SPEEN, ADAM M., M.S. Enhancement of Endogenous Glutathione and NQO1 by Triterpenoid CDDO-Im in SH-SY5Y Cells: Protection Against Neurotoxicant-Mediated Cytotoxicity. (2013)
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Evidence suggests oxidative and electrophilic stress as a major factor contributing to the neuronal cell death in neurodegenerative disorders, especially Parkinson's disease (PD). Early depletion in the levels of thiol antioxidant glutathione (GSH), which may lead to generation of reactive oxygen species, is an important biochemical feature of PD. Many toxins including 4-hydroxynonenal, acrolein, and peroxynitrite are known to cause oxidative stress and contribute to the development of neurodegenerative disorders. This study was undertaken to determine whether CDDO-Im (2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline), a novel triterpenoid compound, could increase endogenous antioxidant defenses protecting against the above neurotoxicant-mediated cytotoxicity in retinoic acid-induced differentiated SH-SY5Y cells. Retinoic acid-induced differentiated human neuroblastoma SH-SY5Y cells are known to possess properties of mature neurons and thus have been a widely used in vitro model for the study of neurotoxicity and neuroprotection. In this study, we showed that incubation of retinoic acid-induced differentiation of human neuroblastoma SH-SY5Y cells with nanomolar concentrations of CDDO-Im (1- 400 nM) for 24 h resulted in a significant increase in the levels of reduced glutathione (GSH) and NAD(P)H:quinone oxidoreductase 1 (NQO1), two critical cellular defenses in detoxification of reactive oxygen species and electrophilic quinone molecules. Pretreatment of the cells with CDDO-Im was found to afford remarkable protection against the neurocytotoxicity elicited by 4-hydroxynonenal, 3-

morpholinosynonimine hydrochloride, xanthine oxidase/xanthine, hydrogen peroxide as well as the environmental neurotoxin acrolein. Acrolein is a ubiquitous unsaturated aldehyde product occurring in the combustion of organic matter including exhausts and pollutants and has been implicated in the pathogenesis of various neurological disorders including Parkinson's disease where acrolein-protein adducts have been observed in post mortem studies. To further determine the role of cellular GSH in CDDO-Im-mediated protection against the acrolein induced cytotoxicity, buthionine sulfoximine (BSO) was used to inhibit cellular GSH biosynthesis. BSO at 25μM dramatically depleted GSH and significantly potentiated acrolein-induced cytotoxicity. Pretreatment of RA-differentiated SH-SY5Y cells with both 25μM BSO and 100nM CDDO-Im was found to prevent the CDDO-Im mediated GSH induction and partially reverse the cytoprotective effects of CDDO-Im treatment on acrolein-induced toxicity.

Taken together, this study demonstrates for the first time that CDDO-Im potently induces the cellular GSH system and NQO1 in RA-differentiated human neuroblastoma SH-SY5Y cells, which is accompanied by dramatically increased resistance of these cells to the damage induced by various neurotoxicants. In addition, we show that the CDDO-Im-mediated up regulation of GSH is a predominant mechanism against acrolein-induced neurotoxicity. The results of this study may have important implications for the development of novel neuroprotective strategies.

ENHANCEMENT OF ENDOGENOUS GLUTATHIONE AND NQO1 BY TRITERPENOID CDDO-IM IN SH-SY5Y CELLS: PROTECTION AGAINST NEUROTOXICANT-MEDIATED CYTOTOXICITY

by

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APPROVAL PAGE

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CHAPTER I

BACKGROUND

Oxidative Stress

The term oxidative stress encompasses many types of cellular damage caused by reactive oxygen species (ROS). ROS include all oxygen-free radicals that can be generated exogenously through radiation, metal-catalyzed reactions, exposure to atmospheric pollutants, as well as produced endogenously by mitochondria-controlled electron transport chain reactions and other metabolic reactions[1]. Mitochondria are known to produce superoxide (0_2-) which degrades into hydrogen peroxide (H_20_2) as part of its electron transport chain and ATP synthesis. H_20_2 is not reactive itself but leads to the formation of other more reactive ROS including peroxinitrite (ONOO-), and the hydroxyl ion (OH-). Exogenous xenobiotics can both directly and indirectly generate ROS. Acrolein is an example of the latter [2-4].

Acrolein is an environmental toxin found in many pesticide products and as a product in the combustion of organic matter.[5]. As a reactive aldehyde, acrolein, has been found to readily damage lipids, making fatty acid rich nerve cell types primary targets [6]. Recent studies show that the resulting oxidative damage leads to decreased cell proliferation and increased apoptosis [7, 8]. Acrolein can also be formed inside the cell as a product of lipid peroxidation by other forms of ROS [4]. Neuronal cell exposure to acrolein and other reactive oxygen species (ROS) generating compounds and the

subsequent cellular damage are found to be associated with the progression of Parkinson's disease (PD) as well as other neurodegenerative disorders [9, 10]. Elevated concentrations of acrolein, up to 40μM, have been found in the brain tissue of PD patients and are considered a specific marker for lipid peroxidation damage [7, 11].

Pathological Effect of Oxidative Stress on the Brain

The brain and neuronal cell types remain some of the most vulnerable cell types to oxidative stressors. Neurons have been shown to perform cellular respiration at a higher rate than other cell types; 20% of the oxygen consumed by the human body is allocated to brain function. More cellular respiration causes more oxygen turn over and increases levels of oxidative stress [12, 13]. The brain's vulnerability also stems from the fact that the cells contain a large amount of polyunsaturated fatty acids which are primary targets for ROS and exogenous toxins [6]. Cellular protection against oxidative damage is associated with antioxidant production; neuronal cells consequently have a limited capacity for antioxidant up regulation and are susceptible to damage [14]. Thus, importance must be placed on the regulation of the antioxidant defense system in order to reduce the neurodegenerative effects of oxidative damage and prevent disorders such as Parkinson's disease.

Parkinson's Disease (PD)

Parkinson's disease (PD) is a prolific neurodegenerative disorder with increasing incidence across the world [15, 16]. The pathogenesis of PD has been related to the degeneration of dopaminergic neuron cells by oxidative stress and can be exacerbated by

exogenous environmental toxins [17, 18]. These environmental toxins, along with ROS, have been implicated in the alteration of essential cellular components leading to cellular dysfunction and ultimately apoptosis of the cells in neurodegenerative disorders [18]. In PD, oxidative damage has been found to specifically target nigrostriatal neurons associated with fine motor control. As previously mentioned, neuronal cell vulnerability stems from the cell's large amount of polyunsaturated fatty acids which have been shown to be primary targets for reactive oxygen species and exogenous toxins [6]. Specifically, unsaturated aldehydes and markers of lipid peroxidation damage have been found in elevated concentrations in brain tissue of patients with neurodegenerative disorders [19]. Due to the environmental factor involved in the pathogenesis of PD, development of novel preventative treatment options has become increasingly important.

Antioxidant Defenses

The cellular antioxidant defense system includes endogenous enzymes as well as exogenous compounds which help promote protection against toxic effects. Glutathione (GSH) is important for cellular defense against ROS and has been found at high concentrations in stressed cells. Increased intracellular concentrations of GSH and its enzymatic derivatives, glutathione-S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPx), result in the detoxification of many types of ROS[20]. GSH and GST have also been implicated in neuronal cell protection against environmental toxins such as acrolein[21]. In addition to endogenous compounds, exogenous antioxidant promoting substances have been shown to increase cellular antioxidant levels, including GSH.

Recently, many studies focus on the use of exogenous antioxidant compounds to prevent oxidative damage to the brain. These treatment options, however, have been shown to be limited in their lack of long term effectiveness and sometimes lead to potentially dangerous side effects making them poor choices for therapeutic nutraceuticals[22-26]. Synthetic indirect protective compounds including triterpenoids, however, have been shown to increase neuron robustness and are associated with increased antioxidant regulation [27].

Triterpenoids and CDDO-Im

Triterpenoids are steroid-like compounds derived from plant extracts which have been shown to have numerous protective/therapeutic effects on various cell types[28]. 2-Cyano-3,12-dixooleana-1,9-dien-28-imidazolide (CDDO-Im) (Figure 1) is a synthetic triterpenoid designed to be more potent and to effect mammalian signaling pathways associated with detoxification and apoptosis [29, 30].

Accordingly, plant-derived triterpenoids, including oleanolic acid CDDO, have shown to increase cell viability and are associated with increased antioxidant regulation [27]. Recently, CDDO has been used as a cancer prevention treatment as well as an anti-inflammatory therapeutic and is currently in clinical trials [31-34]. Despite the recent studies and successes in the therapeutic use of CDDO in human disease, there are limited studies involving its neuroprotective capabilities.

Proposed Study

In this study we proposed to test the potential neuroprotective capabilities of the triterpenoid CDDO-Im against various oxidative and electrophilic species in human SH-SY5Y neuroblastoma cells. The hypothesis we put forth was that treatment with nanomolar levels of CDDO-Im could upregulate endogenous cellular antioxidant enzymes in SH-SY5Y cells, which might then lead to cytoprotective effects against oxidative and electrophilic damage. Accordingly, the study aims were designed to: first, study the induction of endogenous antioxidant defenses by CDDO-Im in human neuroblastoma SH-SY5Y cells and the protective effects of CDDO-Im pretreatment in the cells when exposed to various types of oxidative and electrophilic species. Secondly, we aimed to determine the mechanistic role of GSH in CDDO-Im-mediated protection against acrolein-induced neurocytotoxicity.

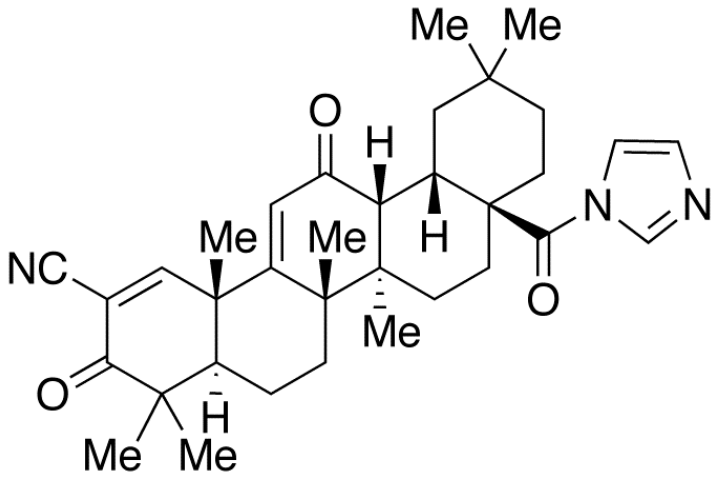

Figure 1. Chemical structure of CDDO-Im. Chemical structure of 2-Cyano-3,12-dixooleana-1,9-dien-28-imidazolide (CDDO-Im) supplied by Toronto Research Chemicals Inc.

CHAPTER II

INDUCTIONS OF ENDOGENOUS ANTIOXIDATIVE ENZYMES BY CDDO-IM AND ITS PROTECTIVE EFFECTS ON VARIOUS OXIDATIVE AND ELECTROPHILIC CELL INJURIES IN SH-SY5Y CELLS

Introduction

Parkinson's disease (PD) is a prolific neurodegenerative disorder with increasing incidence across North America and Europe [15, 16]. The debilitating disorder has been related to the degeneration of dopaminergic neuron cells by oxidative stress and can be exacerbated by exogenous environmental toxins [17, 18]. Oxidative stress is implicated in the alteration of essential cellular components such as nucleic acids, proteins, and lipids leading to cellular dysfunction and ultimately apoptosis of the cells in neurodegenerative disorders [18]. In PD, oxidative damage of neuronal cells, specifically nigrostriatal neurons, has been found as a primary cause leading to the onset of the disease. Neuronal cell vulnerability stems from the cell's large amount of polyunsaturated fatty acids which have been shown to be primary targets for reactive oxygen species and exogenous toxins [6]. Specifically, unsaturated aldehydes such as 4-hydroxy-2-noneal (HNE), which causes lipid peroxidation damage, have been found in elevated concentrations in brain tissue of patients with neurodegenerative disorders [19]. Neuron cell vulnerability is highly dependent on the amount of oxidative damage incurred

overtime. Thus, it is increasingly important to find applicable treatment options for neurodegenerative disorders such as PD.

