. 4a; $F_{4,75} = 303.8$, $p < 0.0001$ against 6-OHDA; $F_{4,75} = 171.9$, $p < 0.0001$ against H_2O_2). Pre-treatment with 100 µM ACDT was effective at lowering ROS levels generated by both toxicants, while the 50 µM ACDT concentration was only effective against oxidative stress induced by 300 µM H_2O_2.

Effect of ACDT on Caspase 3/7 Activity

Increased caspase 3/7 activity is indicative of impending apoptotic cell death. Cells exposed to 40 µM 6-OHDA for 7 h displayed significantly higher caspase 3/7 activity compared to control cells. A 24 h pre-treatment with 50 µM ACDT significantly mitigated this increase (from 4 to 2.6-fold; $F_{3,8} = 19.63$, $p = 0.0005$). ACDT alone had no effect on caspase 3/7 activity (Fig. 4b). The short 7 h exposure to 6-OHDA was selected as this time point appropriately captured the effects of ACDT on this early pro-apoptotic event.

Role of GSH in Modulating ACDT Effects

To explore the role of GSH in the neuroprotective effects of ACDT, the GCLC inhibitor BSO was utilized. The addition of increasing concentrations of BSO (0–20 µM) to the incubation mixture resulted in a concentration-dependent decrease in the level of total GSH induction by ACDT (207% to 37% of control; $F_{6,14} = 235.9$, $p < 0.0001$) (Fig. 5a). In addition, BSO was also found to oppose the neuroprotective effects of 50 µM ACDT against 6-OHDA in a concentration-dependent manner ($F_{7,16} = 113.8$, $p < 0.0001$). BSO had no direct impact on cell viability (Fig. 5b).

Role of the Nrf2 Pathway in ACDT Effects

Protein levels of GCLC and NQO1, enzymes known to be induced by the Nrf2 pathway, were measured in response to increasing concentrations of ACDT. Interestingly, while NQO1 levels showed a concentration-dependent rise ($F_{5,12} = 9.114$, $p = 0009$), GCLC levels did not show a significant change (Fig. 6a). Time points earlier than 24 h also did not depict a change in GCLC levels, while NQO1 levels showed a marked increase as early as 16 h (Fig. 6b; $F_{6,14} = 12.81$, $p < 0.0001$). In a time course study, protein levels of the Nrf2 repressor Keap1 remained unchanged, but levels of Nrf2 itself were found to increase within 2 h of exposure to 50 µM ACDT (Fig. 6c; $F_{6,14} = 2.892$, $p = 0.0476$). The same 2 h time point also depicted a significant increase in protein levels of the excitatory amino acid cysteine transporter EAAT3 in the membrane fraction of ACDT treated neurons (Fig. 6d; $F_{3,8} = 4.953$, $p = 0.0313$). GCLM did not show any changes between the 0 and 24 h exposure window (data not shown).

