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Reactive dopamine metabolites and neurotoxicity : the role of GAPDH and pesticide exposure in Parkinson's disease pathology

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REACTIVE DOPAMINE METABOLITES AND NEUROTOXICITY: THE ROLE OF GAPDH AND PESTICIDE EXPOSURE IN PARKINSON'S DISEASE PATHOLOGY

by

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To Claudia, Andrew, Lola, Christina, Hein, and Zoe

We must embrace pain and burn it as fuel for our journey.

Kenji Miyazawa

1896-1933

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ABSTRACT

Parkinson's disease (PD) is a slow-progressive neurodegenerative disorder affecting 5-6 million people around the globe. The disease is manifested by the rapid deterioration of dopaminergic cells in the substantia nigra portion of the brain; however, the pathological mechanism of selective dopaminergic neuronal death is unknown. A reduction in levels of 3,4-dihydroxyphenylacetaldehyde (DOPAL) is biologically critical as this aldehyde has been shown to be toxic to dopaminergic cells and is a highly reactive electrophile. Investigating neuronal protein targets is essential in determining the cause of toxicity. An essential protein-GAPDH (e.g., glyceraldehyde-3-phosphate dehydrogenase) is an abundantly expressed enzyme known for its glycolytic activity, and recent research has implicated its role in oxidative stress-mediated neuronal death. This work positively shows GAPDH as a target for DOPAL modification, and, for the first time, DOPAL is identified as a potent inhibitor for GAPDH enzymatic activity. LC-MS and other chemical probes (ie. thiol and amine modifiers) show that DOPAL modifies specific amino acids: Lys, Arg, and modifies Cys and Met residues in the cofactor binding-domain of GAPDH. The enzyme inhibition is also time and DOPAL dose-dependent. DOPAL has a unique structure, containing two reactive functional groups: an aldehyde and catechol ring. In-house syntheses of DOPAL analogues, containing the catechol group and lacking the aldehyde, and vice versa have been tested on GAPDH and do not inhibit or modify GAPDH. Therefore, both the catechol and aldehyde groups of DOPAL are specific to binding with GAPDH and are necessary to achieve modification and enzyme inhibition.

In addition to finding a novel enzyme inhibited and modified by DOPAL, this work has also confirmed linking DOPAL levels to a fungicide associated with PD risk. This benzimidazole fungicide, benomyl was shown to inhibit ALDH2 in the SH-SY5Y neuroblastoma cell line via an increase in DOPAL and a decrease in DOPAC. The ratios of DOPAL and DOPAC, the product of ALDH, were measured by HPLC-ECD, and found that benomyl does inhibit ALDH2 in this dopaminergic cell model. The cytotoxicity of benomyl, DA, DOPAL and the combination of DA or DOPAL with benomyl was assessed by MTT assay. Surprisingly, the only toxic combination was the combination of DA or DOPAL with benomyl. In fact, this toxicity appears to be synergistic, as none of the single treatments are significantly toxic to the cells. This synergistic effect also affects GAPDH aggregation. The cell morphology is also drastically different in the presence of the combined treatments, compared to individual treatment of DA, DOPAL or benomyl; cells start to ebb and show apoptotic-like features at just 2h. A second class of pesticides, named chlorpyrifos and chlorpyrifos-oxon were tested for toxicity in PC6-3These compounds were toxic to these cells due to DOPAL accumulation reaching high levels in the 100 µM range.

Exposure to environmental toxins such as pesticides and fungicides has long been linked to PD risk, but only recently to DOPAL levels. This work provides a novel mechanism by which fungicide exposure may stimulate PD pathogenesis.

PUBLIC ABSTRACT

Parkinson's disease (PD) is a slow-progressive neurodegenerative disorder affecting 5-6 million people around the globe. The disease is manifested by the rapid deterioration of dopaminergic cells in the substantia nigra portion of the brain; the pathological mechanism of selective dopaminergic neuronal death is unknown. However, the heightened vulnerability of dopamine neurons is hypothesized to be due to pesticide exposure and the intrinsic presence of dopamine within these neurons. Improper packaging and metabolism of dopamine can be detrimental to these neurons. However, our group and others have shown that a later metabolite of dopamine, 3,4-dihydroxyphenylacetaldehyde (DOPAL) is 1000 times more toxic *in vitro* and 100 times more toxic *in vivo.* Therefore, this metabolite is of great interest and its contribution to PD progression. Given the high toxicity and reactivity of DOPAL, it is essential to investigate potential protein targets. This work positively shows that DOPAL targets an essential protein called glyceraldehyde-phosphate dehydrogenase or GAPDH. DOPAL inhibits GAPDH activity and modifies this protein, drastically changing its three-dimensional structure.

This work also provides a mechanistic link between pesticide exposure, DOPAL levels and PD. A fungicide, benomyl was found to be toxic to neurons due to its ability to inhibit breakdown of the enzyme responsible for metabolizing DOPAL, which therefore leads to an increase of DOPAL within neurons. A sub-group of organophosphate pesticides was also tested and found to be toxic to neurons due to DOPAL accumulation.

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INTRODUCTION

1.1 Parkinson's Disease

Parkinson's disease (PD) is a progressive neurological disorder, and it is the second most common neurodegenerative disorder in the United States. PD affects close to 6.3 million people around the globe, with nearly 60,000 new diagnosed cases each year. In the last 20 years, the disease has had widespread awareness with celebrity campaigning and the founding of the Michael J. Fox Foundation, which has served as an independent source of research and PD therapies. The symptoms and treatments of a disease similar to PD are discussed in a Chinese medical text, Nei Jing, and in the Ayurveda circa 5000BC, an Indian ancient system of medicine[1]. However, PD was properly described in "An Essay on the Shaking Palsy" and the disease was donned a formal name by the English physician, James Parkinson in 1817[2].

PD is an age-related disease, the onset primarily starts at 60 years of age, and the majority of diagnoses occur between 60-79 years. However, there are rare cases of autosomal-dominant and recessive early-onset PD cases, which are genetically linked[3; 4]. As life expectancy has increased in the Western countries, there has been a growing elderly population. As an result, PD and other neurodegenerative disorders will pose a larger socioeconomic burden[5]. It is estimated that the number of individuals over 50 with PD is close to 4.6 million, and will double by 2030 to 8.7 to 9.3 million people[6]. PD pathogenesis is greatly dependent on age, however, PD highly varies among gender[7; 8] and racial groups[9; 10]. The age and gender-adjusted PD rate per 100,000 was highest among Hispanics (16.6%), followed by non-Hispanic Whites (13.6%), and lowest among Asians (11.3%). The incidence rate is much higher among men than women. A comprehensive study showed that the rate is 91% higher among males than females for reasons not yet understood[10].

1.2 Symptoms and Current Treatments

PD is considered a motor disease, and diagnosis is mainly based on the criteria of at least two clinical symptoms, including: resting tremor, bradykinesia, rigidity, or postural imbalance. There is currently no straightforward diagnostic test or biomarker to test for PD. As shown in Figure 1.1 positron emission tomography may be utilized to image existing dopamine (DA) neurons within the midbrain, usually probing for the dopamine transporter (DAT) which is specific for DA neurons. However, it is limited for population-based epidemiological research. Since PD selectively degenerates these DA neurons, DA levels are imbalanced. To restore DA levels, levadopa and tolcapone are commonly administered drugs. Levadopa helps to restore DA levels, as it is a precursor to DA. The drug, tolcapone can also raise DA levels, by inhibition of catechol-o-methyltransferase, an enzyme which metabolizes DA to its methylated metabolite. Another class of drugs that has proven useful in alleviating symptoms is inhibitors of monoamine oxidase (MAO), preventing metabolism of DA as well[11]. Clinical response to these drugs serves as a positive diagnosis marker for PD[12]. A loss of olfactory sense is commonly the first symptom associated with PD, but this is usually overlooked by patients. Another issue is that once clinical symptoms become apparent, nearly 70-80% of dopamine neurons have already been lost. This degeneration is progressive, and there is currently no treatment to stop the cell death.

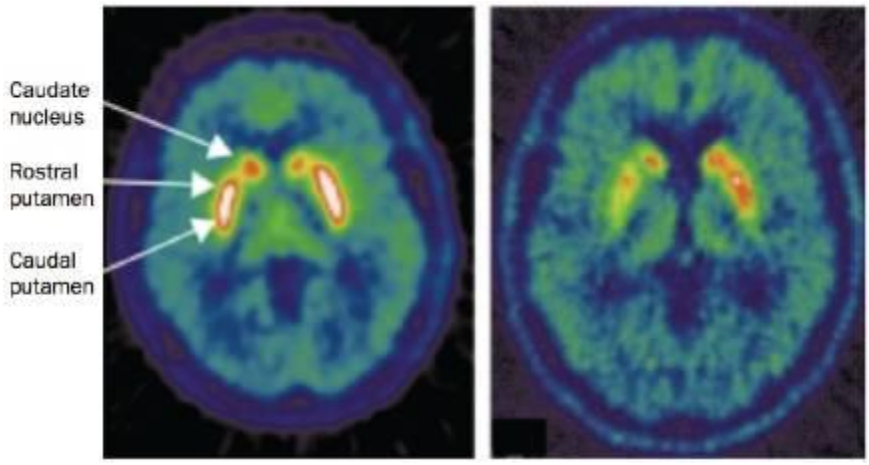


Figure 1.1 PET scan reveals decreased dopamine levels and activity in a healthy brain (left) compared to a Parkinsonian brain (right). Radioactive labelled 18-fluorodopa is injected intravenously and binds to aromatic amino acid decarboxylase (AADC) which is exclusively located in DA terminals. PD brains show an asymmetric loss of tracer uptake[13].

