Dithiolethione ACDT Suppresses Neuroinflammation and Ameliorates Disease Severity in Experimental Autoimmune Encephalomyelitis

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Short title
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Abstract

Multiple sclerosis (MS) is an autoimmune disorder characterized by the central nervous system (CNS) infiltration of myelin-specific pathogenic T cells followed by brain inflammation in association with demyelination. Similarly, experimental autoimmune encephalomyelitis (EAE), the animal model of MS, also exhibits increased CNS infiltration of pathogenic T cells, including Th1 and Th17, leading to detrimental effects of neuroinflammation and demyelination. We previously reported that 3H-1,2-dithiole-3-thione (D3T), the structurally-simplest of the sulfur-containing dithiolethiones, exerted a promising therapeutic effect in EAE. In the current study we report that 5-Amino-3-thioxo-3H-(1,2)dithiole-4-carboxylic acid ethyl ester (ACDT), a substituted derivative of D3T, exhibits anti-inflammatory properties in EAE. ACDT, administered post immunization, delayed disease onset and reduced disease severity in chronic C57BL/6 EAE, and ACDT, administered during disease remission, suppressed disease relapse in relapsing-remitting SJL/J EAE. Further analysis of the cellular and molecular mechanisms underlying the protective effects of ACDT in EAE revealed that ACDT inhibited pathogenic T cell infiltration, suppressed microglia activation, repressed neurotoxic A1 astrocyte generation, lessened blood-brain barrier disruption, and diminished MMP3/9 production in the CNS of EAE.

In summary, we demonstrate that ACDT suppresses neuroinflammation and ameliorates disease severity in EAE through multiple cellular mechanisms. Our findings suggest the potential of developing ACDT as a novel therapeutic agent for the treatment of MS/EAE.

Keywords: MS/EAE; Microglia; Th1/Th17; A1 astrocyte; ACDT; Neuroinflammation; Blood-brain barrier
1. Introduction

Multiple sclerosis (MS) is an autoimmune disorder characterized by the CNS infiltration of myelin-specific pathogenic T cells and subsequent brain inflammation in association with myelin sheath deterioration (Fletcher et al., 2010; Kurschus, 2015; Yadav et al., 2015). Experimental autoimmune encephalomyelitis (EAE) is a commonly used animal model to study MS. Several T cell subsets, including CD4⁺ T cell lineages of Th1 and Th17, CD8⁺ T cells, and IL-17-producing γδT cells, are essential for the pathogenesis of EAE (Camara et al., 2013; Codarri et al., 2011; Grifka-Walk et al., 2015; Jager et al., 2009; McWilliams et al., 2015; Paul et al., 2015). In addition, antigen presenting cells such as peripheral dendritic cells and CNS microglial cells have been shown to play a pivotal role in the promotion of differentiation and reactivation of pathogenic T cells, respectively (Blink et al., 2009; Croxford et al., 2015).

In our previous study, we demonstrated the amelioration of EAE by the agent 3H-1,2-dithiole-3-thione (D3T) (Kuo et al., 2016). The promising in vivo activity of D3T, the structurally-simplest of the sulfur-containing dithiolethiones, is a composite of its physicochemical, pharmacokinetic, and pharmacodynamic properties. Further improvement in the activity of D3T can be made by optimizing these properties through structural modifications. As such, we aimed to determine the influence of modifications of D3T structure on EAE activity. We sought a molecule that retains the dithiolethione pharmacophore, but has functional groups that are easily modified. Our search identified compound 5-Amino-3-thioxo-3H-(1,2)dithiole-4-carboxylic acid ethyl ester (ACDT), a dithiolethione with ethyl ester and primary amine functional groups (Supplemental Fig. 1). We, therefore, decided to investigate the therapeutic efficacy of ACDT in EAE.
In the present study, we report that ACDT exerts anti-inflammatory effects on the amelioration of neuroinflammation in EAE. Our results showed ACDT, administered post immunization, delayed disease onset and reduced disease severity in chronic C57BL/6 EAE, and ACDT, administered during disease remission, effectively suppressed disease relapse in relapsing-remitting SJL/J EAE. Further investigation of cellular and molecular mechanisms underlying the protective effect of ACDT in EAE revealed that ACDT suppressed CNS infiltration of pathogenic T cells, including Th1 and Th17 cells. In addition, ACDT exhibited a suppressive effect on microglia (MG) activation in LPS-stimulated primary MG and in the CNS of EAE. Furthermore, through analyzing neuroinflammatory status in the CNS of vehicle- and ACDT-treated EAE mice, we found that several key inflammatory mediators related to EAE pathogenesis, including TNFα, osteopontin, ICAM-1, CD40, myeloperoxidase, CCL5, and CCL6, were suppressed by ACDT. The modulatory effect on the generation of A1 astrocytes, which have been shown to exert a neurotoxic effect (Liddelow et al., 2017), was also observed in the CNS of ACDT-treated EAE mice compared to that in vehicle-treated EAE controls. Importantly, ACDT treatment lessened blood-brain barrier (BBB) disruption, and that is associated with suppressed MMP3 and MMP9 production in the spinal cord of ACDT-treated EAE mice. In summary, we demonstrate that ACDT alleviates neuroinflammation in EAE through inhibiting pathogenic T cell infiltration, suppressing MG activation, and lessening BBB disruption in the CNS of EAE. Our findings suggest that ACDT could be developed as a potential therapeutic agent for the treatment of MS/EAE.

2. Material and Methods

2.1 Animals
C57BL/6 and SJL/J mice, purchased from The Jackson Laboratory (Bar Harbor, ME), were used in this study. Mice were bred with free accessed food and water, and housed in rooms with controlled temperature, humidity, and 12:12 hour light:dark cycle. All animal studies procedures were approved by the Purdue University Animal Care and Use Committee (PACUC).