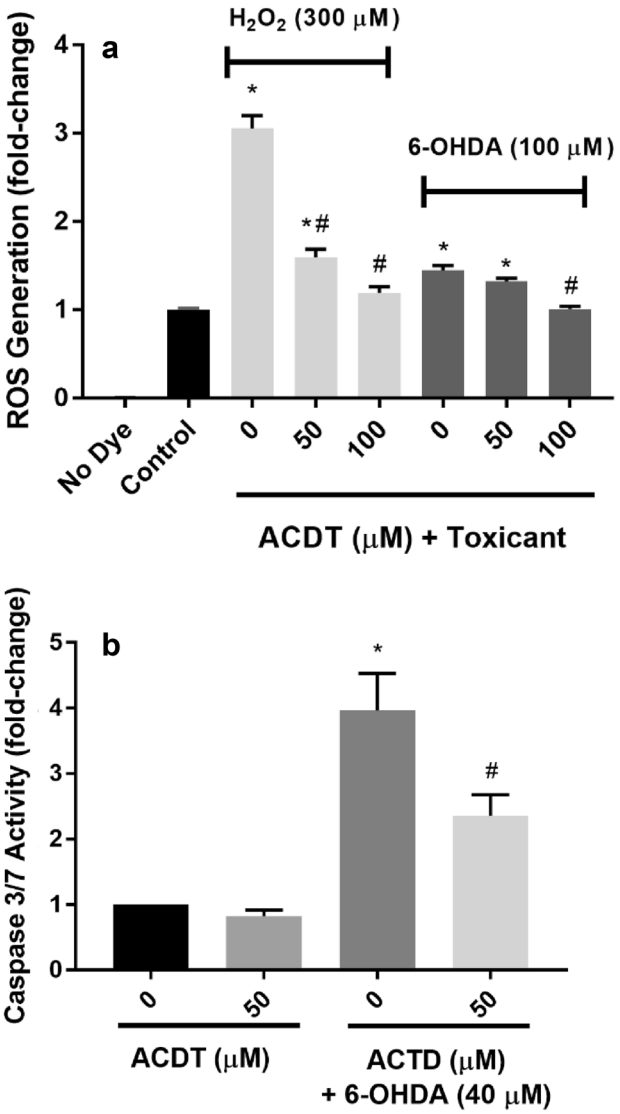

Fig. 4 Mechanisms underlying neuroprotective effects of ACDT against oxidative stress. SH-SY5Y cells were pre-treated with ACDT for 24 h, followed by the addition of oxidative stressors. Fold-change in ROS levels generated by a 2 h exposure to 6-OHDA or H_2O_2 (**a**), and fold-change in caspase 3/7 activity with a 7 h exposure to 6-OHDA (**b**) were measured. Values are expressed as mean ± S.E.M. *$p < 0.05$ versus DMSO control, #$p < 0.05$ versus toxicant alone

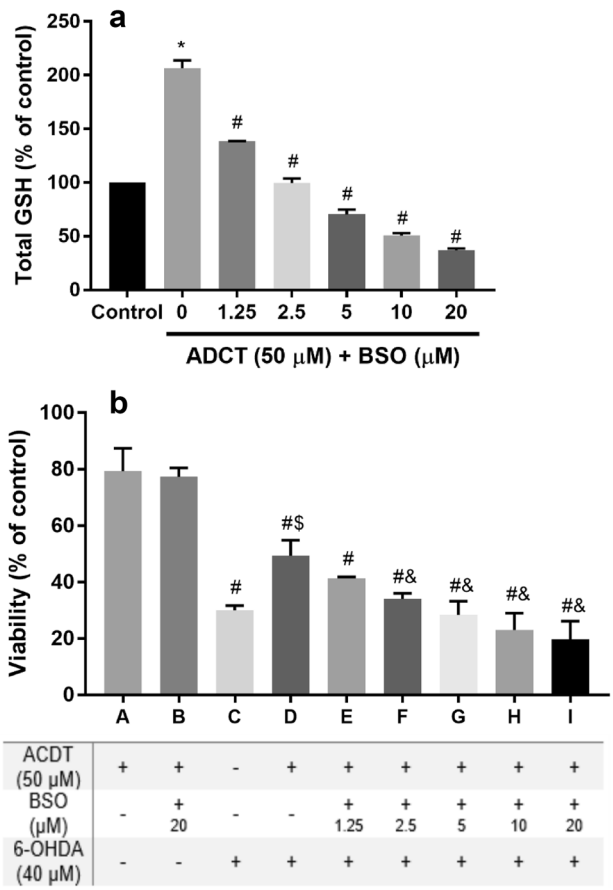

Fig. 5 Role of GSH in modulating ACDT effects. Percent change in total GSH levels in SH-SY5Y cells co-incubated with ACDT and indicated concentrations of GCLC inhibitor BSO for 24 h (**a**). Percent change in cell viability of SH-SH5Y cells co-incubated with ACDT and indicated concentrations of BSO for 24 followed by the addition of 6-OHDA for another 24 h (**b**). Values are expressed as mean ± S.E.M. $^{*}p < 0.05$ versus DMSO control, $^{\#}p < 0.05$ versus ACDT alone, $^{\$}p < 0.05$ versus 6-OHDA alone, $^{\&}p < 0.05$ versus ACDT + 6-OHDA combination