1.3 Etiology

The motor disturbances of PD are attributed to the initial degeneration of DA neurons in the substantia nigra pars compacta. The two major PD hallmarks are the dopaminergic neuron degeneration and the formation of toxic Lewy bodies (Figure 1.2), mainly made up of alpha-synuclein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Genetics plays a role in PD etiology, as several studies have shown a positive family history with PD. The mutated genes involved in PD are classified as *PARK* genes, and are genes that encode for alpha-synuclein, proper protein removal, and protectants against oxidative stress[14; 15]. Oxidative stress is an imbalance between production of reactive oxygen species (ROS) and the cell's ability to detoxify ROS and repair damage. Indeed, antioxidant responses are compromised in PD brains, and mutations among anti-oxidant related genes are present in a subset of PD patients. Glutathione is a common antioxidant in detoxification of ROS, and postmortem PD brains have shown reduced

glutathione levels,[16] and Glutathione-S-transferase polymorphism carriers experience an accelerated onset age of PD[17].

It has been shown that mutations in *PARK* genes are associated with early-onset PD, and cause detrimental changes in proper alpha-synuclein folding, protein removal by ubiquitin proteasome systems, and oxidative stress. However, these genetic mutations only contribute to about 10% of total PD cases[18]. It is clear that PD is a multifactorial disease, and environmental factors play a significant role in the other approximately 90% of the total or sporadic PD cases.

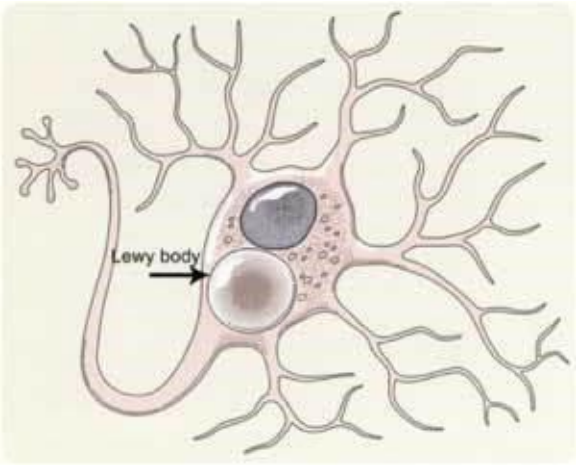


Figure 1.2 Lewy bodies are toxic intracellular aggregates that form within neurons, a major PD disease hallmark. (http://nihseniorhealth.gov/parkinsonsdisease/faq/images/figure003_r.jpg)

There are many risk factors and protective factors proposed in PD etiology, however, detailed specific mechanisms are lacking. Many epidemiological studies concerning PD have also yielded contradictory results. However, the most often replicated results in relation to PD risk have been pesticide exposure and a decreased risk among smokers[19].

1.4 Environmental Factors in Etiology

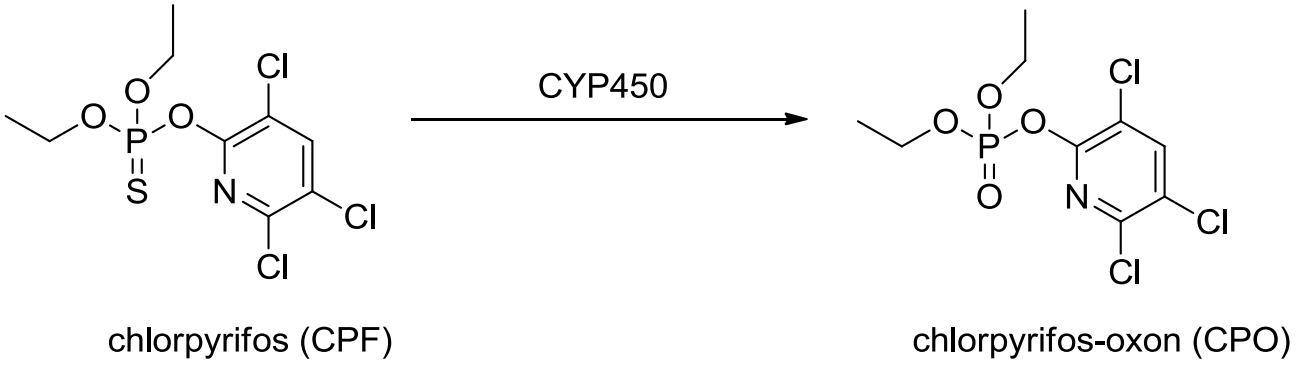
The notion that environmental factors play a role in PD etiology was first supported by the accidental discovery of 1-methyl l-(4-phenyl)-l-1,2,3,6-tetrahydropyridine (MPTP) drug use and its induction of clinically identical PD symptoms[20]. These symptoms were a result of ROS formation due to the MPTP selective toxicity profile towards dopaminergic neurons in the substantia nigra[21].

Over twenty years of epidemiological research highlights a host of environmental compounds, (ie. herbicides, pesticides, fungicides, metals and solvents) that are implicated in PD risk[22-26]. After aging, pesticide exposure is one of the greatest risk factors in PD etiology. Heavy metals such as iron and lead are a toxicology threat due to their steady accumulation in the substantia nigra and generation of oxidative stress. Studies with lead have shown that 7 out of 9 postal workers chronically exposed to lead-sulfate batteries over a 30 year period, developed parkinsonian symptoms[27]. Another study found abnormally high lead concentrations in patients' tibia and calcaneal bones, and these patients had a 2-fold increased risk for PD[28]. Lead exposure also causes DA dysregulation[29], reduces antioxidant cell responses, and decreases the learning ability in rats[30]. Other transition metals such as iron are proposed to contribute to PD pathology due to its pro-oxidant properties, leading to ROS generation via Fenton and Haber-Weiss reactions[31; 32].

It has been reported that rural living and agricultural populated areas have an increased PD risk[33-35]. Agricultural states such as North Carolina and Iowa were used in a prospective study in which exposure was self-reported by pesticide applicators, and was the first report associating PD with increasing lifetime days of pesticide use[36]. A UCLA conducted a study in a rural farming area in central California, utilizing the California Pesticide Use Reporting database and Geographic Information System land-use maps to estimate pesticide exposure since the mid 1970's. This area had been exposed to liberal amounts of pesticides, and neurologists were sent to confirm diagnosis of over 350 PD

cases[37]. Consumption of well water had an increased risk (41-75%), and up to a 2-fold increase in risk for areas containing the highest contaminations of multiple pesticides. Individual pesticides such as the organophosphate, O-O-diethyl-*OI-[3,5,6,-trichloro-2-pyridyl] phosphorothionate)* or chlorpyrifos (CPF) were assessed to contribute to PD by an odds ratio of 1.50[38].

Organophosphate (OP) pesticides are among the most highly used insecticides in agricultural, industrial and domestic locations. Clinical studies have shown that OP exposure caused distal degeneration of the motor neurons[39] and was suggested to cause dysregulation of dopaminergic transmission[40]. The PON1 gene encodes for paraoxonase, a key enzyme which metabolizes OPs. Interestingly, PON1 variants exposed to CPF were also found to be at a 2-fold increased risk for PD compared to wild type or persons heterozygous for the PON1 gene[38]. CPF is metabolized by the liver to form chlorpyrifos-oxon (CPO), which is 3000 times more potent than CPF as an acetylcholinesterase inhibitor[41]. The highly lipophilic CPF and CPO compounds cross the blood brain barrier readily, and are members of the organophosphate class of pesticides. Both of these compounds have been banned for private use, though they are still widely used commercially[42]. Acute doses of CPF caused severe transient parkinsonism in patients, and symptoms were similar to PD such as bradykinesia and muscle rigidity[43]. The proposed mechanisms of toxicity of CPF is via acetylcholinesterase aging and inhibition[39] and ROS generation[44]. However, there are few *in vitro* studies measuring the effects of CPF and CPO on dopaminergic neurons.