2.2 Reagents

5-Amino-3-thioxo-3H-(1,2)dithiole-4-carboxylic acid ethyl ester (ACDT) was purchased from Oakwood Chemicals (West Columbia, SC). Complete Freund’s adjuvant (CFA), Solutol® HS 15, Evans blue, collagenase, deoxyribonuclease I (DNase I), phorbol myristate acetate (PMA), ionomycin, paraformaldehyde, hematoxylin, eosin, cresyl violet, and luxol fast blue (LFB) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for FACS analysis, including Alexa Fluor 488 anti-mouse CD4 (clone: RM4-5), APC anti-mouse IL-17A (clone: TC11-18H10.1), PE/Cy7 anti-mouse IFNγ (clone: XMG1.2), APC anti-mouse CD45 (clone: 30-F11), PE anti-mouse CD11b (clone: M1/70), APC anti-mouse CD40 (clone: 3/23), PE anti-mouse CD80 (clone: 16-10A1), PE/Cy7 anti-mouse CD80 (clone: 16-10A1), PE anti-mouse CD86 (clone: GL-1), and PE/Cy7 anti-mouse CD86 (clone: GL-1) were purchased from BioLegend (San Diego, CA). Antibodies for western blots, including anti-MMP9 (clone: L51/82) and MMP3 (clone: M4405F10) and reagents for intracellular staining, including Brefeldin A solution, fixation buffer, and permeabilization wash buffer, were purchased from BioLegend (San Diego, CA). Mycobacterium tuberculosis H37 RA and antibodies of β-actin (clone: C4/actin) and HRP goat anti-mouse IgG for western blots were purchased from BD (Sparks, MD). Pertussis toxin was purchased from List Biological Labs (Campbell, CA). Percoll was purchased from GE
Healthcare Life Sciences (Pittsburgh, PA). Metaphosphoric acid was purchased from Alfa Aesar (Tewksbury, MA).

2.3 EAE Induction

C57BL/6 mice were immunized with MOG$_{35-55}$ and SJL/J mice were immunized with PLP$_{139-151}$ to induce chronic and relapsing-remitting EAE, respectively. Disease induction was conducted as previously described (Kuo et al., 2016). In brief, 7-9 weeks old female mice were subcutaneously injected with an emulsion of 200μg MOG$_{35-55}$ peptide (C57BL/6 model) or 100μg PLP$_{139-151}$ (SJL/J model) in CFA containing Mycobacterium tuberculosis H37 RA (final concentration 2 mg/ml) on day 0, and followed by intraperitoneal (i.p.) administration of 200ng pertussis toxin in PBS on day 0 and day 2. EAE mice were then randomly grouped into two groups. One group was i.p administered with vehicle (0.5ml 5% Solutol HS 15 / 0.9% saline / DMSO) and the other group was administered with ACDT (0.5ml 5% Solutol HS 15 / 0.9% saline / 20mg/kg ACDT dissolved in DMSO). The clinical scores of vehicle- and ACDT-treated EAE mice were evaluated based on the following criteria; 0: normal mouse, no overt signs of disease; 1: limp tail or hind limb weakness; 2: limp tail and hind limb weakness; 3: partial hind limb paralysis; 4: complete hind limb paralysis; and 5: moribund state. Animals with clinical scores of 5 were euthanized.

2.4 Brain and Spinal Cord Tissue Processing

EAE mice were anesthetized and perfused with ice-cold PBS. The brain and spinal cord were then harvested and homogenized. The brain tissues were homogenized with HBSS buffer only, and the spinal cord tissues were homogenized with HBSS buffer, containing collagenase and
DNase I, followed by 37°C incubation for 45 minutes. Homogenized brain and spinal cord tissue samples were then filtered through 70-μm nylon cell strainers. After centrifugation, cells prepared from brain and spinal cord tissues were subjected to mononuclear cell isolation or RNA extraction. In addition, cells prepared from spinal cord tissues were subjected to cytokine array and western blots.

2.5 Isolation of Mononuclear Cells from Brain and Spinal Cord Tissues
Mononuclear cell isolation was processed as previously described (Kuo et al., 2016). Cells prepared from brain and spinal cord tissues were then resuspended in 8ml 30% Percoll and underlayered with 4ml 70% Percoll followed by centrifugation for 25 minutes at 1000g at room temperature (RT). The mononuclear cells were isolated from the interface between 30% and 70% Percoll.

2.6 FACS Analysis for Intracellular Cytokines and Surface Markers
Mononuclear cells isolated from the spinal cord and brain of C57BL/6 EAE animals were stimulated with PMA (50ng/ml) and ionomycin (750ng/ml) in the presence of Brefeldin A solution (1μl/ml). 5 hours later, cells were then fixed and permeabilized followed by intracellular staining with antibodies of Alexa Fluor 488 anti-mouse CD4 antibody, PE/Cy7 anti-mouse IFNγ, and APC anti-mouse IL-17. IFNγ- and IL-17-expressing CD4+ T cells were then determined by FACS analysis. For MG surface marker analysis, the isolated mononuclear cells were stained with PE anti-mouse CD11b and APC anti-mouse CD45 with PE/Cy7 anti-mouse CD80 or PE/Cy7 anti-mouse CD86, and primary MG were stained with PE anti-mouse CD80,
PE/Cy7 anti-mouse CD86, and APC anti-mouse CD40, followed by FACS analysis (BD FACSVerse).