A time course study to measure the mRNA levels of various Nrf2-associated genes was also performed in cells exposed to 50 µM ACDT. The mRNA levels of NQO1 were found to be significantly higher at 6 and 24 h incubation times ($F_{5,18} = 8.292$, $p = 0.0003$). The mRNA levels of GCLC, Nrf2, Keap1, EAAT3, glutathione-disulfide reductase (GSR), glutathione S-transferase pi 1 (GSTP1), glutathione peroxidase (GPx), superoxide dismutase (SOD), and heme oxygenase 1 (HMOX1) were not found to be significantly altered at any of the time points studied (Table 1). However, immunofluorescence images depicted increased Nrf2 nuclear translocation beginning as early as 2 h with ACDT exposure (Fig. 7a). Additionally, silencing the *Nrf2* gene using siRNA did lead to the suppression of the GSH induction response to 50 µM ACDT (Fig. 7b; $F_{3,8} = 11.96$, $p = 0.0025$).

Discussion

Compared to most other organs, the brain uses a disproportionately higher amount of oxygen per volume of tissue [47] which can lead to an excessive generation oxygen free radicals. The resulting oxidative stress has been implicated in the etiology and progression of numerous neurodegenerative disorders including PD. This condition is associated with the selective loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain. Clinically available treatment options for PD include dopamine precursors, dopamine receptor agonists, and inhibitors of dopamine metabolism. These drugs are associated with both peripheral and central adverse effects. In addition, these drugs offer only symptomatic relief, doing little to alter the course of this chronic, progressively debilitating disease. Unfortunately, exogenous antioxidants such as GSH, NAC, vitamin C, vitamin E, creatine, coenzyme Q10, and silymarin tested in in vitro and in vivo models of PD have provided mixed results [25, 26, 29, 48–54]. Given the high prevalence and economic burden currently associated with PD, the search for novel neuroprotective agents which halt or at least delay the advance of this disease is of prime focus in current scientific inquiry. Recent studies on the sulfur-containing heterocyclic molecules called dithiolethiones have generated promising data. These compounds, particularly D3T and oltipraz, have been shown to cause a coordinated induction of cytoprotective enzymes such as GSH and NQO1 in hepatic, cardiovascular, and cancerous cells/tissues [33, 34, 55–57]. Consequent studies with D3T, the simplest molecule in this chemical series, have established that these effects also extend to neuronal cells [35, 36, 58].

Dithiolethiones have been found to utilize the Nrf2 pathway to activate a cellular cytoprotective machinery [37, 39, 59–62]. Nrf2 is a highly unstable transcriptional factor, normally tethered to the Keap1 protein in the cytoplasm. Keap1 is a cysteine-rich molecule that routes the bound Nrf2 towards ubiquitylation and consequent degradation. In the presence of oxidative stress however, the sulfhydryl groups on the cysteine-rich Keap1 molecule undergo oxidation/alkylation, modifying its structure and thereby diminishing its ability to bind to and repress Nrf2. The free stabilized Nrf2 then migrates to the nucleus, whereupon it binds to the antioxidant response element (ARE) and leads to the transcription of numerous antioxidant genes. These include genes for NQO1 and the GSH-synthesizing enzyme GCLC [63–67]. Disulfides derived from dithiolethiones are hypothesized to react with molecular oxygen to generate a small quantity of H_2O_2. This low degree of oxidative stress produced can then activate the cell's ARE via the Nrf2 pathway, exponentially increasing antioxidant synthesis and initiating a pronounced cytoprotective response [34, 36, 68, 69].