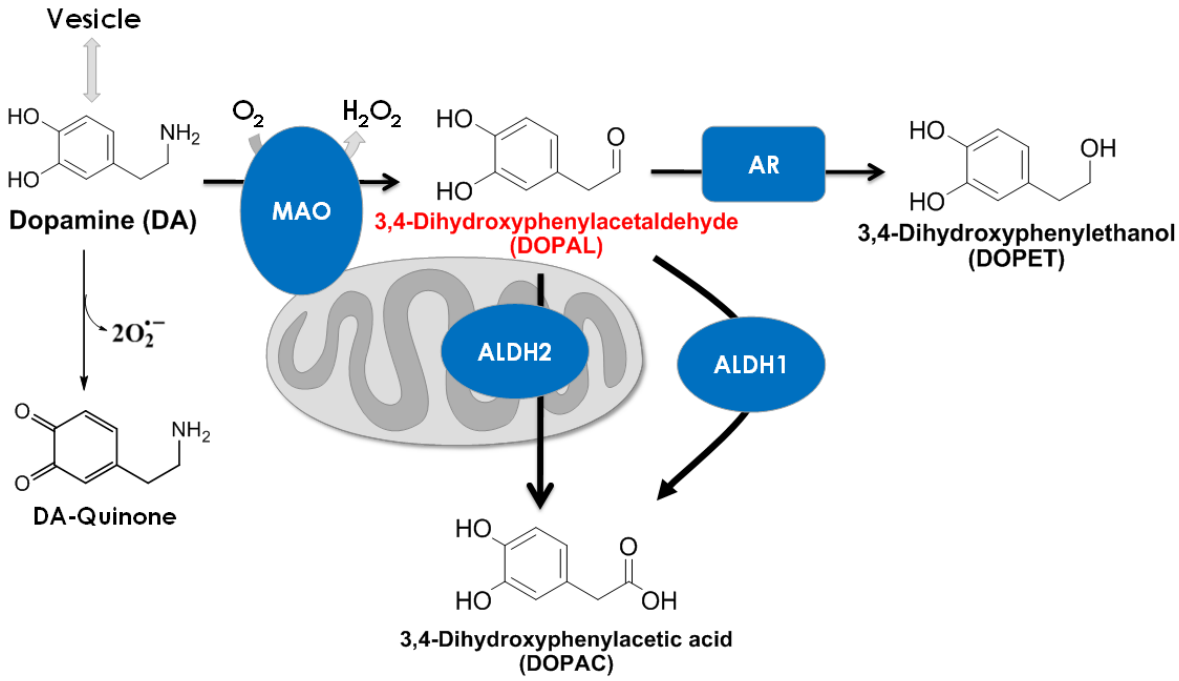


Scheme 1.1 Chlorpyrifos is an organochlorine pesticide. Bioactivation by hepatic metabolism (Cytochrome P450) yields chlorpyrifos-oxon, an irreversible acetylcholinesterase inhibitor. Both compounds are associated with increased PD risk.

1.5 The Catecholaldehyde Hypothesis and Role of 3,4-dihydroxyphenylacetaldehyde in Parkinson's Disease

Catecholamines are pivotal for normal neuronal homeostasis and neurotransmitter function in the central nervous system (CNS). It has been hypothesized that the intrinsic presence of the catecholamine, DA, in dopaminergic neurons contributes to neuronal toxicity observed in PD pathology. Due to the catechol moiety of DA, it undergoes spontaneous oxidation to semi-quinones and orthoquinones, especially when it is not properly sequestered within vesicles[45] (Scheme 1.2). The DA-quinone can covalently modify DNA and damage proteins,[46] and it interferes with proteasomal function[47]. The main route of DA metabolism in dopaminergic neurons is the oxidative deamination by mitochondrial monoamine oxidase (MAO), to form 3,4-dihydroxyphenylacetaldehyde (DOPAL) and hydrogen peroxide as a by-product. DOPAL is reduced by cytosolic aldehyde reductase (AR) to 3,4-dihydroxyphenylethanol (DOPET)[48] and to a greater extent, is oxidized by mitochondrial aldehyde dehydrogenase 2 (ALDH2) and cytosolic ALDH1, to 3,4-dihydroxyphenylacetic acid (DOPAC). Catecholamines are primarily oxidized and metabolized to their respective aldehydes, and these aldehydes are more

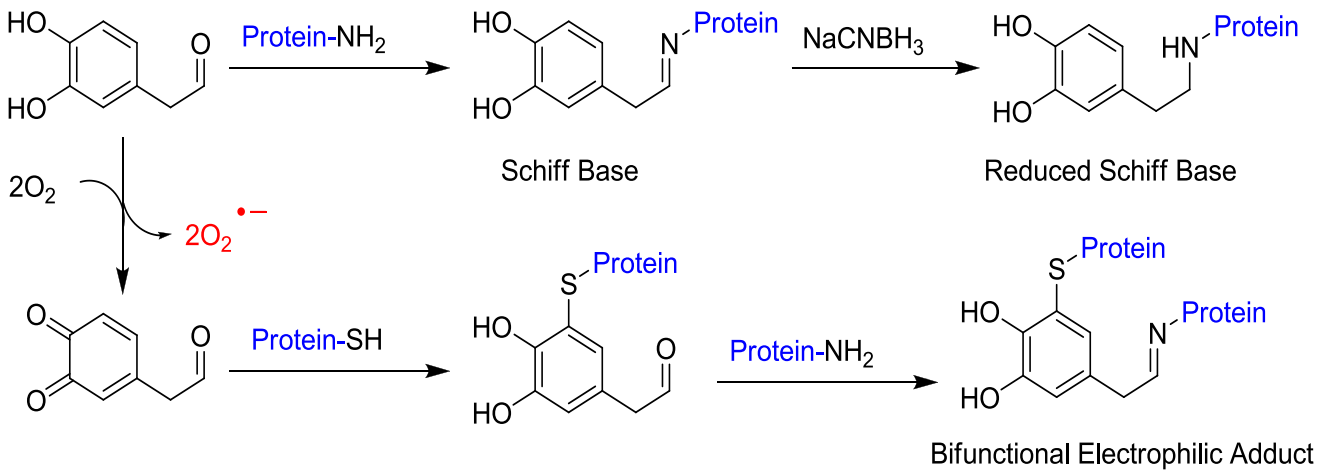
toxic to varying degrees[45; 49; 50]. This leads to the 'catecholaldehyde hypothesis' and the role of these endogenous molecules in mediating toxicity. The aldehyde metabolite of DA, or DOPAL is known to modify proteins and is toxic to cells.[51-53] DOPAL targets a number of proteins such as alpha-synuclein[54], tyrosine hydroxylase[55] and ALDH2[56], key neuronal proteins. Like DA, DOPAL also auto-oxidizes to the ortho-quinone structure, which generates ROS [57] and is several orders of magnitudes more toxic than DA both *in vitro* and *in vivo* [48; 58]. In fact, DOPAL shows cytotoxicity at low micromolar levels of 5-10 µM, which are only slightly higher than physiological levels of 2-3 µM[59].



Scheme 1.2 When DA is not properly stored in vesicles, it can redox-cycle in the cytosol to form the DA-quinone or be metabolized by mitochondrial MAO, forming the aldehyde metabolite, DOPAL and H_2O_2 as a by-product. DOPAL is metabolized to DOPET by AR, and to a greater extent by mitochondrial ALDH2 and cytosolic ALDH1 to form DOPAC.

The two electrophilic functional groups are key in mediating protein binding and toxicity. DOPAL contains both a catechol and an aldehyde group, and both are necessary in illiciting toxicity and inhibition of enzyme activity[60]. Similar to DA and other catecholamines, the catechol of DOPAL can undergo auto-oxidation to the ortho-quinone, and bind to thiols or –Cys via Michael addition[61] and the electrophilic aldehyde has a high affinity for nucleophilic amines such as –Lys residues, and forms a Schiff base. However, *in vitro* studies with model peptides have shown that DOPAL is more reactive toward –Lys (Schiff base) than towards –Cys residues. The DOPAL Schiff base is highly stable in non-reducing conditions, a result that was surprising since Schiff bases are generally unstable if the imine is not reduced to an amine.

As stated before, PD is an age-related disease, and the connection with DOPAL levels is key in determining mechanisms to PD. It was reported that postmortem brain samples showed higher MAO activity in elderly than in young subjects, and that increasing age and enzymatic activity had a direct correlation [62], which may modulate DOPAL levels. In mice, overexpression of MAO-B yielded PD-like symptoms[63]. On the other hand, MAO inhibitors such as Rasagiline and Deprenyl are effective PD drugs[64], causing the inhibition of DOPAL production from DA, and this is also indicative that regulation of DOPAL levels is important.



Scheme 1.3 The top panel shows aldehyde of DOPAL binding to Lys or free amines, forming a Schiff base, reduction of which forms a more stable reduced Schiff base. The catechol ring can also undergo a 2 e^- oxidation to form an ortho-quinone (lower panel) and become a Michael acceptor for Cys, the aldehyde is open to nucleophilic attack by –Lys residues. In this way, DOPAL is considered a linker between two protein monomers.