2.7 Cytokine Array
The Proteome Profiler™ Array - Mouse XL Cytokine Array Kit (R&D Systems, ARY028) was used to determine the relative expression level of cytokines and chemokines in the spinal cord of EAE mice. Experiments were conducted according to the manufacturer's instructions. Cells prepared from spinal cord tissues were lysed in RIPA buffer [50mM Tris-HCl (pH8.0), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.3% SDS, 1mM PMSF, and 1X protease inhibitor cocktail]. Protein concentration was detected by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). 200µg spinal cord protein was incubated with array membrane for overnight at 4°C on a rocking platform shaker. After incubation, the membrane was washed and incubated with the Detection Antibody Cocktail for 1 hour at RT. The membrane was then washed and incubated with 1X Streptavidin-HRP for 30 minutes at RT followed by washing and incubation with Chemi Reagent Mix. Signal dots appeared on the membrane were then detected by X-ray film (FUJIFILM Super RX-N). The fold change of signal dots representing cytokine and chemokine expression was measured and determined by using protein array analyzer running under Image J (National Institutes of Health).

2.8 Measurement of glutathione levels in the Brain and Spinal Cord of EAE
Brain and spinal cord tissues collected from EAE mice were subjected to glutathione (GSH) assay. The total GSH level was measured by using Glutathione (GSSG/GSH) Detection Kit (Enzo Life Sciences, ADI-900-160). The brain and spinal cord tissues were homogenized in ice-
cold 5% (w/v) metaphosphoric acid (20ml/g tissue). Following centrifugation, supernatants were subjected to absorbance reading (405 nm) by BioTek Synergy HT microplate reader (Winooski, VT) to determine GSH levels according to manufacturer’s instruction.

2.9 Western Blot Analysis

Cells prepared from spinal cord tissues were lysed in RIPA buffer [50mM Tris-HCl (pH8.0), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.3% SDS, 1mM PMSF, and 1X protease inhibitor cocktail]. Protein concentration was detected by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Tissue Protein samples were separated on 10% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Blots were then incubated with MMP9, MMP3, or β-actin antibody. Protein signals were detected by using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and X-ray film. The quantification of protein signals was measured by using Image J software.

2.10 Primary MG Cell Culture

Primary MG were generated as previously described (Kuo et al., 2017). In brief, cerebral cortical cells from 1-2 days old neonatal mice were plated in 75-cm² culture flasks in Dulbecco Modified Eagle Medium/F12 (DMEM/F12) (HyClone™) supplemented with 10% heat-inactivated FBS, containing 2mM glutamine and 1X antibiotic/antimycotic (complete medium). On day 3 and 6 after plating, medium was removed and replenished with fresh complete medium containing 10 ng/ml GM-CSF. MG were harvested at Day 13 or 14 by shaking the flasks at 350 rpm for 30 minutes at 37°C. Harvested cells were then seeded in the tissue culture wells. After overnight resting, cells were stimulated with LPS for 24h to induce surface expression of CD40, CD80, and
CD86, or for 1.5h and 3h to induce inflammatory gene expression based on our previous study (Kuo et al., 2017).

2.11 Real-time RT-PCR

Primary MG and cells prepared from brain and spinal cord tissues were subjected to RNA extraction followed by cDNA synthesis. Expression of *Il-1β, Il-12p35, Il-12p40, Il-23p19, Il-6, Gm-csf, Il-1a, Tnfa, Gata1, H2-D1, and Serping1* were detected by real-time RT-PCR as previous described (Yen et al., 2008). The primers used were *Il-1β*: sense 5’-CCCTGCAGCTGAGAGTGGGA-3’ and antisense 5’-TGTGCTCTGCTTGAGGTGCTG-3’; *Il-12p35*: sense 5’-CTTGCTTGGTAGACATCTCTG-3’ and anti-sense 5’-GCAGAGTCTCAGCCATTATGATTC-3’; *Il-12p40*: sense 5’-TGGTTTGGCATCGTTTGCTG-3’ and antisense 5’-ACAGGTGAGGTCACTGTTCT-3’; *Il-23p19*: sense 5’-TGCTGATTTCAGACCTCAGATAAA-3’ and anti-sense 5’-GCATGCAGAATTCCGAGAGA-3’; *Il-6*: 5’-TCCTCTCTGCAAGAGACTTCCATCC-3’ and anti-sense 5’-GGGAAGGCCTGTTGCA-CC-3’; *Gm-csf*: sense 5’-ATGCCTGTCACTGGTGAATGAAG-3’ and anti-sense 5’-GCGGTCTGCACACATGTTA-3’; *Il-1a*: sense 5’-CGCTTGAGTCCGAAAGAAAT-3’ and antisense 5’-CTTCCCCGCTTGAGTCTCT-3’; *Tnfa*: sense 5’-ATGGCCTCCCCTCTCATCAGT-3’ and antisense 5’-CTTGGTTGCTACGACGACGACGTCGCTGACGAGA-3’; *Gata1*: sense 5’-GTGAACAGCATGAGGGGTTT-3’ and antisense 5’-GTTTTTGGCTTCGCTGGTCTG-3’; *H2-D1*: sense 5’-TCCGAGATTGTAAGCGTGAAGA-3’ and anti-sense 5’-ACAGGGCAGTGCAGGAGGATAG-3’; *Serping1* sense 5’-ACAGCCCCCTGATTTCT-3’ and anti-sense 5’-GGATGCTCTCAAGGTTGCTC-3’.
2.12 Evans Blue BBB Permeability Assay

Evans blue extravasation of CNS tissues of EAE was determined as previously described with modifications (Fairless et al., 2012; Lee et al., 2012). Mice were intravenously (i.v.) administrated 4ml/kg 2% (w/v) Evans blue dye. After 2 hours circulation, mice were anesthetized and perfused with PBS. The brain and spinal cord of EAE mice were then carefully dissected to assess Evans blue leakage.