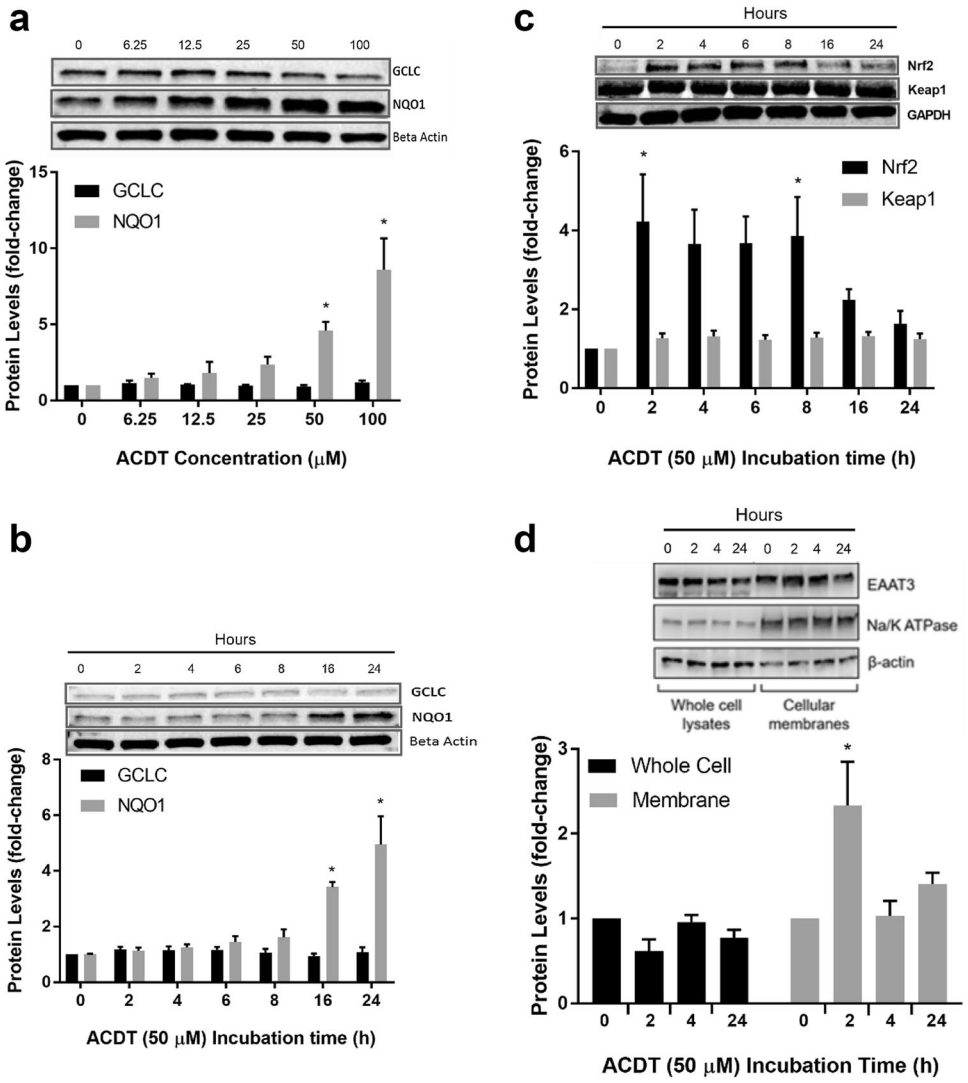

Fig. 6 Effects of ACDT on levels of Nrf2 pathway associated proteins. SH-SY5Y cells were exposed to ACDT at indicated concentrations or exposure times, and Western blot analysis was performed to measure the protein levels of GCLC and NQO1 (**a** and **b**), Nrf2 and Keap1 (**c**), and EAAT3 in whole cell and cell membrane fractions (**d**). Values are expressed as mean ± S.E.M. *p < 0.05 versus DMSO control

With the non-substituted dithiolethione D3T already established to be a promising neuroprotective candidate based on both in vitro [35, 36, 58] and in vivo [37–39] studies, we decided to explore a substituted compound in the same chemical family. We previously identified ACDT to have the optimum chemical structure to retain GSH-inducing properties of D3T with the added advantage of modifiable functional groups to enhance pharmacodynamic-pharmacokinetic (PKPD) properties [42]. To delineate the pharmacological profile of this molecule, we utilized undifferentiated human SH-SY5Y neuroblastoma cells which are generally considered to mimic dopaminergic neurons of the substantia nigra [70]. We also selected 6-OHDA as an exogenous toxicant, which is a widely accepted in vitro model of PD. The neurotoxic effects of 6-OHDA are attributed to the generation of ROS, quinones, and semiquinone radicals [71]. Both GSH and NQO1 play key roles in alleviating the oxidative stress induced by this neurotoxicant. Consistent with a previous studies [38, 42], we found ACDT to be non-toxic at concentrations up to 100 μM. In addition, a concentration–response curve for GSH induction established that 50 μM ACDT caused a significant (more than twofold) increase in total GSH levels with an exposure time as short as 6 h (Fig. 2a and b). The GSH levels measured here are a sum total of reduced (GSH) and oxidized (GSSG) forms of this antioxidant. An increase in this parameter therefore indicates an ACDT-induced increase in de novo GSH synthesis as opposed to a simple amplification of the GSSG recycling response. GSH induction by ACDT is in line with previously published data [35, 36, 38, 42, 72].