Numerous studies focus on the use of exogenous antioxidant compounds to prevent oxidative damage to the brain; however, there are several factors which raise questions to their effectiveness. Many treatment options which have demonstrated the protective effects such as vitamin C and Cu/Zn-superoxide dismutase, are also susceptible to limited bioavailability, inefficient permeability, and potentially more damaging metabolic reactions[26]. All of which make them poor choices as therapeutic nutraceuticals[22-26]. An alternative strategy for reducing oxidative damage is to increase the naturally occurring cellular defenses which suppress ROS generation. Increased intracellular concentrations of the tripeptide antioxidant GSH and its enzymatic derivatives, glutathione-S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPx) are known to contribute to the detoxification of many types of ROS[20]. GSH and GST have also been implicated in neuronal cell protection against environmental toxins such as acrolein[21]. Similarly, the upregulation of natural phase II enzymes such as NQO1 has been shown to contribute to the detoxification of ROS in neuronal cell types [35]. For these reasons it is of great interest to develop therapeutic strategies via increase in the natural endogenous cellular defense mechanisms including GSH and NQO1 to reduce oxidative damage mediated by a bioactive nutraceutical/pharmacological agent

Accordingly, plant-derived triterpenoids, including oleanolic acid and synthetic triterpenoid CDDO, have shown to increase cell viability and are associated with

increased antioxidant regulation [27]. Triterpenoids are steroid like compounds which occur naturally in plant extracts used for medicinal purposes[28]. Recently, CDDO has been used as a cancer prevention treatment as well as an anti-inflammatory therapeutic and is currently in clinical trials [31-34]. Despite the recent studies and successes in the therapeutic use of CDDO in human disease there are limited studies involving its neuroprotective capabilities. One exception is the work of Yang et al who has shown that 2-cyano-*N*-methyl-3,12-dioxooleana-1,9(11)-dien-28 amide (CDDO-MA) is capable of activating transcription factors associated with antioxidant defense and increase some intracellular antioxidant compounds in SH-SY5Y cells and mouse models [36]. These results provide an observation of the potential use of CDDO in neuronal protection; however, a few issues are not addressed. The SH-SY5Y cell model is not differentiated to more closely resemble human dopaminergic neuronal cells [37]. Also, cytoprotection is not addressed when exposed to various types of oxidative stress. Finally, Yang et al utilize high concentrations of CDDO, as low as 500nM, in SH-SY5Y cells to observe increased antioxidant defenses. Overall, the lack of recent studies limits the conclusions that can be drawn from the use of CDDO in human nerve cells and deserves further study.

In our study, we used human SH-SY5Y cells differentiated with retinoic acid (RA) as an in vitro system to investigate the coordinated induction of endogenous antioxidant defenses by 2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline (CDDO-Im) (Figure 1), as well as its potential protective capabilities against oxidative damage induced by various of neurotoxins. RA-differentiated SH-SY5Y cells have been shown to

share similar characteristics of dopaminergic neurons and are a good in vitro model for studying neurotoxicology and neuroprotection (Figure 2) [38, 39]. This study demonstrates for the first time that CDDO-Im as low as 1 nM potently induces the cellular GSH and the phase 2 enzyme NQO1 in RA-differentiated SH-SY5Y cells. Furthermore, the CDDO-Im-mediated coordinated upregulation of cellular defenses is accompanied by remarkably increased resistance of the cells to oxidative and electrophilic stress.

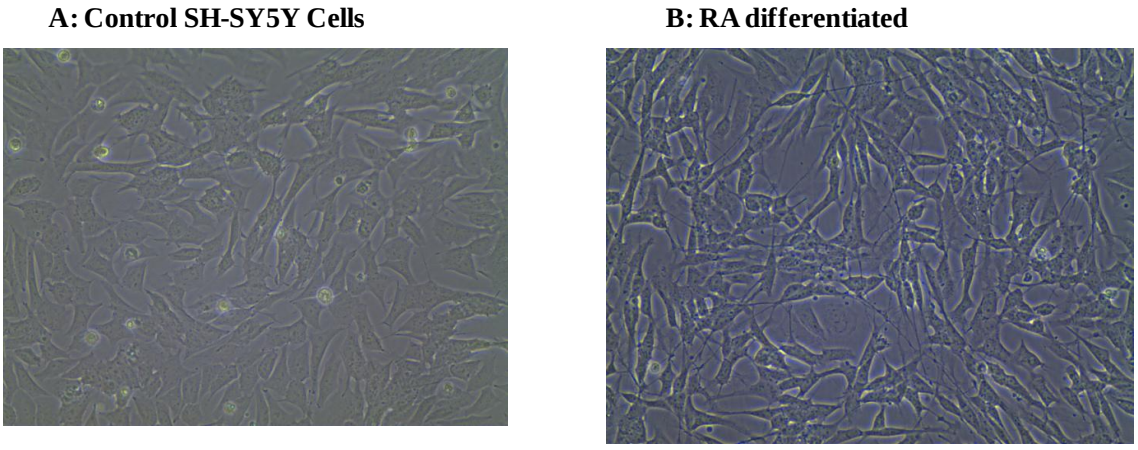

Figure 2. SH-SY5Y cell morphology change when treated with Retinoic Acid (RA). Panel A depicts undifferentiated SH-SY5Y cells grown in 10% FBS DMEM medium. Panel B depicts differentiation of SH-SY5Y cells by all-trans-retinoic acid (RA) treatment (10 μM) for 5 days.

Materials and Methods

Chemicals and Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin streptomycin, and fetal bovine serum (FBS) were obtained from Gibco-Invitrogen (Carlsbad, CA). CDDO-Im [2-Cyano-3,12-dixooleana-1,9-dien-28-imidazolide] was obtained from Toronto Research

Chemicals Inc. (Toronto, Canada). Acrolein, Retinoic Acid (RA), 3-morpholinosynonimine hydrochloride (SIN-1), 4-hydroxy-2-nonenal (HNE), Xanthine Oxidase, Xanthine Sodium Salt, GSH, 1-chloro-2,4dinitrobenzene (CDNB), o-phtalaldehyde, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltertrazolium bromide (MTT) were from Sigma Chemical (St. Louis, MO). Tissue culture flasks and 24-well tissue culture plates were from Corning (Corning, NY).

Cell Culture

Human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FBS, 100U/ml penicillin, and 100ug/ml streptomycin in $75cm^2$ tissue culture flasks at 37^oC in a humidified atmosphere of 5% CO_2. The cells were fed every 2-3 days and subcultured once they reached 80-90% confluence. For experiments, cells were differentiated in medium with 10µM Retinoic Acid (RA) for 5 to 7 days.

Cell Extract Preparation

After experimental treatment, cells were pelleted by centrifugation at 300 x g at 4^oC for 10 min. Cells were then washed once with phosphate buffered saline (PBS) and resuspended in ice-cold 50mM potassium phosphate buffer, pH 7.0, containing 1mM EDTA and 0.1% Triton X-100. The cell suspensions were sonicated, followed by centrifugation at 13,000g for 10 min at 4^oC to remove cell debris. The resulting supernatants were collected and the protein concentrations were quantified with Bio-Rad protein assay dye kit (Hercules, CA) based on the method using bovine serum albumin

(BSA) as the standard [40]. The supernatants were then used for measurement of the antioxidants and phase 2 enzymes.

Preparation of Mitochondrial Fraction

Mitochondria were isolated from freshly harvested SH-SY5Y cells. Briefly, cells were washed once with PBS and centrifuged. The cell pellet was resuspended in 5ml sucrose buffer (0.25 M sucrose, 10mM Hepes, 1mM EGTA and 0.5% BSA, pH 7.4), homogenized in a Dounce tissue grinder on ice. The homogenate was centrifuged at 1500 g for 10min at 4^{o}C. The supernatant was collected and centrifuged at 10,000g for 10min at 4^{o}C. The resulting mitochondrial pellet was washed twice with sucrose buffer and then resuspeneded in ice-cold 50mM potassium phosphate buffer, pH 7.0, containing 1mM EDTA and 0.1% Triton X-100, followed by sonication to lyse the mitochondria. The protein concentrations of mitochondrial lysates were measured as above.

Assay for Glutathione (GSH) Content

Total cellular and mitochondrial GSH content was measured according to the o-phthalaldehyde-based flourometric method, which is specific for determination of GSH at pH 8.0[41, 42]. A volume of the extract was incubated at 4^{o}C with 12.5µl of 25% metaphosphoric acid and a volume of 0.1 M sodium phosphate buffer containing 5mM EDTA for a final volume of 59.5µl for 10 min. The samples were centrifuged at 13,000g for 5min at 4^{o}C. The resulting supernatant was incubated with .1ml of o-phthalaldehyde solution buffer for 15 min at room temperature. Flourescence intensity was then measured at an excitation wavelength of 350nm and an emission wavelength of 420nm.

The sample GSH content was calculated using a GSH (Sigma-Aldrich) standard curve, and expressed as nanomoles of GSH per milligram of sample protein.

Assay for NAD(P)H: Quinone Oxidoreductase (NQO1) Activity

Cellular and mitochondrial NQO1 activity will be measured according to methods previously described [43]. The reaction mix contained 50mM Tris-HCl (pH 7.5), 0.08% Triton X-100, 0.25 mM NADPH, and 80 uM 2,6-dichloroindophenol (DCIP). A volume of sample was added last for a final volume of 0.7 ml. The reaction was conducted in the presence or absence of 60 µM dicumerol a potent inhibitor of NQO1. The two electron reduction of DCIP was monitored over 3 minutes at 25^{o}C at a wavelength of 600nm. The dicumerol-inhibited NQO1 activity was calculated using the extinction coefficient of 21.0 mM^{-1} cm^{-1}, and expressed as nanomoles of DCIP reduced per minute per milligram of sample protein [25, 26].

Assay for Glutathione Reductase (GR) Activity

Cellular GR activity was measured according to the procedures described [43]. This assay is based on NADPH consumption coupled with reduction of the oxidized form of glutathione (GSSG) to GSH by GR. To a cuvette a volume of sample, 60µl of 20mM GSSG and 50mM potassium phosphate buffer (pH 7.0) and 1mM EDTA were added for a final volume of 0.54 mL. The cuvettes were incubated for 3 minutes at 37^{o}C. The reaction was initiated by adding 60ul of 1.5mM NADPH. The subsequent consumption of NADPH was monitored at 340nm, 37^{o}C for 5 min. The sample GR activity was

calculated using the extinction coefficient of 6.22nM^{-1} cm^{-1}, and expressed as nanomoles of NADPH consumed per minute per milligram of sample protein.

Assay for Glutathione Peroxidase (GPx) Activity

Cellular GPx activity was measured based on the formation of GSSG from GPx-catalyzed oxidation of GSH by H_20_2, coupled with NADPH consumption in the presence of exogenously added GR. To a cuvette a volume of sample,50mM potassium phosphate (pH 7.0), 1mM EDTA and 2mM sodium azide, 60ul of 10mM GSH, 60 µl of glutathione reductase (2.4 U/ml), and 60 µl of 1.5mM NADPH for a final volume of 0.54 ml. The cuvette was incubated at 37^oC for 3 min. After addition of 60 µl of 2 mM H_20_2, the rate of NADPH consumption was monitored at 340 nm, 37^oC for 5 min. The sample GPx activity was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1}, and expressed as nanomoles of NADPH consumed per minute per milligram of sample protein.

Assay for Catalase Activity

Cellular catalase activity was measured according to a method previously described. To a quartz cuvette volumes of sample and 50mM potassium phosphate buffer (pH 7.0) were added for a final volume of 0.42 ml. The reaction was initiated by adding 0.18 ml of 30mM H_20_2. The decomposition of H_20_2 was monitored at 240nm, 25^oC for 2 min. The sample catalase activity was expressed as micromoles of H_20_2 consumed per minute per milligram of sample protein.