The levels of DOPAL compared to other DA metabolites are relatively low, as it is efficiently converted by ALR and ALDH to other non-reactive metabolites. Since the DA neurons are decreased in PD pathology, there is a decrease in overall DA metabolites. Thus, it is a misconception that DOPAL levels are higher in PD brains. However, the DOPAL:DOPAC and DOPAL:DA ratios are significantly higher in PD brains than in control subjects[65]. Regular breakdown of DOPAL can be pathological if ALR or ALDH enzymes are compromised.

1.6 ALDH Inhibition and DOPAL

There is a clear aberration of DA metabolism that occurs in PD pathology, and DOPAL is located at the center of these pathways. As stated before, parkinsonian models display increased DOPAL:DOPAC ratios, suggesting that ALDH activity or expression is decreased. The heightened vulnerability of dopamine neuronal cell models such as SH-SY5Y to ALDH inhibition and DOPAL levels has been suggested by Legros et al.[66] The

combined treatment of disulfiram, a potent ALDH inhibitor, and dopamine to stimulate intracellular DOPAL production was toxic to the cells. It was found that only the combined treatment of disulfiram and DA potentiates SH-SY5Y cell death, with minor toxicity at 1h, but significant lost viability at 24h. The combined treatment produced a moderate increase in DOPAL, and the delayed toxic effect of catecholamines is likely due to time-dependent increases in ROS, and mimics a PD-like scenario in which pathology is slow and progressive, rather than a result of one toxic insult. There are other ALDH inhibitors that pose a more relevant threat since they are endogenously produced such as 4-hydroxy-nonenal (4-HNE) and malondialdehyde (MDA), both products of lipid peroxidation. 4-HNE and MDA inhibited ALDH, decreasing DOPAC, and only MDA inhibited aldose reductase, effectively reducing DOPET levels. Given that ALDH and aldose reductase are the two primary enzymes for DOPAL breakdown, normal DOPAL levels were elevated and had a toxic effect at low physiologic levels of 4-HNE and MDA[56].

Pesticide bioaccumulation in the brain may modulate DA synthesis and metabolism, which solely occurs in dopaminergic neurons, and therefore may explain the selective toxicity of dopaminergic neurons. Therefore, it may be relevant to elucidate disease mechanisms connecting pesticide exposure, DA metabolite levels and toxicity of dopaminergic neurons. In an epidemiological study, individuals carrying a variant of the ALDH2 gene were associated with exacerbated PD risk when exposed to ALDH-inhibiting compounds[67]. Additionally, screened pesticides that also inhibited ALDH activity were associated with a 2 to 6-fold increase in PD risk. The most potent ALDH inhibitor from this group of compounds was the benzimidazole fungicide, benomyl, which was banned in 2001 due to accumulation in animal livers and teratogenic properties[68]. However, benomyl bioaccumulates within tissues, and workers exposed to benomyl at work and home locations had an increased PD risk, when compared with exposure at just one location. Additionally, ALDH gene variations in a sub-population

were found to be have a causative role in PD.[69] This link between benomyl exposure and PD was attributed to the alteration of DA homeostasis and ALDH inhibition[70]. Decreased activity or expression of enzymes responsible for detoxifying xenobiotics, such as ALDH has served as an additional link between pesticide exposure and PD risk.

1.7 Protein Aggregation

One of the major hallmarks behind PD pathology is the formation of toxic protein aggregates or Lewy bodies. Post-mortem PD brain samples show an increase of these Lewy bodies, as these samples are immunoreactive against alpha-synuclein. Alpha-synuclein is the major component of Lewy bodies, its exact function is not clearly known, but it is speculated that it may be involved in regulation of dopamine release and transport[71]. Protein misfolding and aggregation is a common theme in PD pathology. Genetic mutations and exposure to heavy metal ions and oxidative stress promotes the misfolding and self-assembly of alpha-synuclein into high molecular weight oligomers and is also characterized by amyloid fibrils[72]. There is recent evidence to show that relatively hydrophobic pesticides such as paraquat, rotenone and dieldrin preferentially bind to intermediate conformations of alpha-synuclein[73]. Improper autophagy and clearance of misfolded proteins are also implicated, as genetic mutations in ubiquitin ligase (UCHL-1) are associated with PD risk[74]. One of the main forms of DOPAL toxicity is the induction of oligomerization of alpha-synuclein[54] and other proteins[55; 56]. One study injected DOPAL into the intranigral region of mouse brains, and resulted in alpha-synuclein oligomers accompanied by dopaminergic neuron death[54]. It has been suggested that DOPAL causes a pathogenetic positive feedback loop, producing increased protein aggregation, which, in turn, increases DOPAL generation[65].

The unique structure of DOPAL, containing catechol and aldehyde, allows for protein aggregation, as shown in Scheme 1.3. For example, the DOPAL analogue, 3,4-dihydroxyphenylacetonitrile (DHPAN) was synthesized to retain the catechol ring, but

the aldehyde was replaced by a nitrile group. Another analogue, 3-methoxy-4-hydroxy-phenylacetaldehyde (MOPAL) retains the aldehyde but the catechol was methylated at the 3-position; both compounds had greatly reduced reactivity towards N-acetyl lysine (NAL), a nucleophilic model for amine-containing amino acids[61]. In addition, it was found that DOPAL binds to protein by Schiff base and Michael addition with amines, and both types of modification appear to be necessary in stabilizing protein oligomers[75]. The crosslinking effect of DOPAL is a likely effect that occurs during PD, and elucidating other protein targets of DOPAL will be important in discovering new therapeutics.

1.8 Glyceraldehyde-3-phosphate Dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase or GAPDH is an enzyme with an essential role in glycolysis. It catalyzes the oxidative-phosphorylation of glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate. The enzyme contains a Cys in the active site, undergoing a hemithioacetal intermediate with the carbonyl of G3P. It also has a highly conserved Rossman fold which utilizes NAD^+ as a hydride ion acceptor, releasing NADH[76]. It is one of the most commonly used 'housekeeping genes' and its constituitive expression allows its utility as a loading control during protein studies. However, recent studies have uncovered diverse functions and implications for neurodegenerative diseases, and alteration of GAPDH structure dictates its intracellular function[77]. GAPDH activity has been shown to be clinically reduced in multiple neurodegenerative diseases[78]. A study showed that GAPDH was the most extensively oxidized metabolic enzyme and activity was impaired in PD brains versus healthy brain samples[79]. Its activity and expression were reduced in a rotenone (PD cell model)[80], and this correlates well with the fact that PD is often accompanied with impaired energetic metabolism pathways. Some reports claim that during high oxidative stress, GAPDH is oxidized and translocates to the nucleus, causing accumulation within the nucleus and activation of apoptosis via Caspase 3[81]. Blocking the oxidative modification on GAPDH and the transfer to the

nucleus is neuroprotective. Interestingly, the anti-parkinsonian drugs, deprenyl and rasagiline were shown to have high affinity for GAPDH and it blocks this oxidative modification[82; 83]. Both of these drugs are MAO inhibitors. A separate study synthesized a deprenyl analog with no affinity to MAO, but which bound tightly to GAPDH and offered neuroprotection, independent of MAO inhibition. These results support the hypothesis that reduction of oxidized-GAPDH and DOPAL levels plays an important role in PD pathology.