To assess if the induction of this cytoprotective molecule also translated to a corresponding neuroprotective effect, we measured the effects of ACDT pretreatment on 6-OHDA-induced changes in cell viability and LDH release. In line with a previous study from our group [42], we found ACDT to exert neuroprotective effects at a concentration as low as 25 μM (Fig. 3a and 3b). In addition, pretreatment with ACDT resulted in a concentration-dependent reduction in

Table 1 Time-dependent effects of ACDT on mRNA levels of Nrf2 pathway-associated factors and other antioxidant enzymes

Incubation period with 50 µM ACDT	mRNA levels (fold-change)								
	GCLC	NQO1	Nrf2	Keap1	EAAT3	GSR	GPx	GSTP1	SOD	HMOX1
0 h	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
2 h	0.83 ± 0.11	1.04 ± 0.08	0.87 ± 0.05	0.75 ± 0.11	0.86 ± 0.07	0.69 ± 0.05	1.08 ± 0.26	0.88 ± 0.08	1.25 ± 0.32	1.05 ± 0.22
4 h	1.36 ± 0.46	1.67 ± 0.31	1.27 ± 0.34	1.32 ± 0.24	1.31 ± 0.16	1.10 ± 0.08	1.17 ± 0.25	0.88 ± 0.03	1.16 ± 0.17	1.67 ± 0.55
6 h	1.86 ± 0.55	2.47 ± 0.33*	1.15 ± 0.12	1.85 ± 0.37	1.33 ± 0.10	1.43 ± 0.21	1.39 ± 0.25	1.01 ± 0.12	1.31 ± 0.04	1.77 ± 0.49
8 h	0.99 ± 0.08	2.19 ± 0.47	0.95 ± 0.01	1.30 ± 0.27	1.17 ± 0.08	1.20 ± 0.06	1.05 ± 0.11	0.97 ± 0.04	1.36 ± 0.21	1.94 ± 0.31
24 h	1.28 ± 0.26	3.78 ± 0.57*	0.85 ± 0.16	1.05 ± 0.25	1.12 ± 0.17	0.99 ± 0.05	1.12 ± 0.17	0.84 ± 0.06	1.24 ± 0.27	2.08 ± 0.14

γ-Glutamylcysteine synthetase catalytic subunit (GCLC); NADPH:quinone oxidoreductase 1 (NQO1); nuclear factor erythroid-2 related factor (Nrf2); Kelch-like ECH-associated protein 1 (Keap1); excitatory amino acid cysteine transporter (EAAT3); glutathione-disulfide reductase (GSR); glutathione peroxidase (GPx); glutathione S-transferase pi 1 (GSTP1); superoxide dismutase (SOD); heme oxygenase 1 (HMOX1). Data are represented as fold-change from untreated control, and values are expressed as mean ± S.E.M

*$p < 0.05$ versus untreated control

6-OHDA-induced GSH production (Fig. 3c). While this data may appear counterintuitive, it likely demonstrates an overall improvement in the health of ACDT-pretreated cells. We hypothesize that pretreatment of cells with ACDT prior to 6-OHDA exposure allowed these cells to proactively synthesize additional GSH. This excess GSH then served as a buffer, hence lowering the degree of protective cellular mechanisms initiated with consequent exposure to an oxidative stress. Prior studies on the non-substituted dithiolethione D3T have reported its neuroprotective effects against various toxicants [35, 36, 72–75]. To the best of our knowledge, this is the first study to establish an in vitro concentration-dependent neuroprotection profile for the disubstituted dithiolethione ACDT.