2.13 Histopathology

EAE mice were sacrificed and perfused with PBS. The spinal cord was then removed from spinal columns, and the lumbar region of spinal cord was then separated and fixed in 4% paraformaldehyde at 4°C for overnight. Fixed tissues were embedded in paraffin for sectioning. Serial 8µm sections were cut on a microtome (Leica RM2155). Cross sections were used for hematoxylin and eosin (H&E) or LFB/cresyl violet staining. Stained spinal cord slides were examined and imaged under magnification (microscope: BX53, Olympus; camera: EXi Aqua, Q Imaging). Image J was used to quantify the area of cell infiltration and the percentage of demyelination by an experimenter blinded to experimental groups.

2.14 Statistical Analysis

Experimental results are given as mean ± SEM. Comparisons between groups were done by using the Mann-Whitney U test, unpaired t test, or one-way ANOVA test. Statistical significance was determined as p values ≤ 0.05. All statistical analysis was performed by using GraphPad Prism 5 software (La Jolla, CA).
3. Results

3.1 ACDT ameliorates disease severity in chronic and relapsing-remitting EAE

To determine whether ACDT exerts a therapeutic potential in EAE, C57BL/6 mice immunized with MOG\textsubscript{35-55} were treated with ACDT every day starting from day 1 post immunization, and the clinical score of vehicle- and ACDT-treated EAE mice was followed for a period of 30 days. Our results showed that ACDT treatment not only delayed disease onset but also reduced disease severity in EAE, with the maximum disease score of 2.2±0.3 compared to that of 4.1±0.2 in vehicle-treated EAE controls (Fig. 1A and Table). When comparing the cumulative score, ACDT-treated EAE mice had a much lower cumulative disease score of 26.6±4.6 compared to that of 64.3±2.8 in vehicle-treated EAE controls (Fig. 1A Table).

We then explored the therapeutic potential of ACDT on the suppression of disease relapse in EAE. Relapsing-remitting EAE was induced in SJL/J mice, and EAE mice were administered with vehicle or ACDT during disease remission (disease score below 1.5). ACDT treatment suppressed disease relapse in EAE, with the maximum disease score of post treatment only reaching 1.4±0.2, whereas vehicle-treated EAE controls developed disease relapse following remission, with the maximum disease score of post treatment reaching 3.0±0.3 (Fig. 1B and Table). When comparing the cumulative score of post treatment, ACDT-treated EAE mice had a much lower cumulative score of 12.6±1.9 compared to vehicle-treated EAE mice of 30.2±2.7 (Fig. 1B Table). Taken altogether, these results demonstrate that ACDT confers protection against EAE through ameliorating disease severity and suppressing disease relapse in EAE.

3.2 ACDT suppresses CNS infiltration of encephalitogenic Th1 and Th17 cells in EAE
The CNS infiltration of encephalitogenic Th1 and Th17 cells plays a pivotal role in the pathogenesis of EAE (Jager et al., 2009; McWilliams et al., 2015). To investigate whether ACDT suppresses encephalitogenic CD4⁺ T cell infiltration of CNS, mononuclear cells were isolated from the brain and spinal cord of vehicle- and ACDT-treated EAE mice at day 13-14 post immunization, and the isolated cells were then subjected to FACS analysis of total CD4⁺ T cells and CD4⁺ T cells expressing IFNγ (Th1) and IL-17 (Th17). We observed a significant increase of infiltrating CD4⁺ T cells in the brain and spinal cord of vehicle-treated EAE mice (Fig. 2A), and those CD4⁺ T cells positively expressed IFNγ (Fig. 2B) or IL-17 (Fig. 2C). In contrast, the number of infiltrating CD4⁺ T cells was largely reduced in the CNS of ACDT-treated EAE mice compared to that in vehicle-treated EAE controls, and that is associated with decreased infiltration of encephalitogenic Th1 and Th17 cells (Fig. 2). Collectively, these results suggest that ACDT-conferred protection in EAE might be mediated through the suppression of CNS infiltration of encephalitogenic Th1 and Th17 cells.

3.3 ACDT suppresses MG activation

MG are the major CNS resident immune cells and function as antigen presenting cells to reactivate pathogenic T cells in lesion sites, thereby exacerbating the disease by epitope spreading (McMahon et al., 2005; Tompkins et al., 2002). MG activation leads to increased secretion of diverse cytokines and enhanced expression of surface maturation markers CD40, CD80, and CD86. To investigate whether ACDT exerts a suppressive effect on MG activation, we cultured primary MG and activated MG with LPS in the presence or absence of ACDT. LPS upregulated surface expression of CD40, CD80, and CD86, whereas ACDT suppressed LPS-induced CD40, CD80, and CD86 upregulation (Fig. 3A). Moreover, LPS upregulated
inflammatory cytokine expression, including IL-23p19, IL-1β, and IL-6 required for Th17 differentiation and reactivation, and IL-12p35 and IL-12p40 required for Th1 differentiation and reactivation. In contrast, ACDT suppressed LPS-induced IL-12p35, IL-12p40, IL-1β, IL-6, and IL-23p19 expression (Fig. 3B). GM-CSF, which was shown to promote neuroinflammation in EAE (El-Behi et al., 2011; Shiomi et al., 2015), was also upregulated in MG activated with LPS, but downregulated in MG activated with LPS in the presence of ACDT (Fig 3B).

To further investigate whether ACDT modulates MG activation in vivo, EAE was induced and mononuclear cells were isolated from the spinal cord and brain of vehicle- and ACDT-treated EAE mice at day 13-14 post immunization. The isolated cells were then subjected to CD45, CD11b, CD80, and CD86 staining followed by FACS analysis. MG were determined based on their positive expression of CD11b and intermediated expression of CD45. Although there was no significant difference of CD86 expression on the surface of CD45intCD11b+ MG isolated from the spinal cord and brain of vehicle- and ACDT-treated EAE mice, CD80 expression was significantly reduced on the surface of CD45intCD11b+ MG isolated from the spinal cord and brain of ACDT-treated EAE mice compared to that in vehicle-treated EAE controls (Fig. 3C).