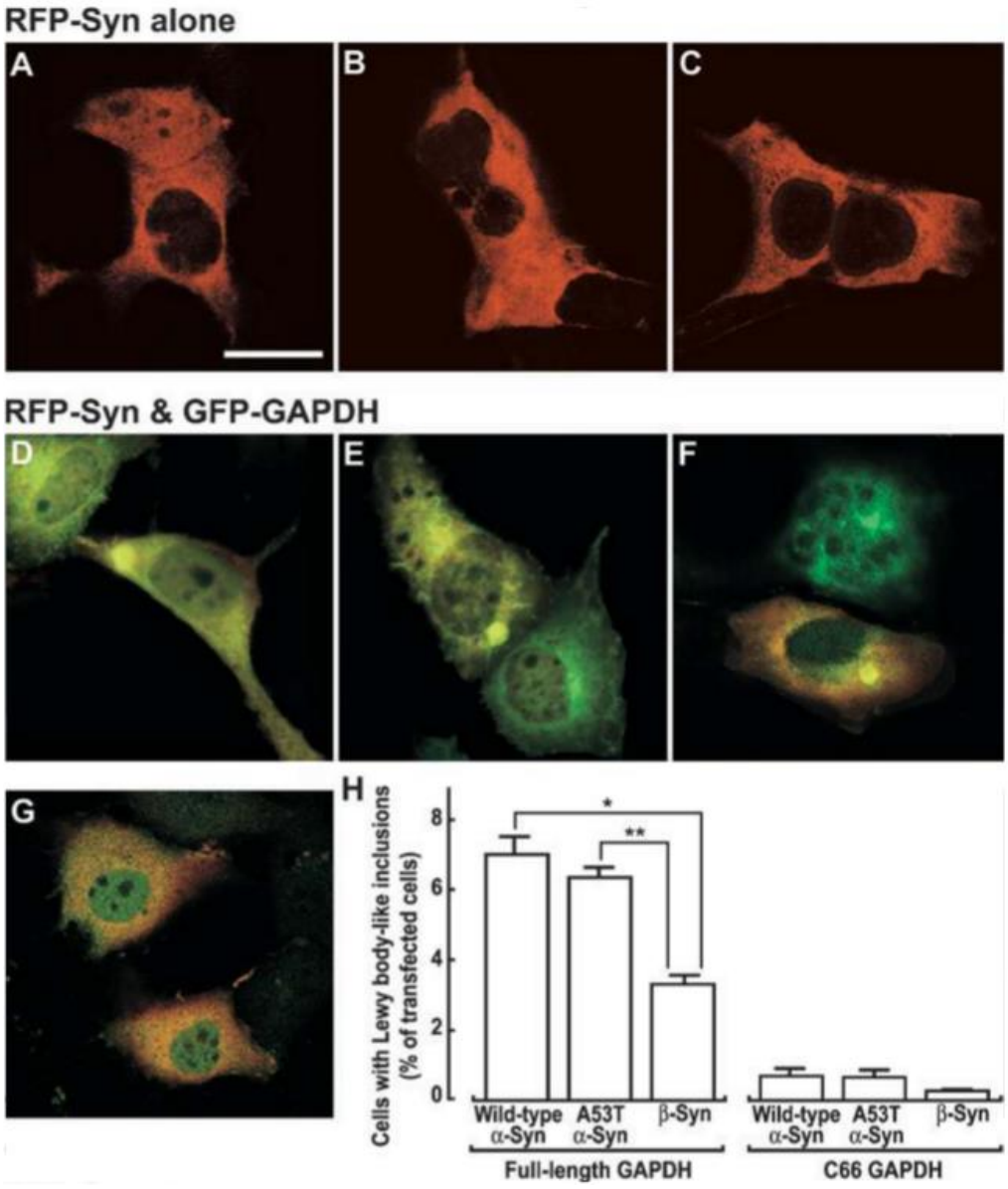


Figure 1.3 COS-7 cells were transfected with RFP-alpha syn fusion proteins: A) wild type alpha-syn B) mutant A53T alpha-syn. C) beta-syn. All three constructs alone show diffuse localization in the cytoplasm. However, transfection of RFP-syn & GFP-GAPDH produced Lewy-body like inclusions shown in yellow. The following are with full length GAPDH and D) wild type alpha-syn, E) mutant A53T alpha-syn, F) beta-syn. With a truncated GAPDH (C66 GAPDH) and RFP-Syn is transfected, there is a dramatic decrease in Lewy-bodies (G). This study was conducted Tsuchiya et al[84].

As mentioned before, the amyloidogenic properties of Lewy bodies are a pathological feature of PD. After alpha-synuclein, GAPDH is the greatest component of Lewy bodies, forming amyloidogenic fibrils. This was first discovered by immunohistochemistry results from post-mortem PD brains, in which GAPDH and alpha-synuclein were co-localized in Lewy bodies[81] (Figure 1.4), and they were also identified via proteomics[85]. It has been suggested that GAPDH promotes aggregation of alpha-synuclein, as aggregates formed in COS-7 cells with co-transfection of alpha synuclein and GAPDH, about 20% of cells formed aggregates with GAPDH alone, and none formed with alpha-synuclein alone. Figure 1.4 shows that transfection of a truncated version of GAPDH with alpha-synuclein, produced little to no aggregates. Additionally, only the GAPDH-alpha-synuclein constructs were thioflavin S positive, which is a dye that stains amyloid-like proteins[84]. The further investigation of subcellular GAPDH-alpha-syn protein interaction may serve as a mechanism to interrelate biochemical abnormalities and clinical phenotypes.

Given the central role of GAPDH in Lewy body formation and its clinical response to anti-parkinsonian drugs, it is likely to play a role in PD pathology. The connection with DA metabolism was not clear until a group discovered that dopamine induced GAPDH aggregation and cellular death in SH-SY5Y neuroblastoma cell[86]. Cell death was also directly correlated to concentration of DA (Figure 1.4). This cell death was accelerated with overexpression of GAPDH, and cell death was significantly reduced with a GAPDH mutant, that retained normal enzyme activity but did not aggregate[86; 87].

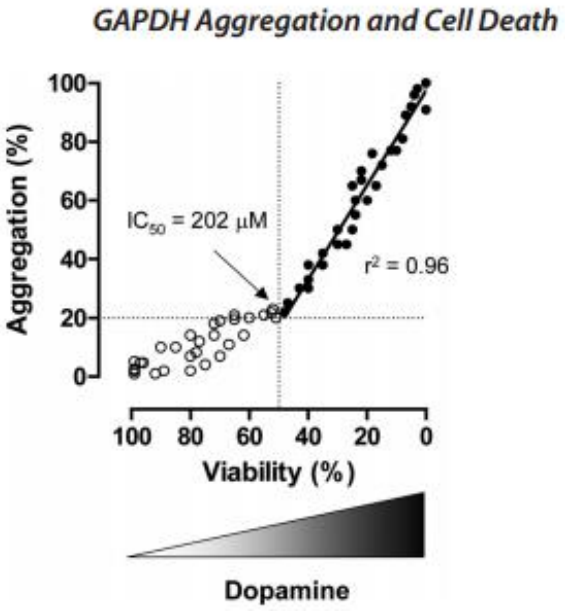


Figure 1.4 There is a direct correlation between GAPDH aggregation and dopamine-induced cell death. The closed circles indicate as above the IC_{50} value. The correlation value ($r^2 = 0.96$) is salculated using linear regression analyses.

As a result of these key studies, clear that it is necessary to maintain a balanced intracellular redox state and blocking GAPDH aggregation can be essential to neuronal health. Thus, abnormal or aggregated GAPDH is suggested to be a PD biomarker[83].

CHAPTER 2

STATEMENT OF HYPOTHESIS

2.1 Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder which results from the loss of dopaminergic neurons. Although the pathogenesis is currently unknown, there is evidence that the presence of dopamine (DA) and other metabolites within these neurons plays an important role in pathogenesis. DA is deaminated by monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetaldehyde (DOPAL), the aldehyde metabolite is much more toxic than DA itself, and is a relevant mechanism for disease progression[59; 61; 88].

DOPAL levels have also been linked to inhibition of aldehyde dehydrogenase (ALDH) and pesticide exposure, which are also major factors in PD pathology[69; 89; 90]. Therefore, the link between these three independent factors will be further examined. The fungicide, benomyl and chlorpyrifos (CPF) and chlorpyrifos-oxon (CPO) and their effects on ALDH activity and neuronal viability will be determined.

The electrophilic nature of DOPAL leads to modification of nucleophilic protein residues, certain proteins have been positively identified[54; 60]. However, the identification of additional protein targets are needed to show the big picture of DOPAL and PD pathogenesis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an essential protein for neuronal homeostasis, and has been shown to be modified and participate in DA-induced cell death[86].

The goal of this study was to determine if GAPDH is a protein target of DOPAL. Additionally, the effects of certain pesticides such as benomyl, CPO and CPF on ALDH activity will be determined and related to neuronal viability.

2.2 Hypothesis

The endogenously produced neurotoxin, 3-4-dihydroxyphenylacetaldehyde (DOPAL), covalently modifies and inhibits glyceraldehyde-3-phosphate dehydrogenase and DOPAL is responsible for the toxicity caused by various pesticides.

2.3 Specific Aims

Specific Aim 1: Investigate GAPDH modification by DOPAL. The extent of GAPDH monomer aggregation will be determined as a function of time and DOPAL dose-dependence. Completion of this aim will determine if GAPDH is a relevant protein target of DOPAL.

Specific Aim 2: Determine the effect of DOPAL on GAPDH activity. Both reactive functional groups of DOPAL will be compared to clearly show which are responsible for inhibition. Completion of this aim is crucial since GAPDH activity is decreased in neurodegenerative diseases[79; 80; 91; 92].

Specific Aim 3: To measure DA metabolism and cell viability as a function of benomyl treatment, ALDH inhibition and DOPAL levels in SH-SY5Y neuroblastoma cells. DOPAL levels will be measured in neuronal models after exposure to benomyl. These findings will address whether ALDH is effectively inhibited in dopaminergic cell models, and if DOPAL is responsible for the proposed toxicity.

Specific Aim 4: To measure changes in DA metabolism, ALDH activity, formation of reactive oxygen species and cell viability as a function of CPO and CPF treatment in PC6-3 cells. Completion of this aim will determine if CPO and CPF compounds affect levels of DA metabolites and provide a novel mechanism for toxicity in cells.