Based on a well-established cell signaling pathway, an increase in cellular ROS levels leads to the disruption of MMP, release of cytochrome c, activation of downstream caspases 3 and 7, and consequently apoptotic cell death [76]. Pretreatment of cells with ACDT was found to significantly reduce the levels of ROS generation (Fig. 4a) and caspase 3/7 activation (Fig. 4b) by known oxidative stressors 6-OHDA and H_2O_2. Based on the aforementioned pathway, assays for these upstream events were performed with exposure times shorter than that for the observed apoptotic cell death (2 h, 7 h, and 24 h respectively). Concentrations for each stressor (100 µM and 300 µM, respectively) were selected to obtain a strong baseline ROS response. While prior results demonstrated that pre-treatment with 50 µM ACDT was enough to oppose the toxic effects of 40 µM 6-OHDA, this particular toxicant concentration was not used in this experiment due to insufficient baseline levels of ROS generated (data not shown). Note that even 100 µM 6-OHDA alone only resulted in a 1.4-fold increase in ROS levels. Overall, the above effects were expected given the significant induction of the powerful antioxidant GSH by this compound, and are in line with previous reports on the dithiolethione D3T [35, 36, 72, 73, 75, 77]. The crucial role played by GSH in ACDT's neuroprotective potential was further validated by measuring the effects of GCLC inhibition on this parameter. This enzyme catalyzes the first rate-limiting step in GSH synthesis. Increasing concentrations of the GCLC inhibitor BSO caused a successful concentration-dependent inhibition of ACDT's GSH-inducing properties (Fig. 5a), which led to a consequent loss of all neuroprotective effects (Fig. 5b). The 21% decrease in cell viability observed here in response to 50 µM ACDT (a previously established non-toxic concentration) can be explained based on the additional 24 h toxicant co-incubation period required in this experimental setup.

Previous studies on D3T have also reported activation of the Nrf2 pathway as its mechanism of action. An increase in mRNA levels of Nrf2 dependent enzymes GCLC and NQO1 was used to explain the increase in GSH content and heightened NQO1 enzyme activity observed in these studies