Altogether, these results suggest that ACDT possesses a suppressive effect on MG activation in vitro as well as partially in vivo.

3.4 ACDT lessens neuroinflammation in EAE

To further investigate whether ACDT-mediated suppression of pathogenic T cell infiltration and MG activation could lead to the alleviation of neuroinflammation in EAE, the spinal cord harvested from vehicle- and ACDT-treated EAE mice was subjected to cytokine array to determine the expression of inflammatory mediators. We found that chemokines, related to the
recruitment of inflammatory immune cells, including CCL5, CCL6, CCL17, and CXCL10, were expressed in the spinal cord of EAE mice, but their expression was suppressed in the spinal cord of ACDT-treated EAE mice (Fig. 4A). In addition, inflammatory mediators that are associated with pathogenesis of EAE, such as osteopontin, CD40, ICAM-1, and myeloperoxidase, were also expressed in the spinal cord of EAE, but suppressed in the spinal cord of ACDT-treated EAE mice (Fig. 4A).

A recent study shows that IL-1α, TNFα, and C1q, produced by activated MG, induce neurotoxic reactive astrocytes that are named “A1 astrocytes”. Importantly, these neurotoxic A1 astrocytes are also present in post-mortem brain tissues of MS patients (Liddelow et al., 2017). In our array results, we found that IL-1α was expressed in the spinal cord of vehicle-treated EAE controls, but suppressed in the spinal cord of ACDT-treated EAE mice. We speculated that ACDT might regulate neurotoxic A1 astrocytes in the CNS of EAE through modulating the expression of inflammatory molecules required for A1 astrocyte generation. We then measured IL-1α, TNFα, and C1q expression in the CNS of vehicle- and ACDT-treated EAE mice. Although we did not detect the expression of C1q in the CNS of EAE mice (data not shown), the expression of TNFα and IL-1α was largely suppressed in the spinal cord and brain of ACDT-treated EAE mice compared to those in vehicle-treated EAE controls (Fig 4B). Subsequently, we determined the levels of A1 astrocytes in the spinal cord and brain of vehicle- and ACDT-treated EAE mice by measuring A1 astrocyte specific genes, including Ggta1, H2-D1, and Serping1. The expression of Ggta1, H2-D1, and Serping1 was suppressed in the CNS of ACDT-treated EAE mice compared to that in vehicle-treated EAE controls (Fig. 4C). Taken altogether, these results demonstrate that ACDT lessens neuroinflammation and potentially suppresses neurotoxic A1 astrocyte generation in EAE.
3.5 ACDT lessens BBB disruption and alleviates spinal cord pathology in EAE

To determine whether ACDT lessens EAE-induced BBB disruption, during the peak of disease vehicle- and ACDT-treated EAE mice were subjected to Evans Blue injection followed by the brain and spinal cord tissue isolation to assess BBB integrity. Evans blue leakage was clearly observed in the CNS especially in the spinal cord of vehicle-treated EAE controls, indicating a compromised BBB integrity. On the other hand, the leakage of Evans blue was significantly reduced in the spinal cord and brain of ACDT-treated EAE mice, suggesting a well preserved BBB integrity in ACDT-treated EAE mice (Fig. 5A). Both MMP3 and MMP9 play a detrimental role in BBB disruption and breakdown in several neurodegenerative diseases (Fanjul-Fernandez et al., 2010; Gerwien et al., 2016; Kim et al., 2011; Konnecke et al., 2013), we thought to determine whether the beneficial effect of ACDT on BBB integrity in EAE is mediated through its regulatory effect on MMP3 and MMP9 production. We measured MMP3 and MMP9 expression in the spinal cord of vehicle- and ACDT-treated EAE mice, and found MMP3 and MMP9 were highly expressed in the spinal cord of vehicle-treated EAE controls. Conversely, the expression of MMP3 and MMP9 was largely suppressed in the spinal cord of ACDT-treated EAE mice (Fig 5B).

We further examined the histopathological features of spinal cord in vehicle- and ACDT-treated EAE mice. The spinal cord harvested from EAE mice was subjected to H&E and LFB/cresyl violet staining to assess cell infiltration and demyelination, respectively. We observed a significant increase of infiltrating inflammatory cells and a moderate level of demyelination in the spinal cord of vehicle-treated EAE controls. In contrast, the histopathology of cell infiltration and demyelination was alleviated in the spinal cord of ACDT-treated EAE mice (Fig. 5C and D).
Altogether, these results demonstrate that ACDT lessens BBB disruption, suppresses MMP3 and MMP9 production, inhibits inflammatory cell infiltration, and alleviates demyelination in the spinal cord of EAE.

3.6 ACDT enhances GSH levels in the CNS of EAE

The inflammatory process in EAE is associated with increased production of reactive oxygen species and reduced production of redox active compounds, such as GSH, in the CNS (Choi et al., 2015; Ljubisavljevic et al., 2012; Mohamed et al., 2003; Morales Pantoja et al., 2016; Ruuls et al., 1995). Our group has previously demonstrated that ACDT strongly induced the production of GSH in SH-SY5Y cells (Brown et al., 2016). To assess whether ACDT induces GSH production in the CNS to modulate neuroinflammation in EAE, the spinal cord and brain were harvested from vehicle- and ACDT-treated EAE mice and subjected to GSH analysis. We found that GSH levels were higher in the brain and spinal cord of ACDT-treated EAE mice than those in vehicle-treated EAE controls (Fig. 6), suggesting that the induction of GSH by ACDT might contribute to its suppressive effect on neuroinflammation in EAE.