CHAPTER 3

DOPAL COVALENTLY MODIFIES GAPDH

3.1 Introduction

Impaired dopamine (DA) metabolism is a common theme in Parkinson's Disease (PD) etiology[63; 69; 93; 94]. DA biotransformation involves the generation of the electrophilic intermediate, 3,4-dihydroxyphenylacetaldehyde (DOPAL) by monoamine oxidase (MAO). Several studies have shown that MAO activity increases with age,[62; 95] which places the aging population at risk for loss of dopamine and augmented DOPAL levels. Pharmacological knockdown of MAO rescued toxicity from PD-related toxins in human neuroblastoma cell models[96]. Similarly, overexpression of MAO-B yielded PD symptoms in mice[93]. In dopaminergic neurons, the vesicles containing DA are mediated by vesicular monoamine transporter (VMAT), and interestingly, VMAT is impaired in PD brains, leaking DA into the cytosol and resulting in faster conversion to DOPAL[97]. Impaired DA metabolic pathways produces abnormal ratios of DA metabolites[50], and it is especially relevant for DOPAL levels since evidence has demonstrated that DOPAL is an endogenous neurotoxin, with normal physiologic levels at approximately 2-3 μM, and at only slightly higher elevations, ~6.6 μM is detrimental to neuronal health[49; 98]. Toxicity of DOPAL is hypothesized to be a result of protein modification and enzyme inhibition, leading to a loss of function. Therefore, it is essential to determine which proteins are relevant in DOPAL-mediated toxicity. To date, two proteins have been determined: tyrosine hydroxylase, which is the enzyme responsible for the rate limiting step of DA synthesis[55] and alpha-synuclein (alpha-syn), forming toxic alpha-syn protein aggregates[54; 75]. However, there are likely many more protein binding targets of DOPAL, such as the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was initially chosen to test DOPAL reactivity with cysteine (Cys) residues, as GAPDH has 4 free Cys per monomer[99]. When the Cys in the enzyme's active site is oxidatively

modified, enzyme activity is lost[100]. The active site Cys is prone to oxidation, participating in disulfide binding with other Cys, and undergoing reversible thiol modification in response to reactive oxygen species (ROS), and thus GAPDH is considered a redox protein switch[77]. It is currently unclear how DOPAL modifies thiols. The incubation of DOPAL with N-acetyl cysteine, a Cys model peptide, did not produce a conjugate and there was no interaction between the two species[57]. However, given DOPAL catechol and oxidation to the quinone, it is likely that oxidized DOPAL binds to Cys, analogous to what occurs with DA-quinone, yielding a 5-S-cysteinyl DA[101]. The Cys of GAPDH can serve as a model for how DOPAL is binding to thiols. It is hypothesized that DOPAL can bind to thiols via a Michael adduct, however, to date, a Cys-DOPAL Michael adduct has not been found via LC-MS and other techniques.

GAPDH was once considered a housekeeping protein with no other function other than its role in the glycolysis pathway. However, GAPDH is also involved in multiple cell processes such as DNA repair[102], tRNA export[103], cytoskeletal dynamics[104] and initiation of apoptosis[105; 106]. Under cellular insult with PD toxins[107] and reactive species, GAPDH translocates to the nucleus and serves as a transcription factor, initiating expression of apoptotic genes,[108; 109]In these studies, knockdown of GAPDH rescued cells from toxicity. Nuclear accumulation of GAPDH and a concomitant increase in cytosolic GAPDH are cellular phenotypes during GAPDH-initiated apoptosis. GAPDH expression levels also increase during rotenone treatment[110], a PD model, and interestingly, GAPDH mRNA and protein levels are also elevated with polychlorinated biphenyl (PCB) cell treatment. Personal colleagues have observed an increase in GAPDH expression, with cell treatment of PCB-153 and PCB-95 (unpublished). Due to the changes in GAPDH caused by various toxins, it is no longer common to use as a protein loading control[111]. Although GAPDH nuclear accumulation is the cell death mechanism for various toxins[108; 112], Nakajima et al. determined that when challenged with DA, the GAPDH cell death mechanism was not due to nuclear GAPDH translocation and transcription of

apoptotic genes, but it was due to the enzyme itself forming toxic aggregates in the cytosol[86]. It is unknown whether DOPAL would also cause GAPDH-mediated toxicity in a similar manner as observed with DA. In regards to toxic protein aggregation, GAPDH is enriched in post-mortem PD Lewy bodies, effectively binding to alpha-syn, and potentiates formation of toxic Lewy bodies[84]. Since alpha-syn is a known protein target of DOPAL, it is of interest to investigate the interaction between DOPAL and GAPDH, and to determine possible synergistic reactivity with these proteins.

GAPDH primarily exists in the cytosol, nucleus and mitochondria; it is one of the most plentiful proteins in neurons, given the abundance and a similar location as DOPAL, this heightens the possibility that GAPDH binds to DOPAL. The interaction of DOPAL and GAPDH will be thoroughly investigated; including the type of binding, the reactivity specificity with other DA metabolites and if DOPAL causes aggregation and changes to the tertiary structure of GAPDH. Conservation of the Rossman fold or NAD^+ binding site of GAPDH is also important for normal GAPDH activity and structure. The effect of DOPAL on NAD^+ binging will also be investigated. These studies will be helpful as DOPAL-protein aggregates could serve as a potential PD biomarker.

3.2 Experimental Procedures

Materials

DOPAL was biosynthesized via a rat liver MAO procedure as previously established[51], and DOPAL stock concentrations were determined using an ALDH assay with nicotinamide adenine dinucleotide (NAD^+) and (HPLC) [52]. MOPAL was biosynthesized according to a similar procedure as DOPAL, outlined in a previously published paper [113]. DA, 3,4-dihydroxyphenylacetic acid (DOPAC), GAPDH from rabbit muscle, glyceraldehyde-3-phosphate (G3P), acetonitrile (ACN), glutathione (GSH) and all other chemicals were acquired from Sigma-Aldrich (St. Louis, MO) unless otherwise

noted. The DOPAL analogue, 3,4-dihydroxyphenylacetonitrile (DHPAN) was synthesized via deprotection of 3,4-(methylenedioxy)-phenylacetonitrile by boron triboromide in methylene chloride[60].

SH-SY5Y Cell Culture

SH-SY5Y neuroblastoma cells were cultured in Opti-MEM supplemented with heat-inactivated fetal bovine serum (10%), penicillin (10 IU/mL), streptomycin (10mg/mL), 2mM glutamine, 1mM sodium pyruvate and 0.1 mM nonessential amino acids. Cells were grown in a 100 mm^2 tissue culture dish at 37^oC in a humid atmosphere containing 5% CO_2. SH-SY5Y cells that were selected for differentiation were seeded into 24-well plates at (1 x 10^5) density, and undifferentiated cells were seeded at (5 x 10^4), and grown in normal cell media for 3 days. Undifferentiated cells were grown an extra 6 days with media change every 3 days. On the 4th day, differentiated cells were treated with 20 µM retinoic acid (RA) for 3 days, media was changed and treated with 80 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for an additional 3 days. On the 10th day, media was changed for undifferentiated and differentiated cells, and 24h after, the cells were treated with DOPAL[114].

RNA Extraction and PCR

RNA was extracted and precipitated by adding 1 mL of TRIzol Reagent (Thermo Fisher Scientific; Waltham, MA) to roughly 100-µl of cell lysis and then following manufacturer's protocol. RNA was incubated with 1 µl DNase (Thermo Fisher Scientific) for 15 mins at 37°C, followed by heat inactivation at 65°C for 15 mins. RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). For complementary DNA (cDNA) reactions, 1 µg of total RNA was added to 0.5 µg random hexamers DNA primers plus 2.5- µl of 10 mM dNTPs (Invitrogen; Carlsbad, CA), and brought to 30-µl with nuclease-free water. Reactions

were heated to 65°C for 5 minutes, and then placed on ice. We then added 19-µl of a master mix containing 10-µl of 5 × First-Strand buffer (Invitrogen), 5-µl of 0.1 M dithiothreitol, 0.5-µl of RNaseOUT (Invitrogen), and nuclease-free water to a volume of 19-µl. Reactions were incubated at 25°C for 10 mins, followed by 37°C for 2 minutes. Then 20-µl of a mix composed of 19-µl master mix and 1-µl Moloney-murine leukemia virus Reverse Transcriptase (New England Biolabs; Ipswich, MA) were added to each reaction. Total reactions were incubated at 37°C for 2 hours followed by 70°C for 15 minutes. qPCR reactions (10-µl) were prepared using the SYBR Green kit (BioRad; Hercules, CA) following the manufacturer's instructions. The following primers were at a final concentration of 200 nM in separate PCR reactions: human SNCA (forward: GCCAAGGAGGGAGTTGTGG; reverse: CCACACCATGCACCACTCC), human GAPDH (forward: TCCCTGAGCTGAACGGGAAG; reverse: GGAGGAGTGGGTGTCGCTGT) or human ACTB (forward: GGCTACAGCTTCACCACCAC; reverse: ACTCCATGCCCAGGAAGG) (IDT; Coralville, IA). Quantitative real-time PCR in 96-well plates (BioRad) was conducted using a BioRad CFX-Connect Real-Time System and the default 2-step amplification protocol with an annealing temperature of 61°C. A standard curve for all primers was performed using a 4-fold dilution series. Human β-actin was utilized as a normalizing control

Protein Analysis by SDS-PAGE

GAPDH protein (0.3mg/ml) was incubated with DOPAL at given time periods in (50 mM sodium phosphate buffer, pH 7.4) at 37° C. Protein samples were then denatured by addition of 6X gel loading buffer (11 mM EDTA, 3.3 mM Tris-HCl, 0.017% SDS, 0.015% bromophenol blue) and heated to 80°C for 3 mins. 9 µg of protein was loaded and 10% polyacrylamide gel was used to separate protein bands via SDS-PAGE. Protein bands from gel were stained with Coomasie blue.