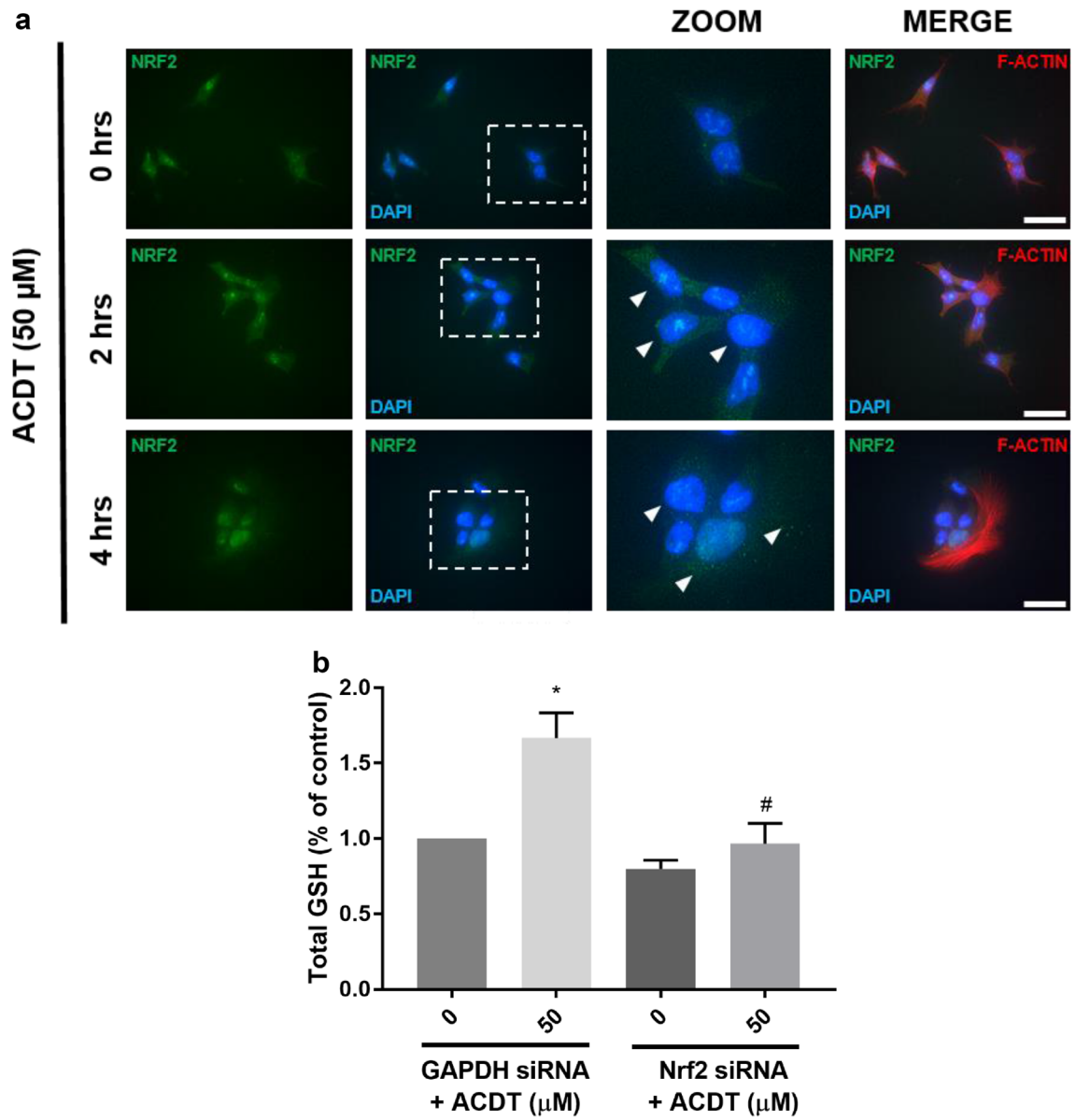

Fig. 7 Role of the Nrf2 pathway in ACDT effects. SH-SY5Y cells were exposed to 50 μM ACDT for increasing incubation times and Nrf2 nuclear translocation was visualized using immunofluorescence (**a**). The effects of *Nrf2* gene silencing on ACDT's GSH-inducing properties were measured (**b**). Data are represented as percent change from scrambled control, and values are expressed as mean ± S.E.M. *$p < 0.05$ versus scrambled control, $^{\#}p < 0.05$ versus scrambled control + ACDT combination

[35, 36, 72]. Interestingly, ACDT exposure did not cause a significant increase in GCLC protein expression (Fig. 6a and b). Given the importance of GCLC in catalyzing the rate-limiting step in GSH synthesis and the significant induction of GSH that was observed with ACDT exposure, an elevation in GCLC protein levels was expected. The activation of the Nrf2 pathway by ACDT could however not be ruled out, as the increase in protein levels of NQO1 was in fact significant. In a property shared with D3T, the protein levels of Nrf2 itself were also found to be increased in response to a short (2 h) exposure to ACDT (Fig. 6c) [37, 39, 40, 78]. This effect was time-dependent, and dissipated after 8 h. A change in Keap1 protein levels was not observed, likely because ACDT caused modifications in the cysteine residues of this molecule as a mechanism to stabilize Nrf2 without causing a direct reduction in actual protein levels. To further investigate the observed rise in total GSH unaccompanied by a corresponding increase in GCLC expression, we measured protein levels of the excitatory amino acid transporters EAAT3. These transporters are used by cysteine molecules to gain entry into the neurons. Cysteine is one of the three building blocks of the tripeptide antioxidant GSH, and its

intracellular levels are rate-limiting in GSH synthesis. The transcription of *EAAT3* is controlled by the Nrf2 pathway [79, 80]. ACDT exposure was found to cause a transient increase in the expression of EAAT3 transporters in the membrane fraction of neuronal cells (Fig. 6d). This effect can at least partially help explain the rise in total cellular GSH circumventing an increase in GCLC levels. In additional attempts to explain the observed increase in GSH levels, we also measured protein levels of GCLM. This subunit controls the level of activity of GCL and is also regulated by the Nrf2 pathway [81]. Elevated levels of GCLM such as those observed with D3T [35] would indicate an increased activity of GCL (as opposed to increased levels), which could help explain the observed increase in GSH production. Again, no significant changes in the levels of GCLM were observed with 0 to 24 h of exposure to ACDT (data not included).