4. Discussion

Dithiolethiones have well-demonstrated protective effects in a number of neurodegenerative and neurological diseases, including Alzheimer disease, UV-induced retinal pigment epithelium cell injury, stroke, and EAE (Cui et al., 2017; Kuo et al., 2016; Kuo et al., 2017; Li et al., 2016; Wang et al., 2017). Our group previously reported the anti-inflammatory properties of D3T, the structurally simplest of the dithiolethiones, in EAE (Kuo et al., 2016). As a continuation of our study of D3T, we sought to assess the effect of modifying the dithiolethione core structure on
EAE activity. ACDT structurally retains the dithiolethione pharmacophore, but has two versatile functional groups (carboxylic acid ethyl ester and primary amine) that are readily amendable for future modifications. As the therapeutic effect of ACDT in EAE is unknown, we therefore investigated its activity in two well-established EAE models, chronic C57BL/6 EAE and relapsing-remitting SJL/J EAE. We observed that in the chronic EAE, ACDT treatment delayed disease onset for an average of 4 days compared to controls, minimized maximum disease score to the average of 2.2 as opposed to 4.1 for controls, and ameliorated disease severity with cumulative score of 26.6 compared to 64.3 for controls. More importantly, in the relapsing-remitting EAE, ACDT, administered during disease remission, prevented disease relapse, and the cumulative score of post treatment significantly reduced to 12.6 compared to 30.2 for controls. Based on these findings, we concluded that ACDT exerts a therapeutic effect in chronic and relapsing-remitting EAE by ameliorating disease severity and preventing disease progression.

We further investigated the cellular mechanisms underlying the protective effect of ACDT in EAE. The infiltration of pathogenic Th1 and Th17 cells has been shown to play a critical role in the pathogenesis of EAE (El-Beih et al., 2011; Jager et al., 2009). ACDT treatment suppressed the infiltration of Th1 and Th17 cells in the spinal cord and brain of EAE mice compared to those in vehicle-treated EAE controls. In addition, our observation of reduced total infiltrating CD4+ T cells in the CNS of ACDT-treated EAE mice led us to speculate that ACDT might lessen disease-induced BBB disruption. Indeed, we found that EAE mice treated with vehicle had a compromised BBB integrity and EAE mice treated with ACDT exhibited a well preserved BBB integrity, as the profound leakage of Evans blue was only observed in the spinal cord of vehicle-treated EAE controls but not in the spinal cord of ACDT-treated EAE mice. Further analysis of the spinal cord of EAE revealed that metalloproteinases MMP3 and MMP9, essential
proteases contributing to the cleavage of BBB basement membrane and degradation of tight junction proteins (Lakhan et al., 2013; Yang et al., 2007), were largely expressed in the spinal cord of vehicle-treated EAE controls but dramatically suppressed in the spinal cord of ACDT-treated EAE mice. These results correlate with the histopathology of spinal cord in which increased cell infiltration and enhanced demyelination were observed in vehicle-treated EAE controls but not in ACDT-treated EAE mice. CNS resident cells, including MG and brain endothelial cells, and infiltrating inflammatory immune cells are the cell populations, producing MMP3 and MMP9 under neuroinflammatory conditions (Amtul et al., 2014; Brkic et al., 2015; Yang et al., 2007). Further investigation would be required to dissect which cell types that secret MMP3 and MMP9 following EAE are modulated by the treatment of ACDT.

A recent study shows that activated microglia induce neurotoxic reactive astrocytes in neurodegenerative diseases (Liddelow et al., 2017). Cytokines TNFα and IL-1α and complement component C1q released by activated microglia promote the generation of reactive astrocytes that exert a toxic effect on neurons. These reactive astrocytes were subsequently named as “A1 astrocytes”. A1 astrocytes are rapidly generated in vivo following CNS injury and present in many human neurodegenerative diseases, including Huntington’s disease, Alzheimer’s disease, ALS, Parkinson’s disease, and MS (Liddelow et al., 2017). A1 astrocytes express a spectrum of signature genes that can be used for screening or detecting these neurotoxic reactive astrocytes in the CNS of neurodegenerative diseases. In the present study, we examined whether ACDT-mediated amelioration of neuroinflammation could lead to reduced A1 astrocyte generation in EAE. We observed ACDT inhibited a spectrum of cytokines, chemokines, and inflammatory mediators in the CNS of EAE. Importantly, essential cytokines required for the generation of neurotoxic A1 astrocytes, including TNFα and IL-1α, were suppressed in the CNS of ACDT-
treated EAE mice, although we did not detect the expression of C1q in the CNS of EAE. Subsequent analysis of A1 astrocytes in the CNS of EAE revealed that ACDT suppressed the expression of A1 astrocyte signature genes, including Ggta1, H2-d1, and Serping1. Thus, our results demonstrated that ACDT ameliorated neuroinflammation and that might subsequently reduce neurotoxic A1 astrocyte generation in EAE. However, a comprehensive study through measuring additional A1 markers is required to further confirm the suppressive effect of ACDT on the generation of A1 astrocytes in the CNS of EAE. Moreover, whether the reduction of A1 astrocyte generation by ACDT could lead to decreased neuronal injury in EAE would require further investigation to reveal potential neuroprotective effects of ACDT in EAE.