MTT Reduction Assay

Cell viability was determined by a modified MTT reduction assay, as described previously [42]. Cells were plated into 48-well tissue culture plates ($1x10^6$ cells per well). After incubation of the cells in the presence of oxidative and electrophilic species for 24h at 37^oC, the media was discarded followed by the addition of 0.4 ml of fresh DMEM supplemented with 0.5% FBS containing 0.2mg/ml MTT. The plates were incubated at 37^oC for another 2 h. The media was completely removed and to each well a mixture of dimethyl sulfoxide, isopropanol, and deionized water (1:4:5) was added so solubilize the formazan crystals. The amount of dissolved formazan was quantified at an absorbance of 570nm.

Statistical Analysis

Statistical analysis was performed using SAS software (Cary, NC). All data are expressed as means ± SEM from at least three independent experiments unless otherwise indicated. Differences between mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. Differences between two groups were analyzed by Student t-test. Statistical significance was considered at $p < 0.05$.

Results

Effect of CDDO-Im on Cellular Antioxidants and Phase II Enzymes

Shown in Figure 3A and 3B, SH-SY5Y cells express measurable basal levels of both GSH and NQO1. Incubation of the cells with CDDO-Im at 1, 10, 25, 100, and 400nM for 24 h led to induction of cellular GSH and NQO1. CDDO-Im at 10, 25, 100, and 400nM caused statistically convincing concentration dependent induction of GSH protein. Treatment of the cells with CDDO-Im at a concentration of 10nM resulted in a 41% increase in cellular GSH. CDDO-Im treatment at 1nM caused a statistically significant increase of 48% in cellular NQO1 levels. In contrast to the results observed for GSH and NQO1, incubation of SH-SY5Y cells with CDDO-Im did not result in any statistically significant changes in the activities of cellular GPx, GR, or Catalase, as noted in Table 1. Similarly, no cellular GSH or NQO1 induction was observed in cells treated with therapeutic concentrations of Vitamin E (Fig 3c and 3d).

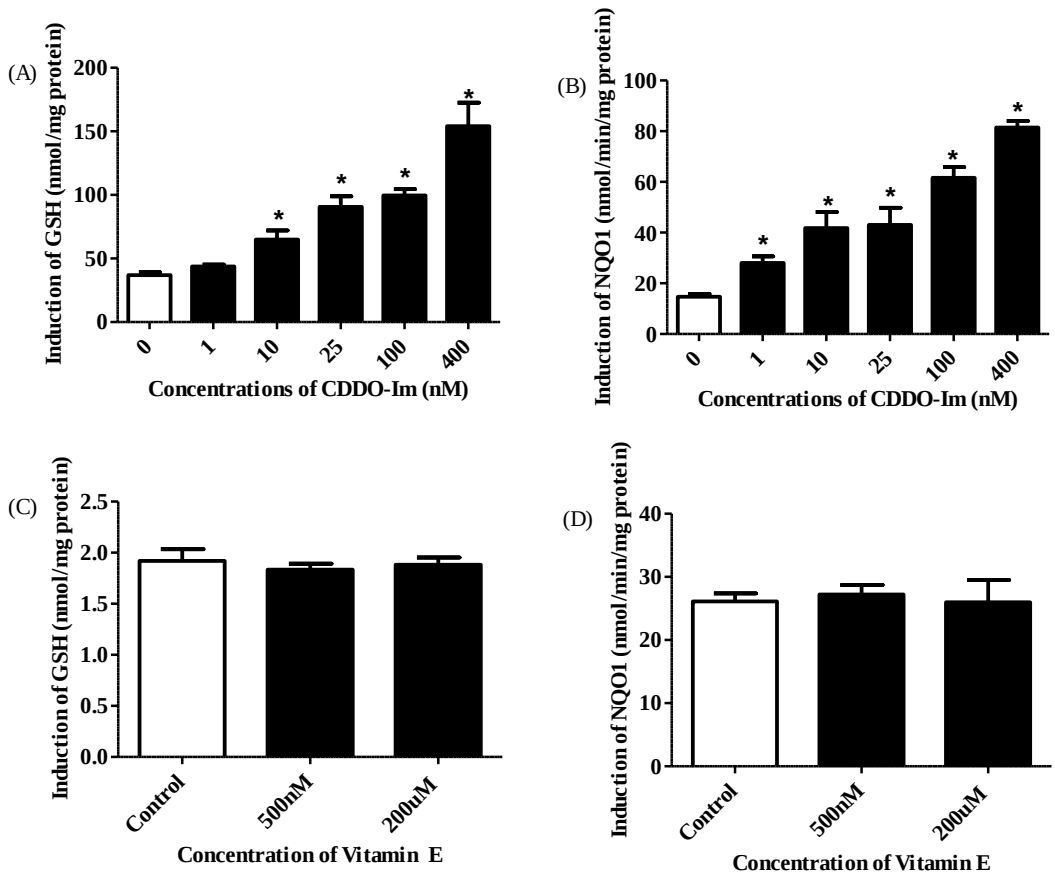

Figure 3. Cellular induction of GSH and NQO1 by CDDO-Im and Vitamin E. Concentration-dependent induction of GSH, NQO1 by CDDO-Im in RA-differentiated SH-SY5Y cells. Cells were incubated with the indicated concentrations of CDDO-Im for 24 h, followed by measurement of cellular GSH content (A) and activities of NQO1 (B). For contrast, cells were incubated with 500nM and 200μM Vitamin E for 24 h and evaluated for GSH and NQO1 levels (C and D). Values represent means ± SEM from at least three separate experiments. *Indicates statistically convincing evidence ($p < 0.05$) of a difference compared to the control treatment.

Treatment	GR (nmol/min/mg protein)	GPx (nmol/min/mg protein)	Catalase (nmol/min/mg protein)
Control	25.70 ± 1.79	26.14 ± 1.42	4.87 ± 0.17
1uM CDDO-Im	26.24 ± 3.87	27.27 ± 1.08	5.32 ± 0.29
10uM CDDO-Im	27.75 ± 3.74	28.92 ± 1.09	4.42 ± 0.31
25uM CDDO-Im	28.54 ± 2.67	25.55 ± 2.43	4.87 ± 0.20
100uM CDDO-Im	27.75 ± 3.66	26.48 ± 1.95	5.36 ± 0.32
400uM CDDO-Im	27.85 ± 3.73	24.58 ± 3.41	5.13 ± 0.21

Table 1. Effects of CDDO-Im treatment on levels of GR, GPx and Catalase. Values represent means ± SEM from at least three separate experiments. No statistically convincing evidence of a difference was observed.

Induction of Mitochondrial GSH and NQO1 by CDDO-Im

GSH and NQO1 are known to be present in the mitochondria of mammalian cells. Additionally, the mitochondria are predominant targets of ROS damage. For these reasons we examined the levels/activities of both GSH and NQO1 in the isolated mitochondria of SH-SY5Y cells after treatment with CDDO-Im for 24 h. As shown in Figure 4, incubation of the cells with concentrations as low as 10nM of CDDO-Im led to a statistically significant induction of mitochondrial GSH and NQO1.

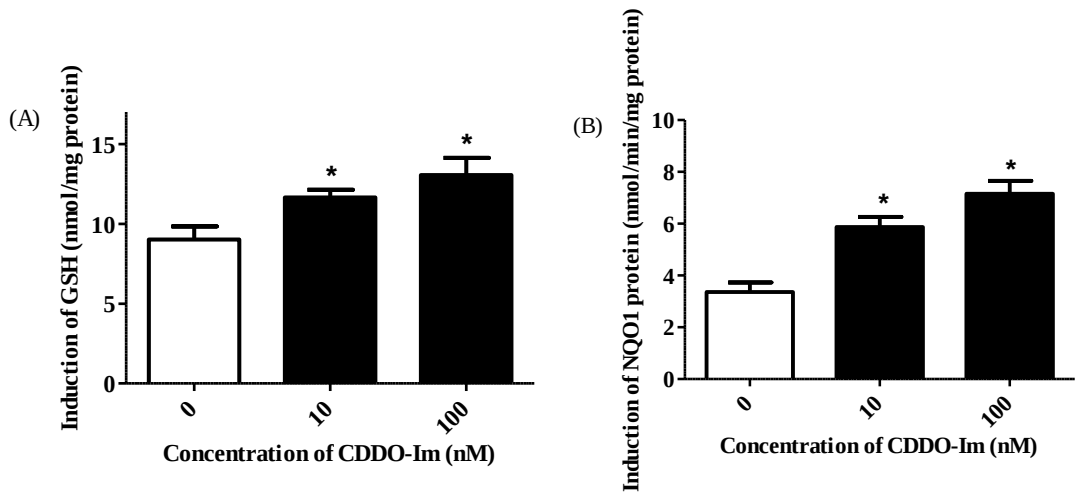

Figure 4. Mitochondrial induction of GSH and NQO1. Concentration dependent induction of mitochondrial GSH and NQO1 in RA-differentiated SH-SY5Y cells by CDDO-Im. The cells were incubated with 100 nM CDDO-Im for 24h, and then the mitochondria were isolated. Mitochondrial GSH level (A) and activities of NOQ1 (B) were measured as described in the "Materials and methods" section. Values represent means ± SEM from at least three separate experiments. *Indicates statistically convincing evidence ($p < 0.05$) of a difference compared to the control treatment.

Protective effect of CDDO-Im pretreatment on cytotoxicity by Xanthine Oxidase/Xanthine and H_20_2

XO/Xanthine and H_20_2 are toxic exposures which are known ROS-generating compounds. XO/Xanthine is a known donor of superoxide radicals while H_20_2 is a preliminary substrate in the creation of various ROS including superoxide and hydroxyl radicals. Antioxidants and phase 2 enzymes have been shown to play a critical role in the mitigation of ROS-induced damage. To determine if the elevated levels of GSH and NQO1 by CDDO-Im in RA-differentiated SH-SY5Y is correlated to cytoprotection against oxidative stress the cells were pretreated with CDDO-Im and then exposed to toxins: XO/Xanthine and H_20_2. In Figure 5A, incubation of the control cells with various

concentrations of XO in the presence of 0.5mM xanthine for 24h resulted in significant decreases in cell viability in a concentration-dependent manner. Similarly, Figure 5B depicts that incubation of control SH-SY5Y cells with various concentrations of H_20_2 for 24 h resulted in a significant decrease in cell viability in a concentration-dependent manner. Through our study, it was determined that at a concentration of 50µM XO/Xanthine there was a 70% reduction in cell viability. When cells were pretreated with 100nM CDDO-Im, 50µM XO/Xanthine resulted in only a 25% reduction in cell viability. Similarly, in cells treated with 150µM H_20_2, we observed a 70% reduction in cell viability, while cells pretreated with 100nM CDDO-Im for 24 h only experienced a 50% reduction in cell viability. The results indicate that pretreatment of the cells with CDDO-Im at 100nM for 24 h afforded concentration dependent protection against the above ROS-mediated cell injury from both toxins.