To check if a response generated at the level of transcription was somehow diluted during translation, we proceeded to measure upstream mRNA levels of these biomolecules. In line with D3T [36], NQO1 mRNA levels showed a statistical increase which explained the corresponding elevation in NQO1 protein levels observed (Table 1). Interestingly Nrf2 and EAAT3, which also showed significant increase based on Western blot analyses, did not depict corresponding changes in mRNA levels. In addition to the modification of Nrf2-binding cysteine residues on Keap1 as a mechanism to increase Nrf2 stability, dithiolethiones have also been proposed to utilize a second pathway for this effect. A dithiolethione-initiated post-translational phosphorylation of the Nrf2 molecule itself by various intracellular kinases has been hypothesized to disrupt its interaction with the keap1 repressor protein [78, 82–86]. The lack of change in Nrf2 mRNA levels accompanied by an increase in Nrf2 protein expression suggests the latter mechanism to be at play. In other words, the increase in Nrf2 protein levels was likely due to the decreased degradation (not increased synthesis) of this transcriptional factor [87]. The extent of involvement of two aforementioned pathways in the Nrf2 stabilization by ACDT was not assessed in the current study, but would be of interest to delineate in the future.

Most importantly, contrary to D3T effects, we were unable to detect any significant changes in GCLC even at the transcriptional level. The mRNA levels of other antioxidant molecules such as GSR, GSTP1, GPx, SOD, and HMOX1 also did not depict any significant changes. Although mRNA levels for the first four enzymes listed above were also not found to change in SH-SY5Y cells exposed to 100 μM D3T [36], recent animal studies have reported an induction in HMOX1 upon exposure to D3T [37, 39, 40]. Based on differences between the two more well-studied dithiolethiones D3T and oltipraz, it has already been established that the effects of one dithiolethione cannot be extrapolated to all others [56, 88]. A lack of change in mRNA levels of GSR in response to ACDT further corroborates that the observed GSH induction is based on an upregulated de novo synthesis of this biomolecule as opposed to an increased recycling from its oxidized form. Within all the cytoprotective enzymes modulated by the Nrf2 pathway, NQO1 and GCLC are the two most inducible. While increased levels of NQO1 likely played an important role in the neuroprotective effects of ACDT, the role of GSH was shown to be key. Increased Nrf2 translocation into the cell nucleus was visibly captured using immunocytochemistry (Fig. 7a). As a final experiment, silencing the *Nrf2* gene was found to suppress ACDT-dependent GSH induction (Fig. 7b). This is in agreement with data from D3T treated *Nrf2* knockout animals showing a lack of drug response [37, 39]. Taken together, these results point towards the central role of GSH and the Nrf2 pathway in ACDT's neuroprotective effects.

Future studies include the measurement of protein levels of GCL as a whole (not separate subunits) and the enzyme activity of GSR, both of which have been reported to increase in response to D3T [35, 74, 75]. Another major avenue open for investigation is the hydrogen sulfide (H_2S) hypothesis. D3T has been proposed to be a H_2S donor, with this gasotransmitter indicated to contribute to D3T's neuroprotective effects [89]. In addition to activating the Nrf2 pathway [90], H_2S has been shown to serve as a positive allosteric modulator for GCL and an up-regulator of cystine transport into the cell [91]. The cysteine dimer cystine upon entering the cell can then be broken down into cysteine monomers to be used in the synthesis of GSH. H_2S has also been found to lower the activity of gamma-glutamyl transpeptidase [92], a Nrf2-dependent enzyme involved in the metabolic degradation of GSH [93, 94]. A recent study demonstrated a D3T-induced increase in Bcl-2 coupled with a decrease in Bax and caspase-3 protein levels in PC12 cells exposed to amyloid β_{1-42} [73]. The extracellular regulated protein kinase 1/2 (ERK1/2) pathway was proposed to be involved in these effects, which is a mechanism yet to be explored for the dithiolethione ACDT.

In conclusion, our studies demonstrated ACDT to be a promising candidate utilizing the Nrf2 pathway for its GSH inducing and neuroprotective effects. The current study identifies some key differences between D3T and ADCT with respect to protein- and mRNA-induction profiles of various Nrf2-associated biomolecules. Recent data from animal studies on D3T indicate a strong neuroprotective potential in a wide array of neurodegenerative conditions. Retaining the D3T pharmacophore, ACDT offers the advantage of containing two functional groups amenable for chemical modification. Even though a favorable in vivo PKPD profile for ACDT has been established [38], using these functional groups to further enhance these properties and to crosslink ACDT with other neuroprotective/antiparkinsonian

drugs has unexplored potential. With the clinical success of dimethyl fumarate as the only FDA-approved Nrf2 activator commercially available [95], further exploration of dithiolethiones as a potential novel therapy for PD is justified.

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