Western blot analysis

GAPDH protein (0.3mg/ml) was incubated with DOPAL at given time periods in (50 mM sodium phosphate buffer, pH 7.4) at 37° C. Protein samples were then denatured by addition of 6X gel loading buffer (11 mM EDTA, 3.3 mM Tris-HCl, 0.017% SDS, 0.015% bromophenol blue) and heated to 80° C for 3 mins. 1 μg of protein was loaded and 10% polyacrylamide gel was used to separate protein bands via SDS-PAGE. After electrophoresis, protein was transferred to nitrocellulose membrane at 20V for 45 mins. at room temperature, and blocked overnight in 5% BSA+TBST (bovine serum albumin and tris buffered saline with Tween-20) as a blocking buffer. Primary rabbit anti-GAPDH antibody was diluted at 1:10,000 (Sigma Aldrich) for 2 h at room temperature. Membranes were washed with 0.05 M Tris, 0.9% NACl containing 0.05% Tween-20 (TBST). A hoseradish-peroxidase-conjugated secondary goat-anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:20,000 and incubated for 1.5 h at room temperature. Protein band signals were detected with an Amersham ECL-plus Western Blotting Detection kit according to the manufacturer's instructions.

Catechol Protein analysis

GAPDH and DOPAL, MOPAL or DHPAN were incubated as stated before for 4h. The protein samples containing catechol adducts were stained with a redox-cycling sensitive dye nitroblue tetrazolium (NBT) which stains catechols[115]. GAPDH samples (5 ug) were subjected to SDS-PAGE 10% acrylamide and transferred to a nitrocellulose membrane. The membrane was then placed in 0.24 mM NBT with 2 M potassium glycine buffer (pH 10) and incubated overnight at 4°C.

GAPDH Analysis of free thiols

GAPDH (0.5 mg/ml) was incubated with 25 or 125 μM DOPAL in (50 mM sodium phosphate buffer at pH 7.4) for 2 or 4 h. At the end of the incubation period,

samples were titrated with 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) for 20 mins at room temperature, and heated for 3 mins at 80°C. Samples were centrifuged at 1000g for 3 mins, 100-µL of supernatant were transferred to a 96-well plate, and absorbance was measured for the reduced 2-nitro-5-benzoic acid product at 412 nm. Formation of this product is directly correlated to the number of free thiols in solution via its absorptivity constant.

LC-MS Identification of DOPAL-modified GAPDH peptides

GAPDH and GAPDH (0.3 mg/mL) treated with 50 µM DOPAL for 4h were incubated in glass vials at 37°C. Excess DOPAL was removed by Biorad filtration columns. Final concentrations of $NaCNBH_3$ (1 mM) and ACN (0.1%) v/v were added to each sample and samples were briefly heated for 3 mins at 70°C, the trypsin (Promega) digested in solution for 8h at 37°C. at a 1:50 ratio. A 10-µL aliquot was taken and diluted 1:10 with 50 mM ammonium carbonate and 15 µL of sample was injected onto an LC-MS Phenomenex Aeris Widepore XB-C18 column. Mobile phase A was 0.1% formic acid in water, mobile phase B was 0.1% formic acid in ACN. The gradient elution was 0 m: 5% B, 5-15 min: 5-50% B, 15-30 min: 50-90% B, 31 min: 50%, 40 min: 5%. The MS scans were performed on a Shimadzu LCMS (IT-TOF) and set to positive ion scanning mode from 200-2000 m/z in 3.3 msec. MS spectra were analyzed by comparison to UCSF Prospector *Digest* command which matches the experimental m/z masses to theoretical sequenced GAPDH peptides. The database was set to assume trypsin digest, maximum missed cleavages as 1, peptide mass 200-5000 and ion mass tolerance of 1.00 Da. And the variable modifications were set to Gln>pyro-Glu (N-term Q) and oxidation of methionine. The sequence coverage was determined by dividing the number of amino

acids that matched with the database by the total number of amino acids in GAPDH ~333. In order to identify DOPAL adducts, the mass of a DOPAL Schiff-base adduct (134 Da) or reduced schiff base (136 Da) was added to each matched peptide and scanned in the GAPDH-DOPAL sample.

3.3 Results

GAPDH is covalently modified by DOPAL

Purified GAPDH was incubated with DA, DOPAL, DOPAC (0, 50 μM) for 4 h. Although DA, DOPAL and DOPAC covalently modified GAPDH as evident by NBT staining (Figure 3.5C) only DOPAL causes significant GAPDH aggregation. The monomer band at 37 kDa is intact in the untreated GAPDH. There is some loss of the monomer band density with DA ~10% and DOPAC ~10% (Figure 1.1). However, it was to a lesser extent compared to the loss observed with DOPAL at 38%. Conversely, the band density in the high molecular weight (MW) region greatly increased with DOPAL but negligibly changed with DA and DOPAC. The high molecular weight region includes cross-linked monomers observed only in the presence of DOPAL. Since NAD^+ is a cofactor for GAPDH, NAD^+ (1 mM) was co-incubated along with DOPAL to measure any difference in GAPDH modification. However, the presence of NAD^+ did not significantly affect protein reactivity and aggregation with DOPAL or metabolites.

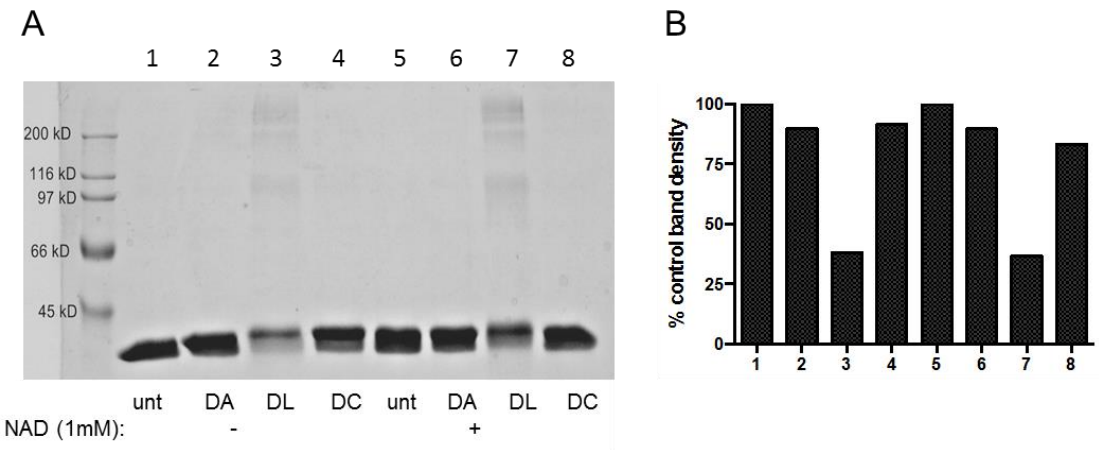
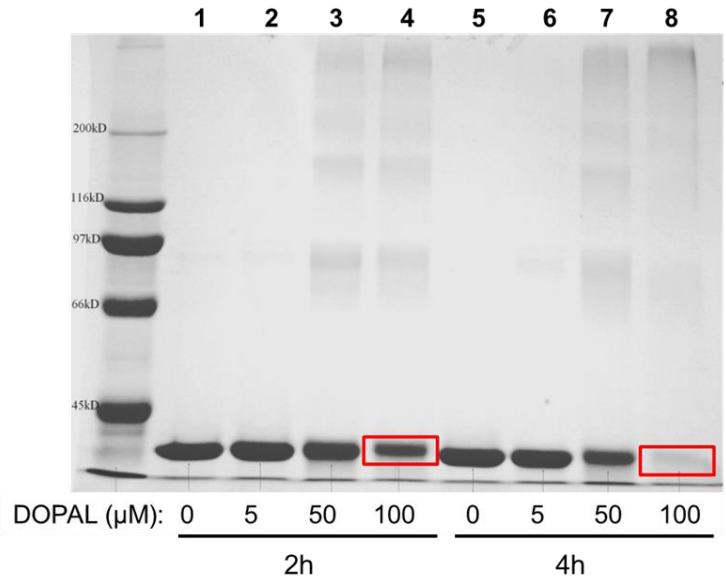


Figure 3.1 DOPAL modifies GAPDH in a concentration-dependent manner yielding protein aggregation. 0.3 mg/ml GAPDH was incubated with DA, DL, DC +/- NAD^+ (1 mM) for 4 h at 37º C. (A) Samples were subjected to SDS-PAGE and stained by Coomasie stain, 9 µg of GAPDH loaded per lane. Coomassie protein staining showing the loss of 37 kDa parent protein band and formation of high molecular weight oligomers with 50 µM DOPAL. (B) The corresponding band densities at 37 kDa were quantified on a UVP Imager, n=1. unt = untreated, DOPAL = DL, DOPAC = DC.