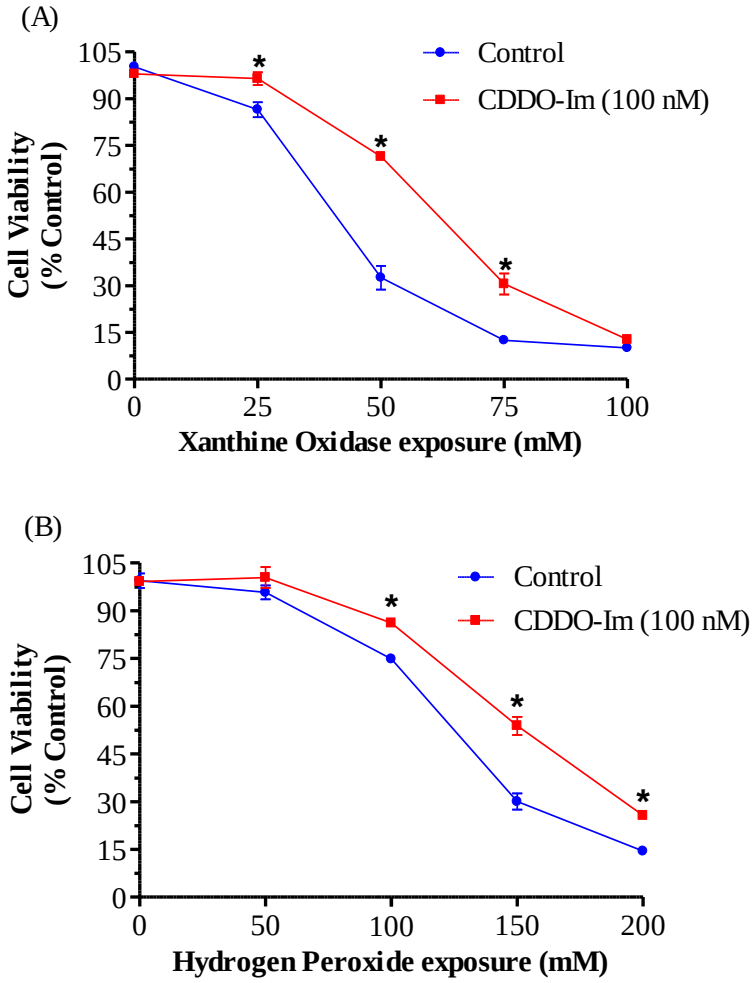

Figure 5. CDDO-Im mediated protection against XO/Xanthine and H_20_2 cytotoxicity. Protective effects of CDDO-Im-pretreatment on ROS -mediated cytotoxicity in RA-differentiated SH-SY5Y cells. The cells were pretreated with or without 100nM CDDO-Im for 24 h, followed by incubation with various concentrations of XO (in the presence of 0.5 mM xanthine) or H_2O_2 for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent means ± SEM from 4 separate experiments. *Indicates statistically convincing evidence ($p < 0.05$) of a difference between Control treatment and 100nM CDDO-Im treatment at each toxin concentration.

Protective effect of CDDO-Im pretreatment on cytotoxicity by 3-Morpholinosydnonimine (SIN-1)

The compound, SIN-1 is a known generator of peroxynitrite and nitric oxide during biological metabolism [44]. As noted in Figure 6, exposure of RA-differentiated SH-SY5Y cells to SIN-1 for 24 h resulted in a significant decrease in cell viability in a concentration-dependent manner. It was observed that the concentration-dependent cytotoxicity was attenuated by pretreatment of the cells with CDDO-Im at 100nM for 24 h. In our study we observed that treatment of RA-differentiated SH-SY5Y cells with SIN-1 at a concentration of 400μM caused a 21% reduction in cell viability and a concentration of 800μM showed a 73% reduction in cell viability. In contrast, when cells were pretreated with 100nM CDDO-Im, the cells showed only a 4% reduction and 26% reduction in cell viability respectively.

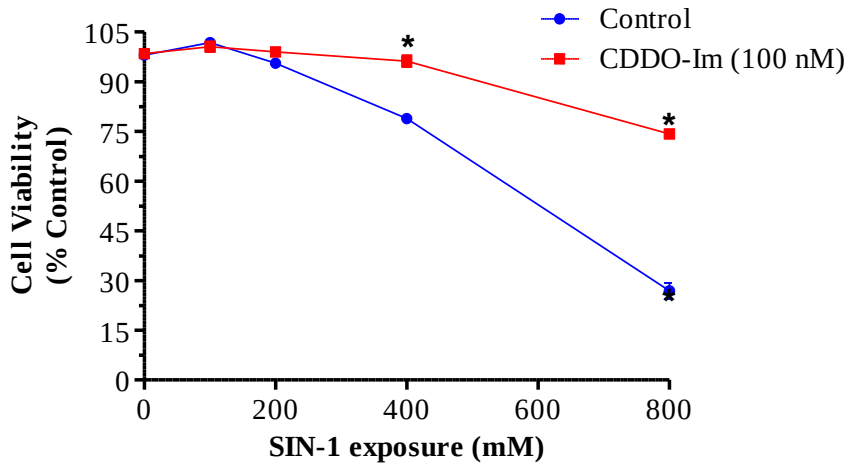

Figure 6. CDDO-Im mediated protection against SIN-1 cytotoxicity. Protective effects of CDDO-Im-pretreatment on SIN-1-mediated cytotoxicity in RA-differentiated SH-SY5Y cells. The cells were pretreated with or without 100nM CDDO-Im for 24 h, followed by incubation with various concentrations of SIN-1, a known peroxynitrite donor for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent means ± SEM from 4 separate experiments. *Indicates statistically convincing evidence ($p < 0.05$) of a difference between Control treatment and 100nM CDDO-Im treatment at each toxin concentration.

Protective effect of CDDO-Im pretreatment on cytotoxicity by 4-Hydroxy-2noneal (HNE)

HNE is a lipid peroxidizer and a neurotoxic marker found in some individuals with neurodegenerative disorders [19]. Figure 7 shows that exposure of RA-differentiated SH-SY5Ycells to HNE for 24 h also significantly decreased cell viability in a concentration-dependent manner. When pretreated with 100nM CDDO-Im for 24 h, we have shown increased cell viability in SH-SY5Y cells. In our study, cells were exposed to 10, 20, 40, 60, and 80 µM HNE and cell viability was measured using MTT assay. Our results in Figure 7 show that cells exposed to 60 µM HNE experienced an 87% reduction in cell viability while cells pretreated with 100nM CDDO-Im only showed a 46% reduction in cell viability.

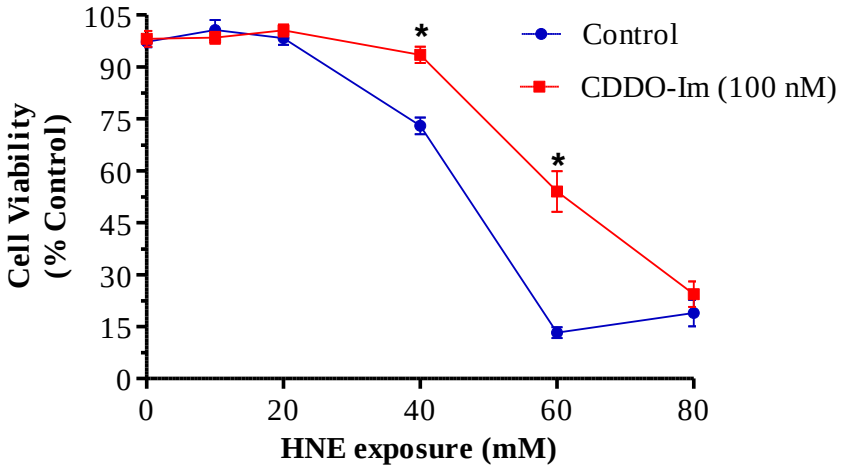

Figure 7. CDDO-Im mediated protection against HNE cytotoxicity. Protective effects of CDDO-Im-pretreatment on HNE-mediated cytotoxicity in RA-differentiated SH-SY5Y cells. The cells were pretreated with or without 100nM CDDO-Im for 24 h, followed by incubation with various concentrations of HNE, a known lipid peroxidizer for another 24 h. After this incubation, cell viability was determined using MTT reduction assay. Values represent means ± SEM from 4 separate experiments. *Indicates statistically convincing evidence ($p < 0.05$) of a difference between Control treatment and 100nM CDDO-Im treatment at each toxin concentration.

Discussion

It has become increasingly known that oxidative stress including environmental toxicant-mediated ROS damage in nervous tissue has contributed to neurodegenerative disorders such as PD [45, 46]. Studies have established that the brain and neuronal cell types remain some of the most vulnerable cell types to oxidative stress due to the fact that a high percentage of the oxygen consumed by the human body is allocated to brain function, and dopaminergic neurons have been shown to perform cellular respiration at a higher rate than other cell types [12, 47]. The high levels of respiration in these cells results in the creation of various types of ROS capable of damaging cellular lipids, proteins, DNA, and carbohydrates [48, 49]. ROS, such as super oxide radical, hydrogen

peroxide, and peroxynitrites have been found to be elevated in damaged and stressed neuronal tissue. Concurrently, depletion of antioxidant defense mechanisms, such as GSH, has become an early marker for PD [49]. Thus, it has become increasingly important to study therapies to induce antioxidant defense mechanisms such as GSH.

In this study we utilize human SH-SY5Y cells differentiated with retinoic acid (RA) as an in vitro system to determine if the synthetic triterpenoid CDDO-Im can mediate the induction of key endogenous antioxidant defenses and provide a cytoprotective effect against various electrophilc and oxidative species [38, 39]. The design of this study shows that nanomolar concentrations of CDDO-Im can upregulate the antioxidant defenses GSH and NQO1 at both the cellular and mitochondrial levels. However, at the same time, CDDO-Im pretreatment did not result in any significant induction of GPx, GR, or Catalase. These results indicate that GSH and NQO1 may be regulated by distinct signaling pathways. In this regard the nuclear factor E2-related factor 2 (Nrf2) and its binding to the antioxidant response element have been found to be critical transcription steps for many cytoprotective genes in various cell types including CDDO-MA-treated SH-SY5Y cells [36]. Further study should be performed to determine if the Nrf2 signaling pathway is also responsible for the coordinated induction of antioxidant and phase 2 defenses by CDDO-Im in SH-SY5Y cells.

GSH and NQO1 are two critical cellular defenses that protect neuronal cells against oxidative and electrophilic insults. GSH is a tripeptide molecule which at increased concentrations has been shown to assist in the detoxification of many types of ROS including those associated with the pathophysiology involved in neurodegenerative

disorders such as PD [20, 50]. One of the earliest biochemical markers observed in patients with PD is a depletion of GSH content [51]. GSH behaves as a compound capable of direct removal of ROS as well as a substrate for a number of associated enzymes including GR, GPx, and GST. Ultimately, the network of GSH protective efforts has been shown to contribute to DNA repair, protein function, and synthesis of supportive enzymes and amino acids [52]. Many recent studies have shown that the regulation of GSH is a key component in normal cell function and the mitigation of oxidative stress.

In addition to GSH, NQO1 is another endogenous compound associated with detoxification of ROS in neurodegenerative disorders [53]. NQO1 is an important cellular phase II enzyme, the main function of which is the metabolism of quinones to a more water-soluble derivative for removal from the body [54, 55]. More recently, NQO1 has also been implicated in direct ROS scavenging [56]. Specifically, NQO1 has been shown to directly autoxidize and detoxify superoxide radicals and hydrogen peroxide in various cell types [57]. Thus, our findings suggest that the coordinated induction of the endogenous antioxidant GSH and enzyme NQO1 by CDDO-Im may be a promising strategy for protecting against oxidative and electrophilic neuronal cell degeneration (Figure 3A and 3B).

It is important to note that in addition to cellular antioxidant and phase 2 enzymes, marked induction of mitochondrial GSH content and NQO1 by CDDO-Im were also observed. Mitochondria are the predominant site of cellular respiration in the cell and are also extremely vulnerable to oxidative stress [58]. The high levels of respiration produce

excess ROS as a byproduct including superoxide, hydrogen peroxide, and hydroxyl radical [59, 60]. Mitochondrial dysfunction has also been found as a common symptom in PD pathogenesis [61-63]. Mitochondria do have their own endogenous ROS scavenging compounds, but during the etiology of the disease, the amount of ROS generation inevitably overwhelms the natural defenses of the cell [64]. Depleted amounts of mitochondrial GSH have been shown to directly result in increased levels of H_20_2 and oxidative damage [65]. Thus, increasing the ROS defenses available to the cell and mitochondria is an important step in preventing dysfunction. Our study provides the important observation of the potential for mitochondrial antioxidant and phase II defenses since mitochondria dysfunction is a precursor to decreased cell viability and degenerative disorders such as PD (Figures 4A and 4B) [61-63].