Our previous study demonstrated that D3T, the structurally-simplest dithiolethione, ameliorated disease of EAE (Kuo et al., 2016). In the current study, we showed ACDT, a dithiolethione with ethyl ester and primary amine functional groups, alleviated disease severity to a similar degree as D3T in EAE. These results suggest a potential use of dithiolethiones as novel therapies for EAE/MS. As dithiolethiones are known to potently activate Nrf2 (Jia et al., 2008; Tran et al., 2009), the protective effect of D3T and ACDT in EAE might be mediated through Nrf2 defense pathway. Currently, there is only one FDA-approved Nrf2 activator on the market, dimethyl fumarate (DMF) (Alroughani et al., 2017). Based on the success of this drug in the clinic, the development of further Nrf2 activators is warranted. Additionally, DMF activates Nrf2 via the depletion of GSH, which generates oxidative stress (Lehmann et al., 2007; Xie et al., 2015). This has the potential effect of inducing cellular injury. In contrast, dithiolethiones activate Nrf2 in a process that does not trigger oxidative stress onto the cell (Brown et al., 2014; Giustarini et al., 2014; Kwak et al., 2003). Thus, dithiolethiones, such as D3T and ACDT, may represent better
therapeutic agents for the treatment of EAE/MS through direct activation of the Nrf2 pathway without compromising the beneficial effect of GSH.

5. Conclusions

We reported for the first time that ACDT conferred protection against the autoimmune inflammatory disease EAE. We showed ACDT inhibited CNS infiltration of pathogenic CD4+ T cells, including Th1 and Th17 cells, and ACDT suppressed MG activation through inhibiting the expression of surface maturation markers and the production of inflammatory cytokines. In addition, ACDT lessened BBB disruption in the spinal cord of EAE through inhibiting MMP3 and MMP9 production that resulted in a better preserved BBB integrity with decreased CNS infiltration of inflammatory immune cells and alleviated demyelination in the spinal cord of EAE. In summary, our study demonstrated that ACDT exerts a therapeutic potential for the treatment of MS/EAE by targeting several pathological pathways related to the development and progression of EAE.

Acknowledgements

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References


Figure Legends

Fig. 1 ACDT ameliorates disease severity in chronic and relapsing-remitting EAE
(A) C57BL/6 mice (n=18/group) immunized with MOG35-55 were administered i.p. with vehicle or ACDT (20mg/kg) every day starting from day 1 post immunization. (B) SJL/J mice (n=9/group) were immunized with PLP139-151 to induce EAE. Following the first remission and before the onset of second relapse, EAE animals were administered i.p. with vehicle or ACDT (20mg/kg) every day. The clinical score of EAE animals was followed, and EAE mortality rate, onset of disease, maximum score, and cumulative score were assessed. Data represent mean ± SEM. Statistical significance was determined as: *p<0.05, **p<0.01, and ***p<0.001 by Mann-Whitney U test.

**Fig. 2 ACDT suppresses CNS infiltration of encephalitogenic Th1 and Th17 cells in EAE**

C57BL/6 mice (n=9/group) were subjected to EAE induction followed by i.p. administration with vehicle or ACDT (20mg/kg) every day starting from day 1 post immunization. At day 13 or 14 post immunization, mononuclear cells were isolated from the brain and spinal cord of vehicle- and ACDT-treated EAE mice. The cell numbers of total CD4+ T cells (A), IFNγ- (B), and IL-17-expressing CD4+ Th cells (C) in the brain and spinal cord were determined by FACS analysis. Isotype controls (ISO) were used to determine cells positive for surface expression of CD4 (A) and CD4+ T cells positive for intracellular expression of IFNγ (B) or IL-17 (C). Data represent mean ± SEM. Statistical significance was determined as: ***p<0.001 by unpaired t test.

**Fig. 3 ACDT suppresses MG activation**

Primary MG were left untreated (medium) or pretreated with vehicle or ACDT 100µM for 1 hour followed by LPS (100ng/ml) stimulation. (A) 24 hours after LPS treatment, cells were subjected to FACS analysis for surface expression of CD40, CD80, and CD86. Dashed lines represent isotype control. (B) 1.5 and 3 hours after LPS treatment, cells were collected and subjected to RNA extraction followed by Q-PCR analysis for mRNA expression of IL-23p19,
IL-12p35, IL-12p40, IL-1β, IL-6, and GM-CSF. One representative of three independent experiments is shown. Statistical significance was determined as: *p<0.05, **p<0.01, and ***p<0.001 by one way ANOVA with post hoc Bonferroni’s multiple comparison test. (C) C57BL/6 mice (n=8/group) were subjected to EAE induction followed by i.p. administration with vehicle or ACDT (20mg/kg) every day starting from day 1 post immunization. At day 13 or 14 post immunization, mononuclear cells were isolated from the brain and spinal cord of vehicle- and ACDT-treated EAE mice, and the isolated cells were then stained with antibodies of CD45 and CD11b with CD80 or CD86 followed by FACS analysis. MG were determined as CD45^intCD11b^+ cells, and the expression of CD80 and CD86 on the surface of CD45^intCD11b^+ cells was determined. Isotype controls (ISO) were used to determine MG positive for CD80 or CD86 expression. Statistical significance was determined as: ***p<0.001 by unpaired t test.

**Fig. 4 ACDT lessens neuroinflammation in EAE**

C57BL/6 mice were subjected to EAE induction followed by i.p. administration with vehicle or ACDT (20mg/kg) every day starting from day 1 post immunization. (A) At day 13 post immunization, the spinal cord of vehicle- and ACDT-treated EAE mice was harvested and subjected to cytokine array analysis (n=3/group). (B and C) At day 13 or 14 post immunization, the spinal cord and brain of vehicle- and ACDT-treated EAE mice (n=10/group) were harvested and subjected to Q-PCR analysis for mRNA expression of IL-1α, TNFα (B), Ggta1, H2-D1, and Serping1 (C). Statistical significance was determined as: *p<0.05, **p<0.01, and ***p<0.001 by unpaired t test.