In addition to induction of antioxidant and phase II enzymes in RA-differentiated SH-SY5Y cells treated with CDDO-Im, this study, for the first time, observes the cytoprotective effect pretreatment with CDDO-Im can have on RA-differentiated SH-SY5Y cells (Figure 2A). SH-SY5Y cells are generally classified as dopaminergic neurons, which possess many qualities of substantia nigra neurons with high levels of dopamine hydroxylase and tyrosine hydroxylase [39, 66]. The pretreatment of these cells with retinoic acid further matures them to resemble neuronal cell types by slowing their proliferation and changing their morphology to more closely resemble dopaminergic neurons. We observed that the coordinated induction of cellular defenses was accompanied by augmented resistance of these cells to oxidative damage induced by

super oxide donors such as XO/xanthine and H_20_2, nitric oxide/peroxynitrate derived from SIN-1, and lipid peroxidation caused by HNE.

XO/xanthine and H_20_2 are widely used superoxide radical donors and have previously been shown to induce oxidative stress in various cell types [67, 68]. In our study we used these two exogenous toxins as a way to increase the oxidative stress on RA-differentiated SH-SY5Y cells. H_20_2 is a prolific and yet stable form of ROS making it able to pass through cellular barriers before being metabolized to the more reactive superoxide and hydroxyl radicals [69]. H_20_2 is of particular interest since it is a byproduct of dopamine turnover in dopaminergic neurons and may be associated with the accelerated cell degeneration associated with PD [70]. XO/xanthine acts as a single electron donor to normal 0_2 molecules forming the superoxide radical [71]. The superoxide radical has been implicated in disrupting enzyme function and damaging nucleic acids [72, 73]. In our study we utilize H_20_2 and XO/xanthine as systems to increase oxidative stress in SH-SY5Y cells. Our results show statistically convincing evidence that treatment with CDDO-Im reduces the cytotoxicity of ROS generated by XO/xanthine and H_20_2 (Figures 5A and 5B).

Cellular dysfunction causing ROS also includes nitrogenous compounds including nitric oxide (NO) which readily reacts with the superoxide radical to form the highly reactive peroxynitrite compound [74]. SIN-1 is a known peroxynitrite donor in cellular metabolism and has been found to cause direct oxidative damage to various cell types [44]. Peroxynitrite molecules cause oxidative damage to key cellular proteins and nucleic acids, altering cell signaling function and decreasing cell viability [75].

Peroxynitrite and NO have also been found to induce apoptosis in SH-SY5Y cells [76-78]. In our study, these results show that CDDO-Im pretreatment is correlated to a mitigation of SIN-1-mediated cytotoxcity (Figure 6).

In addition to SIN-1, we tested the impact exposure HNE would have on RA-differentiated SH-SY5Y cells and the effect pretreatment with CDDO-Im would have on cell viability. HNE is a reactive aldehyde compound capable of lipid peroxidation damage which disrupts the integrity of the lipid membrane of the cells and ultimately reduces cellular viability [79, 80]. Specifically, HNE has been implicated in a range of cytopathological effects including inhibition of protein function, DNA transcription, and cellular signaling [79]. Routinely, autopsied brain tissue from PD patients has been shown to contain increased levels of protein carbonyl damage and HNE, both indicative of oxidative damage to the central nervous system (CNS) [81, 82]. Our treatment concentrations were chosen to be biologically relevant since HNE adducts have been found at concentrations up to 40 μM in the CNS. Our results indicate that pretreatment with CDDO-Im is capable of protecting against HNE-mediated toxicity in a concentration dependent manner (Figure 7).

In conclusion, pretreatment of RA-differentiated SH-SY5Y cells with CDDO-Im significantly elevates both cellular and mitochondrial concentrations of GSH and NQO1. More importantly, the pretreatment has been shown to effectively mitigate a reduction in cell viability caused by ROS-generating compounds: XO/xanthine, H_20_2, SIN-1, and HNE. Overall, our results demonstrate that CDDO-Im may be a potent and novel

treatment option for mitigating neurodegeneration and ultimately slowing the progression of disorders such as Parkinson's disease (Figure 8).

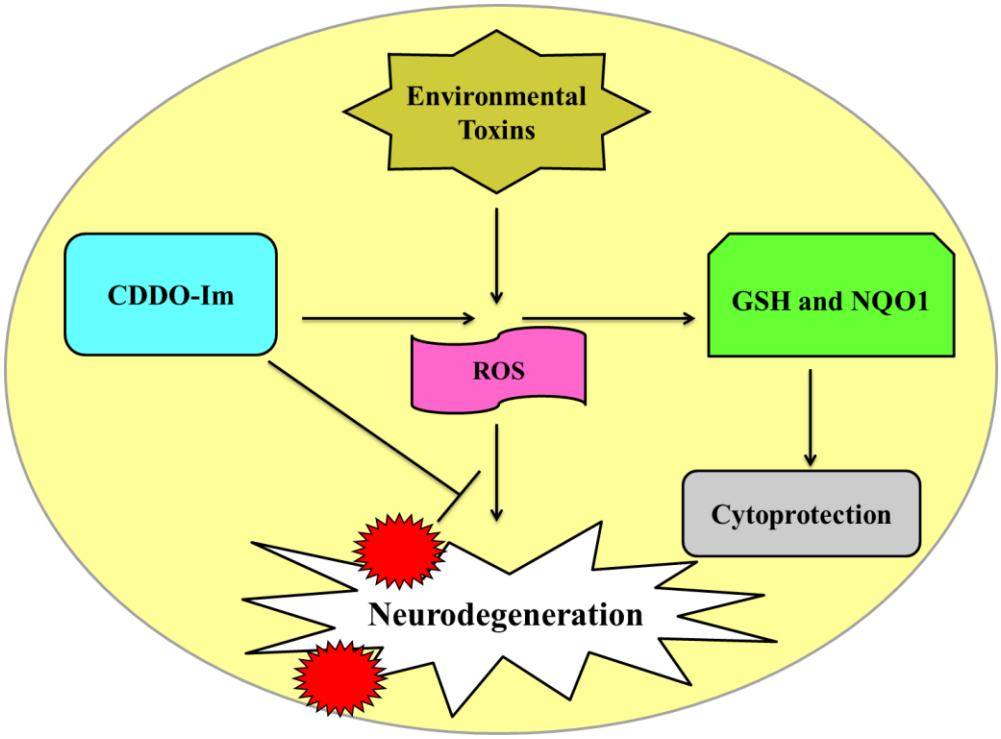

Figure 8. Diagram of proposed cytoprotection by CDDO-Im.

CHAPTER III

THE MECHANISTIC ROLE OF GSH IN CDDO-IM MEDIATED PROTECTION AGAINST ACROLEIN-INDUCED NEUROCYTOTOXICITY

Introduction

Acrolein is a ubiquitous environmental toxin formed during the combustion of organic matter. The toxin is found in many pesticide products and in all forms of smoke, including automobile exhaust and cigarettes[5]. An exogenous reactive aldehyde, acrolein, has been found to readily peroxidize lipids including the fatty acid rich nerve cell types [6]. Recent studies show that the resulting oxidative damage leads to decreased cell proliferation and increased apoptosis [7, 8]. Acrolein can also be formed inside the cell as a product of lipid peroxidation by other forms of ROS [4]. Neuronal cell exposure to acrolein and other (ROS) generating compounds and the subsequent cellular damage are found to be associated with the progression of Parkinson's Disease as well as other neurodegenerative disorders [9, 10]. Elevated concentrations of acrolein, up to 40μM, have been found in the brain tissue of PD patients and are considered a specific marker for lipid peroxidation damage [7, 11]. Formation of these acrolein-protein adducts have been implicated in the pathogenesis of neurodegenerative disorders including PD [9, 10]. Acrolein causes damage to biomolecules including lipids, protein, and DNA damage leading to cell death and neurodegeneration [4]. In addition to direct oxidative damage, acrolein also plays a role in the depletion of beneficial antioxidant defenses such as

cellular glutathione (GSH) [8, 83]. Thus, treatment options designed to upregulate the natural cellular antioxidant defenses were found to partially protect against substantia nigra degeneration in animal models [84, 85].

Studies have shown that GSH, glutathione-s-transferase (GST), and aldose reductase (AR) play important roles in the detoxification of acrolein [21, 86]. Foremost among these is GSH, a tripeptide thiol consisting of glutamate, cysteine, and glycine with higher levels in neuronal cells [87]. GSH is capable of conjugating reactive species and is also aided by other enzymatic antioxidants to detoxify peroxides in the cell [88]. It has been suggested that GST utilizes GSH as a cofactor in its detoxification process of eledtrophilic aldehydes [89]. Similarly, AR has been shown to utilize a binding site on GSH to detoxify the GSH-adlehyde conjugates [90, 91]. Due to the limited successes using exogenous antioxidants, many studies have been focused on the increase of the endogenous antioxidant capabilities of the cells, including GSH, against oxidative injury. Thus, regulation of GSH, GST, and AR could be an important defense mechanism against acrolein-induced neurological disorders. In our study we proposed a novel strategy for protective intervention of PD through the upregulation of endogenous antioxidant defenses in neuronal SH-SY5Y cells mediated by the synthetic triterpenoid compound 2-Cyano-3,12-dixooleana-1,9-dien-28-imidazolide (CDDO-Im). Triterpenoids are steroid like compounds derived from plant extracts which have shown to have numerous protective/therapeutic effects on various cell types[28]. Various forms of CDDO have also been utilized in clinical trials as an anti-cancer proliferation treatment and an anti-inflammatory agent for rheumatoid arthritis [31-34].

We have recently found that CDDO-Im at low concentrations, within an achievable plasma range, significantly induces GSH and NQO1 in RA-differentiated SH-SY5Y cells conferring protection against reactive oxidative and electrophilic species. However, the effects of CDDO-Im in ameliorating the toxic effects of acrolein remain unknown. Furthermore, it remain unclear the roles of GSH, GST, and AR in CDDO-Im mediated cytoprotection against acrolein toxicity. In this study, we determined the protective effect of CDDO-Im against acrolein-induced cytotoxicity and its potential for induction of GSH, GST, and AR to elicit its protective effect in RA-differentiated SH-SY5Y cells. Additionally, we further identified the mechanism by which CDDO-Im mediates protection through the prevalence of GSH.

Materials and Methods

Chemicals and Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin streptomycin, and fetal bovine serum (FBS) were obtained from Gibco-Invitrogen (Carlsbad, CA). CDDO-Im [2-Cyano-3,12-dixooleana-1,9-dien-28-imidazolide] was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Acrolein, GSH, Retinoic Acid (RA), 1-chloro-2,4dinitrobenzene (CDNB), o-phtalaldehyde, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltertrazolium bromide (MTT) were from Sigma Chemical (St. Louis, MO). Tissue culture flasks and 24-well tissue culture plates were from Corning (Corning, NY).

Cell Culture

Human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FBS, 100U/ml penicillin, and 100ug/ml streptomycin in $75cm^2$ tissue culture flasks at 37^oC in a humidified atmosphere of 5% CO_2. The cells were fed every 2-3 days and subcultured once they reached 80-90% confluence. For experiments, cells were differentiated in medium with 10µM Retinoic Acid (RA) for 5 to 7 days.

Cell Extract Preparation

After experimental treatment, cells were pelleted by centrifugation at 300 x g at 4^oC for 10 min. Cells were then washed once with phosphate buffered saline (PBS) and resuspended in ice-cold 50mM potassium phosphate buffer, pH 7.0, containing 1mM EDTA and 0.1% Triton X-100. The cell suspensions were sonicated, followed by centrifugation at 13,000g for 10 min at 4^oC to remove cell debris. The resulting supernatants were collected and the protein concentrations were quantified with Bio-Rad protein assay dye kit (Hercules, CA) based on the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The supernatants were then used for measurement of the antioxidants and phase 2 enzymes.

Assay for Glutathione (GSH) Content

Total cellular and mitochondrial GSH content was measured according to the o-phthalaldehyde-based flourometric method, which is specific for determination of GSH at pH 8.0[41, 42]. A volume of the extract was incubated at 4^oC with 12.5µl of 25%

metaphosphoric acid and a volume of 0.1 M sodium phosphate buffer containing 5mM EDTA for a final volume of 59.5µl for 10 min. The samples were centrifuged at 13,000g for 5min at 4^{o}C. The resulting supernatant was incubated with 0.1ml of o-phthalaldehyde solution buffer for 15 min at room temperature. Fluorescence intensity was then measured at an excitation wavelength of 350nm and an emission wavelength of 420nm. The sample GSH content was calculated using a GSH (Sigma-Aldrich) standard curve, and expressed as nanomoles of GSH per milligram of sample protein.

Assay for Glutathione S-transferase (GST) Activity

Cellular GST activity was measured according to the method described previously using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate[92]. The reaction mix was made containing 1 mM GSH, 1mM CDNB, and 3 mg/ml bovine serum albumin in 0.1 M sodium phosphate buffer (pH 6.5). The above reaction mix was added to a cuvette with a volume of sample for a final volume of 0.6 ml. The subsequent rate of formation of CDNB-GSH conjugate was monitored at 340 nm, 25^{o}C for 5 min. The sample GST activity was calculated using the extinction coefficient of 9.6 nM^{-1} cm^{-1}, and expressed as nanomoles of CDNB-GSH conjugate formed per minute per milligram of sample protein.

Assay for Aldehyde Reductase (AR) Activity

Cellular AR activity was measured using a modified method as described previously [93, 94]. To a cuvette 50mM potassium phosphate buffer, pH 6.0, 0.4 M Li2SO4, 10mM D,L-glyceraldehyde and 100uM NADPH was added. The reaction was initiated by adding 50µl sample. Activity was measured by monitoring of the subsequent

consumption of NADPH spectrophotometrically at 340nm, 25^{o}C for 5 min. The sample AR activity was expressed as nanomoles of NADPH consumed per minute per milligram of cellular protein.

MTT Reduction Assay

Cell viability was determined by a modified MTT reduction assay, as described previously [42]. Cells were plated into 48-well tissue culture plates (1x10^{6} cells per well). After incubation of the cells in the presence of oxidative and electrophilic species for 24h at 37^{o}C, the media was discarded followed by the addition of 0.4 ml of fresh DMEM supplemented with 0.5% FBS containing 0.2mg/ml MTT. The plates were incubated at 37^{o}C for another 2 h. The media was completely removed and to each well a mixture of dimethyl sulfoxide, isopropanol, and deionized water (1:4:5) was added so solubilize the formazan crystals. The amount of dissolved formazan was quantified at an absorbance of 570nm.

Carbonyl Reduction Assay

Cell extract was prepared as described above and carbonylation of cell extract was performed according to methods described previously [95]. Firstly, 300 µL of DNPH reagent was combined with 75 µL sample in a micro centrifuge tube and incubated at room temperature in the dark and mixed by vortex every 15 minutes. At the same time a control tube of 75 µL sample and 300 µL 2M HCL was prepared in exactly the same manner. Next added 375 µL 20% TCA and mixed by vortex, incubated on ice for 5 minutes. The cells were centrifuged for 10 min at 4^{o}C, supernatant discarded, and the

supernatant resuspended in 375 µL 10% TCA on ice for 5 min. Repeat previous wash sequence and discard supernatant. The pellet was then resuspended in 500 µL of ethanol: ethyl acetate (1:1) mixture and resuspended pellet with a spatula. After repeating wash resuspend the pellet with 500 µL 6 M guanidine hydrochloride and vortex. Centrifuge again and remove any leftover debris. Transfer 220 µL of supernatant into a 96 well plate and measure at wavelength 370 nm wavelength.

TBARS Assay

Cell extract was prepared as above with the exception that the whole homogenate will be used for testing in a modified method measuring TBARS[96]. Firstly, 50 µL cell extract, 50 µL 8.1 % SDS, 375 µL 20% acetic acid, 375 µL 0.8% TBA and 150 µL deionized water. The tubes were then placed in an incubator at 95^{o}C for 60 min. After cooling, samples and standards were read at 532nm. Results were expressed in nanomoles MDA per milliliter of protein.

Assay for Lactate Dehydrogenase (LDH) Release

LDH is an intracellular enzyme which leaks out from cells due to plasma membrane damage and is widely used to determine cytotoxicity as described previously [97]. RA-differentiated SH-SY5Y cells were treated with DMEM medium with 1% FBS. After incubation the cell culture medium was collected from the cell culture and centrifuged. The supernatant was collected in a new tube for assay. To an assay cuvette, 50µl of the collected culture medium 30µL 5.5mM sodium pyruvate, and 490µl PBS were added. To initiate the reaction 30µl 3mg/ml NADH was added. The LDH-catalyzed

NADH activity was expressed as nanomoles of NADH consumed per minute per milliliter of culture medium.

Statistical Analysis

Statistical analysis was performed using SAS software (Cary, NC). All data are expressed as means ± SEM from at least three independent experiments unless otherwise indicated. Differences between mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test. Differences between two groups were analyzed by Student t-test. Statistical significance was considered at $p < 0.05$.

Results

Protective effects of CDDO-Im pretreatment on acrolein-induced cytotoxicity

Cellular antioxidants/enzymes play important roles in protecting cells against oxidative stress induced neuronal damage. We recently found that various cellular antioxidant/phase 2 enzymes can be induced by CDDO-Im in RA-differentiated SH-SY5Y cells. Because of the critical involvement of acrolein and antioxidant defenses in the pathogenesis of neurodegenerative diseases, we investigated the cytoprotective effects of CDDO-Im pretreatment on acrolein-induced toxicity in RA-differentiated SH-SY5Y cells. RA-differentiated SH-SY5Y cells were tested in response to exposure to acrolein for 24 h with or without a 24 h pretreatment of 100nM CDDO-Im. Control cells showed a decrease in cell viability when treated with acrolein at concentrations of 20, 40, and 80 μM for 24 h. Conversely, the cells pretreated with 100nM CDDO-Im for 24 h showed a

concentration dependent significant increase in cell viability when treated with acrolein under the same conditions, as determined by the MTT assay. Pretreatment of SH-SY5Y cells with CDDO-Im afforded a dramatic protection against acrolein-induced toxicity when compared to treatment with Vitamin E (Fig. 9). We also visualized the cellular damage to RA-differentiated SH-SY5Y cells and observed that pretreatment with 100nM CDDO-Im resulted in a less dramatic change in cell morphology when exposed to 40 µM acrolein(Fig 10).

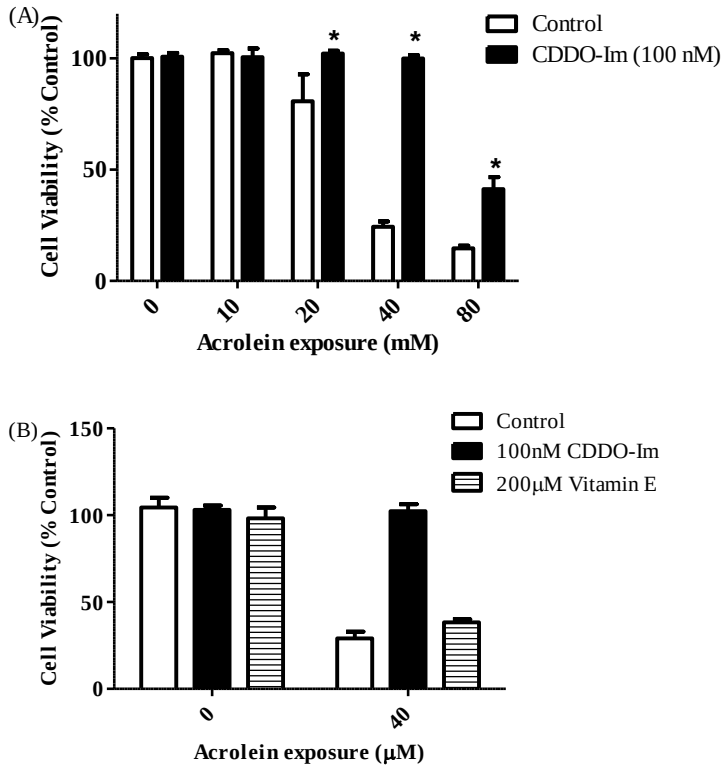

Figure 9. Effects of CDDO-Im pretreatment on acrolein-induced cytotoxicity. In panel A, RA-differentiated SH-SY5Y cells were treated with and without 100nM CDDO-Im for 24 h followed by 10-80 µM acrolein. In panel B, cells were pretreated with 100nM CDDO-Im or 200 µM Vitamin E for 24 h followed by exposure to 40 µM acrolein for 24 h. After incubations, cytotoxicity was determined by MTT reduction assay Values represent means ± SEM from at least three separate experiments. *Indicates significantly convincing difference ($p < 0.05$) from the respective control group.

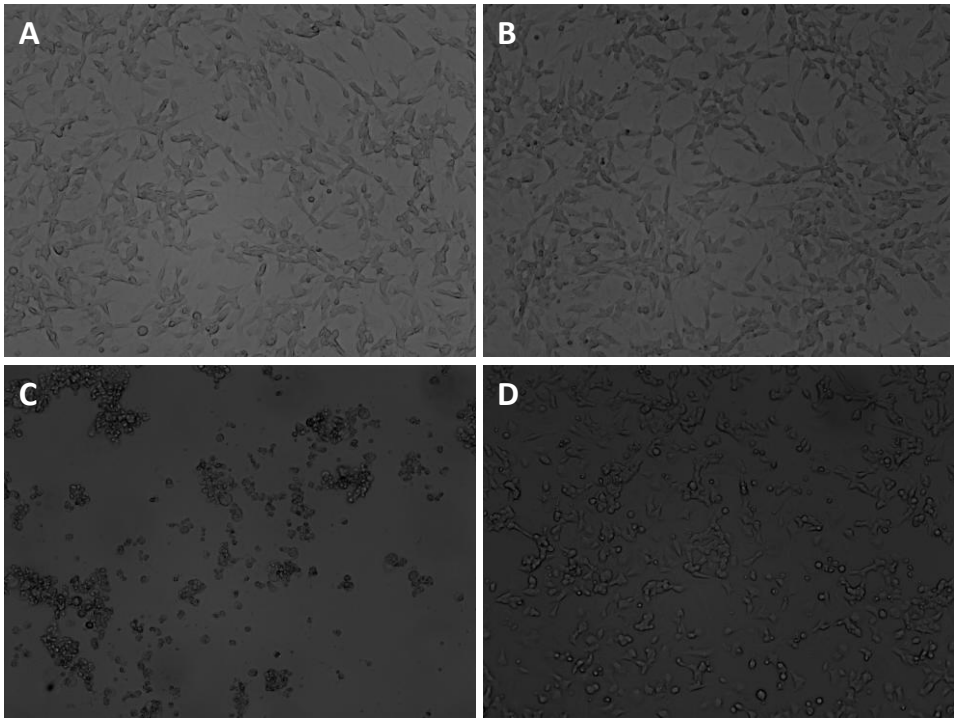

Figure 10. Phase-contrast microscopy. Panel A shows control RA-differentiated SH-SY5Ycells, panel B shows cells treated with 100nM CDDO-Im for 24 h, panel C shows cells treated with 40 µM acrolein for 24 h and panel D shows cells pretreated with 100nM CDDO-Im for 24 h followed by 40 µM acrolein for 24 h.

Effects of CDDO-Im treatment on GSH content and GST and AR activity

We recently showed that incubation of RA-differentiated SH-SY5Y cells with 10, 25, 100, and 400 µM CDDO-Im for 24 h resulted in significant increases in total cellular GSH content in a concentration dependent fashion. Because, GST and AR play a critical role in detoxification of acrolein, we next determined whether the activities of GST and AR are altered by CDDO-Im. As shown in Figure 11, panel A depicts that RA-differentiated SH-SY5Y cells treated with 100nM CDDO-Im for 24 h resulted in a significant increase in cellular GSH but not in GST or AR. In panel B, the mediated

induction of GSH by 100nM of CDDO-Im is further determined to occur in a time dependent manner.