**Fig. 5 ACDT lessens BBB disruption and alleviates spinal cord pathology in EAE**
C57BL/6 EAE animals were i.p. administration with vehicle or ACDT (20mg/kg) every day starting from day 1 post immunization. At day 13 post immunization, vehicle- and ACDT-treated EAE mice (n=5/group) were subjected to i.v. administration of Evans blue. (A) 2 hours after Evans blue administration, animals were sacrificed, and the brain and spinal cord of vehicle- and ACDT-treated EAE mice were dissected to assess the leakage of Evans blue. (B) The spinal cord tissues were homogenized and subjected to western blot analysis of MMP3 and MMP9 production. (C and D) The lumbar regions of spinal cord tissues were fixed and subjected to H&E and LFB/cresyl violet staining to assess cell infiltration and demyelination, respectively. The area of cell infiltration and the percentage of demyelination were quantified. Arrow indicates cell infiltration (C) and demyelination (D). Statistical significance was determined as: *p<0.05 and **p<0.01 by Mann-Whitney U test.

**Fig. 6 ACDT enhances GSH levels in the CNS of EAE**

C57BL/6 EAE animals were i.p. administration with vehicle or ACDT (20mg/kg) every day starting from day 1 post immunization. At day 13 post immunization, the spinal cord and brain of vehicle- and ACDT-treated EAE animals (n=12/group) were harvested, and GSH levels of spinal cord and brain were determined. Statistical significance was determined as: *p<0.05 and **p<0.01 by unpaired t test.
### Fig. 1

**A**

- **Clinical Scores Post Immunization**
  - Vehicle
  - ACDT 20mg/kg

- **Onset of Disease**
  - Vehicle: 10.7±0.4 days
  - ACDT 20mg/kg: 14.8±0.7 days ***

- **Maximum Score**
  - Vehicle: 4.1±0.2
  - ACDT 20mg/kg: 2.2±0.3 ***

- **Cumulative Score**
  - Vehicle: 64.3±2.8
  - ACDT 20mg/kg: 26.6±4.6 ***

**B**

- **Clinical Scores Post Treatment**
  - Vehicle
  - ACDT 20mg/kg

- **Onset of Disease**
  - Vehicle: 8.8±0.3 days
  - ACDT 20mg/kg: 9.3±0.3

- **Maximum Score Post Treatment**
  - Vehicle: 3.0±0.3
  - ACDT 20mg/kg: 1.4±0.2 **

- **Average Score Post Treatment**
  - Vehicle: 2.0±0.2
  - ACDT 20mg/kg: 0.8±0.1 ***

- **Cumulative Score Post Treatment**
  - Vehicle: 30.2±2.7
  - ACDT 20mg/kg: 12.6±1.9 ***
Fig. 2

(A) ISO, Vehicle, ACDT

(B) ISO, Vehicle, ACDT

(C) ISO, Vehicle, ACDT
Fig. 3

(A) CD40, CD80, CD86 fluorescence intensity as measured by flow cytometry.

(B) Bar graphs showing the expression levels of Il-12p35, Il-23p19, Il-1b, Il-6, Il-12p40, and Gm-csf in response to LPS treatment with or without ACDT. 

Vehicle vs ACDT comparison with statistical significance indicated by asterisks: * p < 0.05, ** p < 0.01, *** p < 0.001.
Fig. 3

(C) 

Brain

Spinal Cord

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Brain

- CD80⁺CD45⁻CD11b⁺ Cells (%)
- CD86⁺CD45⁻CD11b⁺ Cells (%)

Spinal Cord

- CD80⁺CD45⁻CD11b⁺ Cells (%)
- CD86⁺CD45⁻CD11b⁺ Cells (%)

*** N.S.
Fig. 4

(A) 

Vehicle | ACDT
---|---
CCL5 | CCL6 | CCL17 | CXCL10 | IL-1α | Osteopontin | CD40 | Chitinase 3-like 1 | C-Reactive Protein | ICAM-1 | Myeloperoxidase | Pentraxin 3

High | Low

(B) 

**Graphs showing expression levels of proteins in the brain and spinal cord under Vehicle and ACDT conditions.**

- **IL-1α**: 
  - Brain: Vehicle: 1.0, ACDT: 1.0
  - Spinal Cord: Vehicle: 1.5, ACDT: 1.5

- **Tnfa**: 
  - Brain: Vehicle: 1.5, ACDT: 1.5
  - Spinal Cord: Vehicle: 2.0, ACDT: 2.0

- **Ggta1**: 
  - Brain: Vehicle: 1.0, ACDT: 0.5
  - Spinal Cord: Vehicle: 1.0, ACDT: 0.5

- **H2-D1**: 
  - Brain: Vehicle: 1.0, ACDT: 0.5
  - Spinal Cord: Vehicle: 1.0, ACDT: 0.5

- **Serping1**: 
  - Brain: Vehicle: 1.0, ACDT: 0.5
  - Spinal Cord: Vehicle: 1.0, ACDT: 0.5

(C) 

**Brain** 

- **Ggta1**: 
  - Vehicle: 1.0, ACDT: 0.5

**Spinal Cord** 

- **H2-D1**: 
  - Vehicle: 1.0, ACDT: 0.5
Fig. 5  (A)  

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<td>% of Demyelination</td>
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Fig. 6

**Total GSH Level (µmoles/g of tissue)**

**Brain**
- Vehicle: ~33 µmoles/g
- ACDT: ~42 µmoles/g

**Spinal Cord**
- Vehicle: ~6 µmoles/g
- ACDT: ~12 µmoles/g

* indicates a significant difference for Brain, and ** indicates a significant difference for Spinal Cord.
Highlights

- Dithiolethione ACDT ameliorates disease severity in both chronic and relapsing-remitting EAE.
- ACDT inhibits the CNS infiltration of pathogenic T cells in EAE.
- ACDT suppresses MG activation *in vitro* and *in vivo*.
- ACDT alleviates neuroinflammation and reduced neurotoxic A1 astrocyte generation in EAE.
- ACDT lessens blood-brain barrier disruption and alleviates spinal cord pathology in EAE.