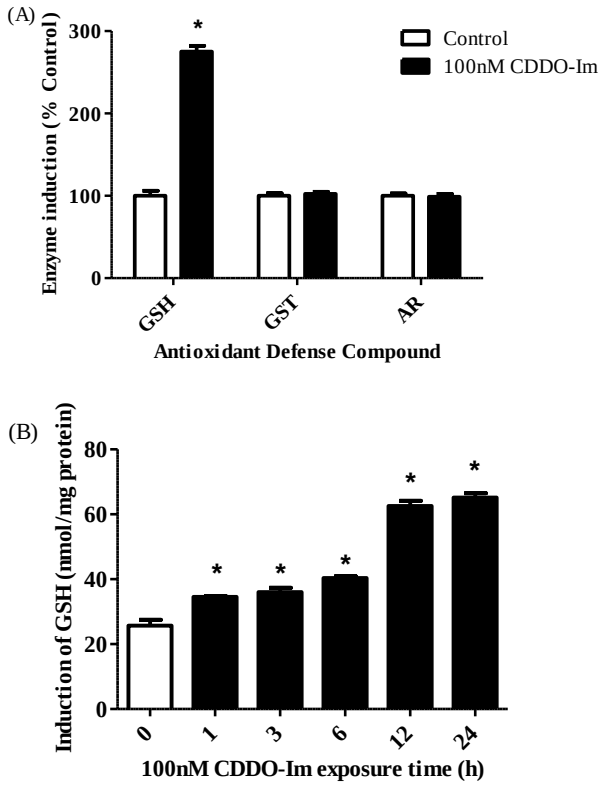

Figure 11. Effect of CDDO-Im treatment on GSH, GST, and AR. Effects of CDDO-Im treatment on cellular levels of GSH, GST, and AR (A) in RA-differentiated SH-SY5Y cells. Cells were incubated with 100 nM CDDO-Im followed by measurement of cellular GSH content and activities of GST and AR (panel A). Panel B depicts a time dependent increase in cellular GSH in RA-differentiated SH-SY5Y cells treated with 100nM CDDO-Im. Values represent means ± SEM from at least three separate experiments. *Indicates significantly convincing difference ($p < 0.05$) from the respective control group.

GSH depletion by acrolein

Next we investigated the role of GSH in detoxification of acrolein. To this end we determined if acrolein could cause depletion of cellular GSH content in RA-differentiated SH-SY5Y cells. Figure 12A indicates that cells incubated with 40 µM acrolein for 0.5-6

h resulted in a significant depletion of cellular GSH. While Figure 12B indicates the GSH depletion precedes the decrease in cell viability as noted by the LDH release into cell culture media. Significant depletion of cellular GSH is observable at the 0.5 h time point while significant LDH increase is observed at the 2 h time point.

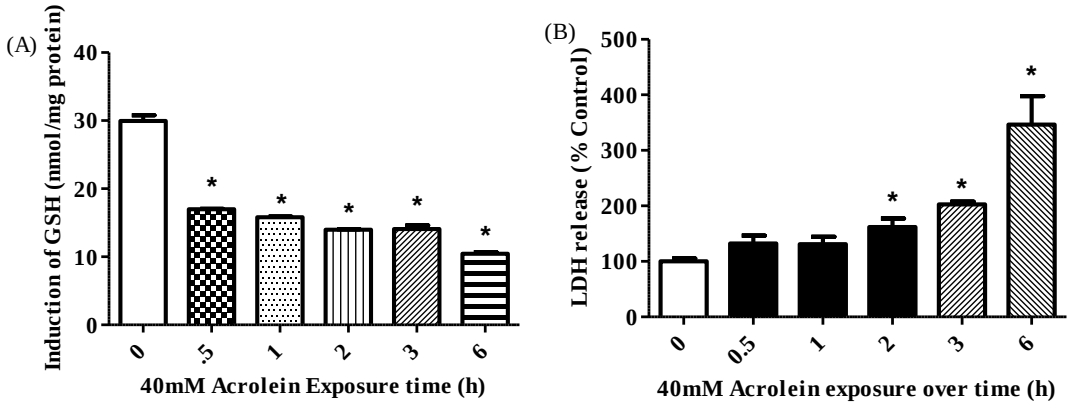

Figure 12. Time dependent acrolein-induced GSH depletion and LDH release. Panel A shows that RA-differentiated SH-SY5Y cells treated with 40µM acrolein exhibit a time dependent depletion of cellular GSH levels. Panel B depicts treatment with 40µM acrolein results in a time dependent increase in cellular LDH released. Values represent means ± SEM from at least three separate experiments. *Indicates significantly convincing difference ($p < 0.05$) from the respective control group.

Effects of BSO pretreatment on acrolein-induced cytotoxicity

BSO is a known specific inhibitor of the GSH subunit gamma-glutamylcysteine synthetase (GCLC) and has been widely used for depleting intracellular GSH in various cell types. As shown in Figure 13A, incubation of RA-differentiated SH-SY5Y cells with 25, 50, and 100 µM BSO for 24 h caused a 50-80% decrease in total cellular GSH without any change in cell viability as seen in LDH release and MTT assay results (Figure 13B and 13C). In Figure 13D cells were pretreated with and without 25 µM BSO

for 24 h, and then exposed to various concentrations of acrolein for another 24 h. The observed MTT assay indicates that pretreatment with BSO for 24 h resulted in a significant potentiation of acrolein-induced cytotoxicity when compared to cells without BSO, particularly at the 20µM concentration.

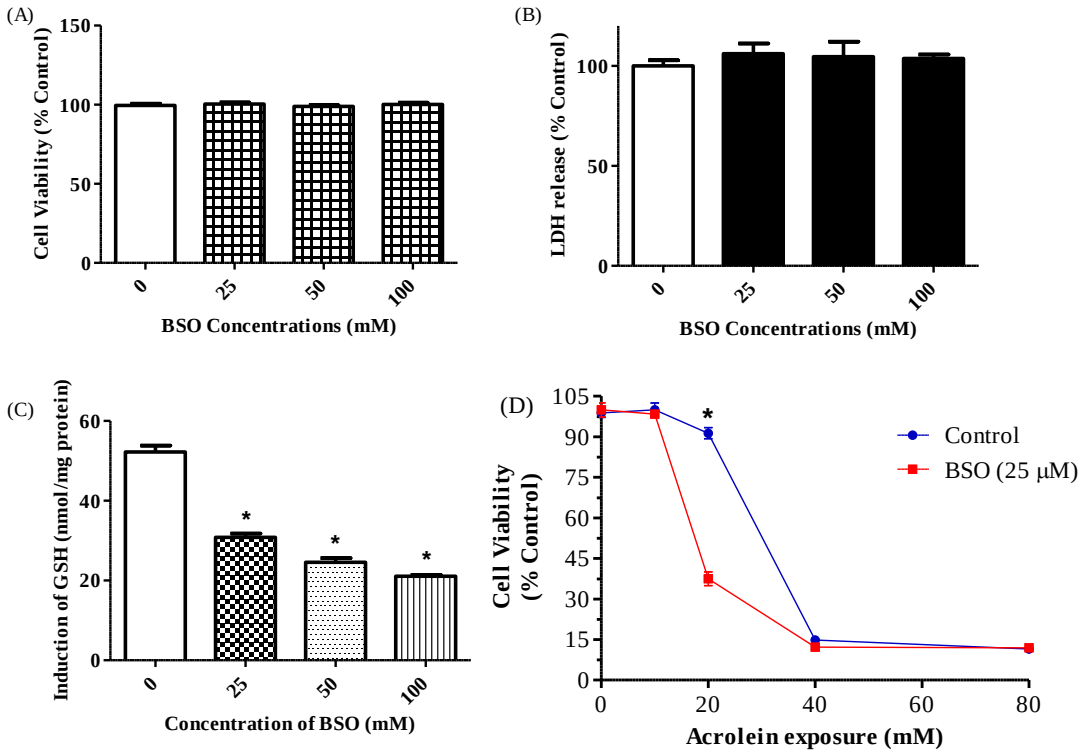

Figure 13. BSO depletion of cellular GSH and reduced cell viability. Effects of BSO treatment on cellular GSH and acrolein-induced cytotoxicity in RA-differentiated SH-SY5Y cells. Panel A and B depict cell viability of SH-SY5Y cells in the presence of BSO at the indicated concentrations for 24 h as measured by MTT (A) and LDH (B). Panel C depicts the effect of BSO treatments at various concentrations on GSH level. In panel D the cells were incubated with or without 25µM BSO for 24h prior to the media being removed and the cells exposed to the indicated concentrations of acrolein for another 24h, followed by the determination of cell viability by MTT reduction assay. Values represent means ± SEM from at least three separate experiments. *Indicates significantly convincing difference ($p < 0.05$) from the respective control group.

Effects of BSO cotreatment on CDDO-Im mediated cytoprotection against acrolein-induced cytotoxicity

To further examine the causal role of GSH in CDDO-Im mediated protection against acrolein toxicity, we examined the resultant GSH and cell viability from co treatment with CDDO-Im, BSO, and then Acrolein. RA-differentiated SH-SY5Y cells were pretreated with 100nM CDDO-Im in the presence or absence of 25 µM BSO followed by exposure to 40 or 20 µM acrolein. 40µM acrolein alone or 20µM acrolein in the presence of 25 µM BSO were chosen as they were the lowest concentrations to induce at least a 65% decrease in cell viability (Figures. 9A and 13D). Shown in Figure 14A, cotreatment of cells with CDDO-Im and BSO prevented the GSH induction observed with CDDO-Im alone resulting in a reduced cellular concentration level. Figure 14B represents a comparative study in GSH induction in the presence of acrolein as observed in the MTT assay. Incubation of cells treated with 40µM acrolein alone for 24 h led to an 83% decrease in cell viability. Secondly, incubation of cells pretreated with CDDO-Im for 24 h, followed by 40 µM acrolein for 24 h resulted in significant cellular protection against acrolein-induced cytotoxicity. Incubation of cells pretreated with 25 µM BSO followed by 20 µM acrolein resulted in a significant decrease in cell viability. Similarly, pretreatment of cells with 100nM CDDO-Im with 25 µM BSO followed by 20 µM acrolein resulted in a significant 51% decrease in cell viability, reducing the protective effect of CDDO-Im. Depletion of cellular GSH by BSO dramatically prevents the CDDO-Im mediated cytoprotective effects on acrolein-induced toxicity.

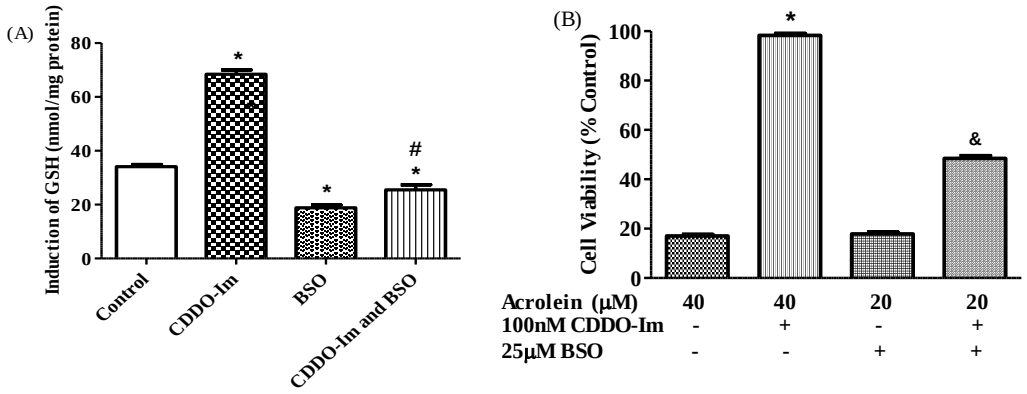

Figure 14. Effects of BSO co-treatment on CDDO-Im mediated cytoprotection. Effects of cotreatment with BSO on CDDO-Im mediated induction of GSH (A) and protection against acrolein cytotoxicity (B) in SH-SY5Y cells. In panel A, Cells were incubated with 100nM CDDO-Im, 25μM BSO, or 25μM BSO + 100nM CDDO-Im for 24 h, followed by measurement of cellular GSH content. In panel B, cells were cultured in a 48 well plate incubated with 100nM CDDO-Im, 25μM BSO, or 25μM BSO + 100nM CDDO-Im for 24 h. The media was removed and the cells were then cultured in the presence or absence of 40μM or 20μM acrolein for 24 h, followed by determination of cell viability by MTT reduction assay. Values represent means ± SEM from at least three separate experiments. *Indicates significantly convincing difference ($p < 0.05$) from the respective control group. #Indicates a significant difference from the 100nM CDDO-Im group. &Indicates a significant difference from 40μM Acrolein +100nM CDDO-Im group.

Effect of CDDO-Im co-treatment with BSO on acrolein-mediated lipid peroxidation and protein damage.

In order to further investigate the cellular damage associated with acrolein exposure, we studied the amount of protein and lipid damage in RA-differentiated SH-SY5Y cells exposed to 40 μM acrolein for 24 h. In Figure 15, panel A depicts Protein Carbonyl concentration, a marker of protein damage incurred by cells exposed to acrolein and pretreated with and without CDDO-Im and BSO for 24 h. Panel B depicts the amount of Thiobarbituric Acid reactive substances (TBARS), a maker of lipid peroxidation, in cells treated with 40 μM acrolein for 24 h and pretreated with and without CDDO-Im and

BSO for 24 h. In both cases an elevated amount of protein carbonyl and TBARS is measured in cells treated with 40 µM acrolein compared to control cells and cells pretreated with 100nM CDDO-Im for 24 h. Pretreatment of SH-SY5Y cells with CDDO-Im afforded a dramatic protection against acrolein-induced lipid peroxidation and protein damage. When cells were pretreated with 25 µM BSO in addition to CDDO-Im, the protein carbonyl and TBARS again was observed to be significantly elevated. These results further confirmed that GSH played a predominant role in CDDO-Im-mediated cytoprotection against acrolein toxicity.

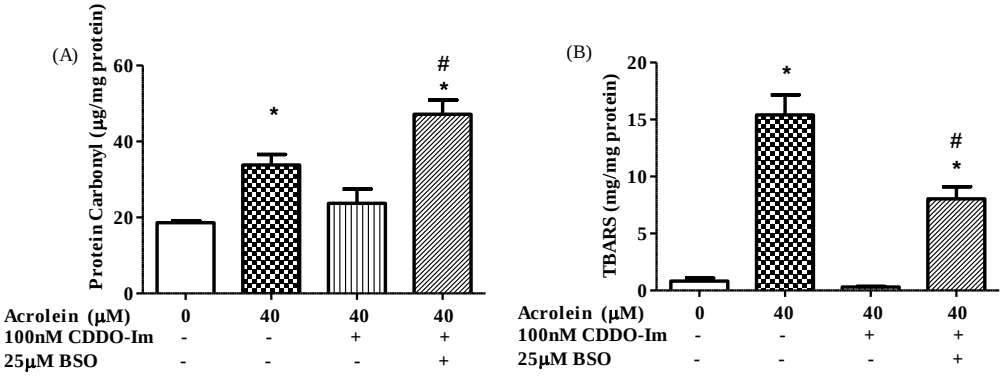

Figure 15. Acrolein induced protein and lipid damage. BSO prevents the CDDO-Im mediated cytoprotection against acrolein-induced lipid peroxidation and protein damage in RA-differentiated SH-SY5Y cells. Protein Carbonyl (A) and TBARS (B) was measured in cells treated with 40 µM acrolein alone for 24 h, and with pretreatment with and without 100nM CDDO-Im and 25 µM BSO for 24 h. Values represent means ± SEM from at least three separate experiments. *Indicates significantly convincing difference ($p < 0.05$) from the untreated control group. #Indicates a significant difference from the 100nM CDDO-Im treated group.

Discussion

The reactive aldehyde acrolein is a major byproduct of oxidative stress and particularly lipid peroxidation, which has been implicated in the pathogenesis of neurodegeneration [9, 10]. Acrolein is a ubiquitous and potent reactive aldehyde and acrolein-protein adducts have been established as an important cellular marker in autopsies of PD patients [8, 83]. Several endogenous cellular factors, including GSH, AR, and GST, have been suggested to play important roles in the detoxification of acrolein [21, 86]. Our results indicate that RA-differentiated SH-SY5Y cells are capable of expressing basal levels/activities of GSH, AR, and GST, and CDDO-Im pretreatment protects cells against acrolein induced cytotoxicity. Incubation with low nanomolar concentrations of CDDO-Im resulted in significant induction of GSH. Conversely, the same concentrations of CDDO-Im failed to significantly induce activities of GST and AR, indicating that in SH-SY5Y cells, these two aldehyde-detoxifying enzymes may be regulated via distinct signaling pathways (Figure 9). Our results further indicate that elevation of cellular GSH, while not GST or AR is a primary mechanism underlying CDDO-Im-mediated protection against acrolein toxicity in RA-differentiated SH-SY5Y cells.

The main tripeptide antioxidant GSH has been suggested by many studies to attenuate the oxidative damage underlying various neurodegenerative disorders, including PD[98]. At the same time a depletion of cellular GSH has been discovered as an early cellular marker for PD. In this study we demonstrate that acrolein-induced GSH depletion preceded the loss of cell viability (Figure 12A and B). This result suggests that

cellular GSH depletion may be an important early event in acrolein-induced cytotoxicity in RA-differentiated SH-SY5Y cells. Since it has been established that GSH is able to directly react with acrolein to form less reactive protein conjugates, the direct GSH-aldehyde interaction would account for the early depletion of GSH after initial exposure while the decrease in cell viability occurs after the GSH levels are depleted [99]. The initial depletion of GSH preceding the loss in viability also corroborates the report that GSH is an imperative cellular component in detoxification of reactive aldehydes including acrolein [100]. To further study the involvement of GSH depletion on acrolein cytotoxicity in SH-SY5Y cells, BSO was utilized to deplete cellular GSH. According to our results, BSO is a potent inhibitor of GSH and does not cause a decrease in cell viability or cause any damage to the cell membrane (Figures 13A, 13B, and 13C). Pretreating cells with BSO for 24 h prior to acrolein for 24 h resulted in a concentration dependent potentiation of acrolein-induced toxicity at a faster rate than cells not treated with BSO (Figure 13D). To provide evidence of GSH involvement CDDO-Im-mediated detoxification of acrolein, RA-differentiated SH-SY5Y cells were pretreated with CDDO-Im in the presence and absence of 25μM BSO followed by exposure to 20 or 40 μM acrolein. In this experiment, cells co-treated with BSO and CDDO-Im showed a reduced induction of GSH, significantly less than CDDO-Im alone (Figure 14A). When then treated with acrolein, the BSO inhibition of GSH reduced the CDDO-Im mediated cytoprotective effects by more than 50% (Figure 14B). Our observations indicate that elevated levels of cellular GSH are a primary mechanism underlying CDDO-Im-mediated protection against acrolein toxicity in RA-differentiated SH-SY5Y cells.

To further demonstrate the role of GSH involvement in in CDDO-Im-mediated cytoprotection against acrolein toxicity, we measured the protein and lipid damage incurred on RA-differentiated SH-SY5Y cells exposed to acrolein (Figure 15A and 15B) in presence of CDDO-Im. The main damage to cells occurs as a result of acrolein-induced alterations to macromolecules such as membrane lipids and proteins which are responsible for maintaining normal cell function. In our observations, treatment with CDDO-Im significantly reduces the amount of protein carbonyl and TBARS measured in cells treated with 40 μM acrolein for 24 h. Also, we observed that co-treatment with CDDO-Im and BSO resulted in elevated levels of both markers similar to those observed in cells exposed to 40 μM acrolein alone. Similar to our cell viability results above, our observations indicate that elevated levels of cellular GSH are a primary mechanism underlying CDDO-Im-mediated reduction of acrolein induced protein and lipid damage in RA-differentiated SH-SY5Y cells.

It is important to note that these studies cannot exclude the possibility that other mechanisms may be also involved in CDDO-Im-mediated protection as the BSO inhibition did not completely reverse its protective effects. Thus, more study is needed to fully understand the CDDO-Im protective mechanisms. Acrolein research has established that the primary observed cause for cell death occurs via apoptosis and impacts various transcription factors. In lung tissue, acrolein toxicity has been found to activate the p53 apoptosis pathway leading to cell death [101]. Similarly, the activation of the growth related transcription factor NF-kB is also affected by ROS and acrolein[102, 103]. GSH is necessary for the activation of the NF-kB signaling pathway and acrolein depletion of

GSH may contribute to varying activation depending on dose and duration of exposure[104]. In a study using SK-N-SH human neuroblastoma cells, acrolein exposure resulted in a biphasic depletion/increase in GSH accompanied by a similar decrease/increase in NF-kB activation [105]. In a SH-SY5Y cell, Yang et. al. has shown that the CDDO-Methyl Amide has been shown to interact with the Keap-1, Nrf2, and ARE elements to influence gene transcription for endogenous antioxidant enzymes[36]. However, whether acrolein cytotoxicity occurs in a similar fashion and protection by CDDO-Im is regulated by similar pathways in neuronal cells remain unknown.

Increased oxidative stress by acrolein combined with the reduced endogenous GSH and other atioxidants is an important underlying risk factor for the development of neurodegenerative disorders such as PD. Accordingly, acrolein induced toxicity has been linked to many neurodegenerative disorders such as Parkinson's disease [51, 106, 107]. The potential treatment option of GSH itself is limited since it does not readily pass through the blood brain barrier and may be limited by glial cell metabolism if it is able to enter the CNS[108]. Thus, a great deal of research has been conducted regarding potential therapeutic treatments focused on the use of exogenous antioxidant compounds [109-111]. However, both in vitro and in vivo models have shown that treatment with exogenous vitamins and antioxidant compounds are limited in their bioavailability, permeability, and are metabolized at a rate exceeding that of the oxidative damage [112, 113]. While studies with transgenic models have yielded the most convincing evidence for the role of a particular antioxidant enzyme in protecting against oxidative injury, using such an approach in humans is not yet feasible [114-119]. CDDO-Im is a lipophilic

compound and most likely would be able to penetrate the blood brain barrier as it has been shown in other CDDO compositions[36]. This study for the first time indicates that CDDO-Im can be used at nanomolar concentrations to induce cytoprotection against the reactive aldehyde acrolein toxicity, suggesting it is a promising agent in ameliorating lipid peroxidation damage and neurodegeneration. Our results indicate that cellular GSH can be potently elevated through the use of CDDO-Im at nanomolar concentrations. Additionally, the upregulation of the GSH system appears to be the predominant factor in protection against acrolein-induced toxicity. In this regard, our results suggest that the increase in endogenous GSH by CDDO-Im could be a novel strategy against reactive aldehyde-induced neurotoxicity.

The acrolein concentrations used in our study range from 10 to 80 μM. These concentrations have been found to be biologically relevant in various cell types. Studies have reported that human plasma can normally contain acrolein-protein adducts at concentrations of 30 to 50 μM [120]. Respiratory toxicity has been shown to occur at approximately 40 μM [121]. Additionally, Alzheimer's patients have been found to have increased concentrations of acrolein-protein adducts in several brain regions at 10 μM [11]. Therefore, the concentrations of acrolein used in our study are achievable in vivo. We are the first to utilize RA-differentiated SH-SY5Y cells as a model for studying neurotoxicity induced by acrolein and neuroprotection by CDDO-Im against acrolein induced-oxidative damage. These cells are classified as dopaminergic neurons because the RA differentiation ensures they have more developed neurotransmission capabilities including high levels of dopamine hydroxylase and tyrosine hydroxylase [37, 66, 122,

123]. Thus, these cells represent a widely used in vitro model for the study of human neurodegenerative disorders. Given that CDDO-Im shows potency at nanomolar concentrations for induction of endogenous GSH that affords protection against acrolein-induced cytotoxicity, it is likely that target of induction of endogenous GSH by CDDO-Im may afford a protective influence against neurodegenerative diseases.

In conclusion, this study demonstrates for the first time that cellular GSH can be potently induced by nanomolar concentrations of CDDO-Im in RA-differentiated SH-SY5Y cells and afford protection against acrolein induced oxidative stress. We report that GSH, while not induction of AR or GST plays a predominant role in CDDO-Im mediated cytoprotection. These new findings may have implications for the development of novel therapies against reactive aldehyde-induced neurotoxicity.

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