Reaction of DOPAL with GAPDH yields protein aggregation

The protein studies to determine the reactivity of DOPAL and GAPDH were measured by SDS-PAGE and staining GAPDH protein bands with Coomasie blue. The monomer band of GAPDH is at ~37 kDA, and this band is greatly reduced with increasing DOPAL concentration. This effect is accompanied with an increase in protein staining in the high molecular weight region. For example, at DOPAL concentrations above 50 µM, there is a clear formation of high molecular weight GAPDH oligomer protein bands which correspond to the dimer (~96 kDa) and the tetramer (~192 kDa).



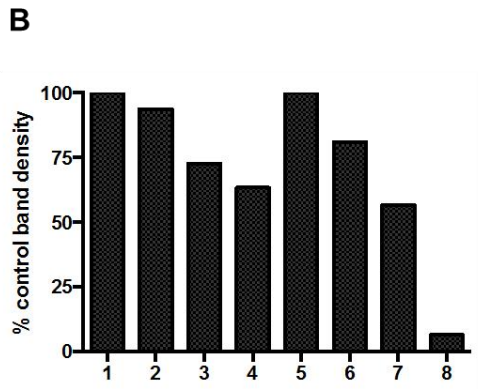


Figure 3.2 DOPAL modifies GAPDH in a concentration and time-dependent manner yielding protein aggregation. 0.3 mg/ml GAPDH was incubated with DOPAL for 2 or 4 h at 37ºC. (A) Samples were subjected to SDS-PAGE and stained by Coomasie stain, 9 µg of GAPDH loaded per lane. Concentration and time-dependence of high molecular weight oligomer formation and loss of parent band with increasing [DOPAL] 0-100 µM. (B) The corresponding 37 kDa band densities were quantified on a UVP Imager, n=1.

DOPAL increases co-aggregation of GAPDH and alpha-syn

As previously shown, DOPAL caused global GAPDH aggregation, and decreased the density of the monomer band of 37 kDa. However, when DOPAL was co-incubated with GAPDH and alpha-syn, there was more of a dramatic decrease in the GAPDH

monomer band density (Figure 3.3) than with GAPDH and DOPAL alone. The high MW band density also increased in the presence of alpha-syn and GAPDH versus with GAPDH alone.

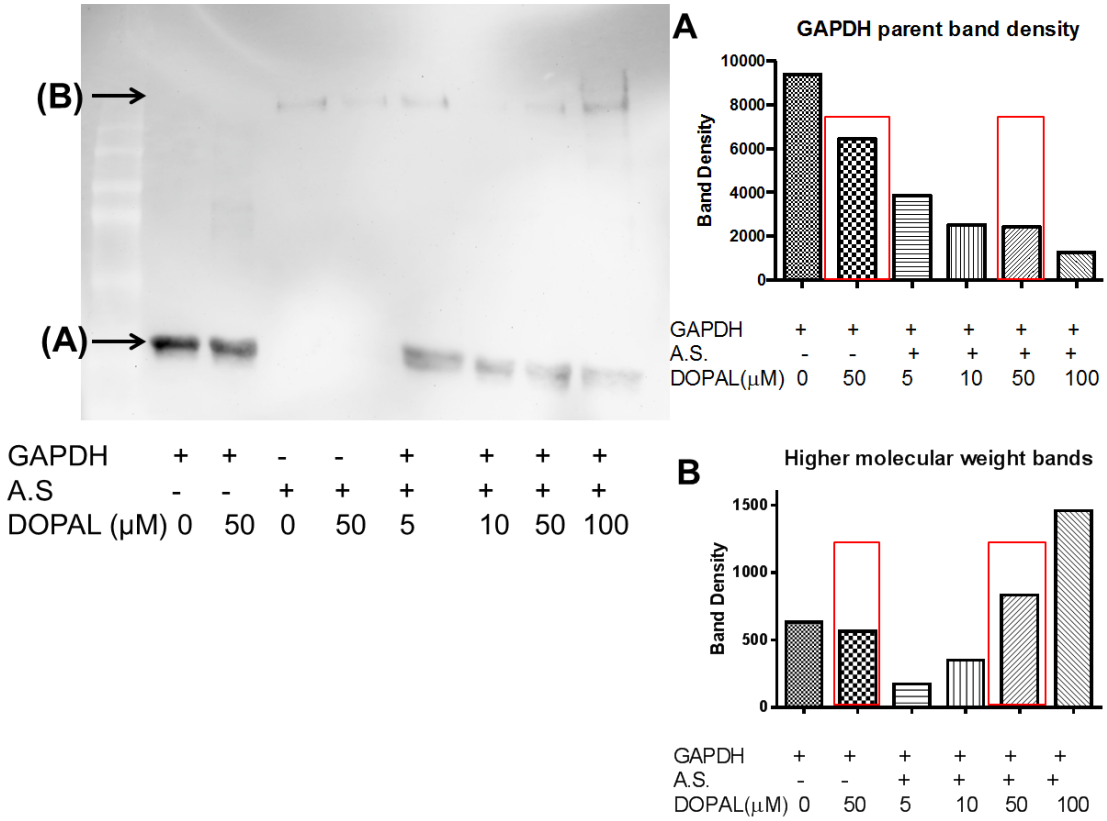


Figure 3.3 The co-incubation of DOPAL and GAPDH + alpha-syn. A) The western blot shows the decrease in monomer band as a function of DOPAL concentration. However, the red squares show the difference of the band densities when alpha-syn was present. B) The parent band density decreased when alpha-syn is present, C) and high molecular weight band density increased. Band density quantification is based on n=1.

DOPAL induces mRNA expression of GAPDH and alpha-syn

Since the aggregation of GAPDH and alpha-syn is a pathogenic mechanism which occurs in DA neurons in PD, it was of interest to test whether DOPAL treatment

modulated expression levels of GAPDH and alpha-syn. SH-SY5Y cells treated with 50 µM DOPAL for 24 h, produced a slight increase of alpha-syn mRNA and a significant increase or GAPDH mRNA. GAPDH mRNA was only slightly increased (5 µM DOPAL) over untreated, but produced nearly a 1.5-fold increase in mRNA with 50 µM DOPAL. The RNA extraction and DNA amplification were processed in Robert Cornell's laboratory.

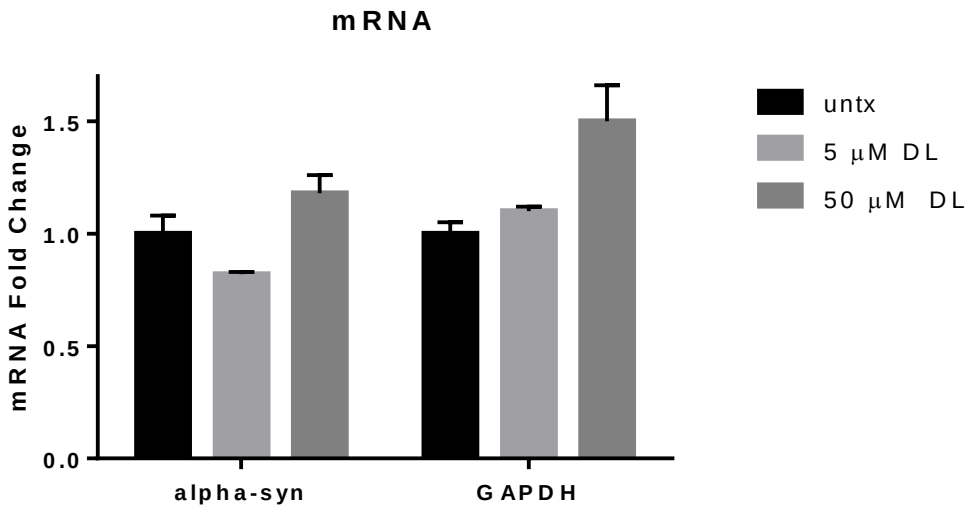


Figure 3.4 mRNA levels change after 24 h treatment with DOPAL. SH-SY5Y cells were treated with 5, 50 µM DOPAL and RNA was extracted from cell lysate. 50 µM DOPAL increased alpha-syn and GAPDH mRNA. B-actin was the loading control

DOPAL is responsible for catechol adducts on GAPDH

The protein samples containing catechol protein adducts were stained with the redox-cycling sensitive dye nitroblue tetrazolium (NBT). In Figure 3.5, GAPDH treated with DOPAL showed that there is redox cycling/staining of GAPDH. At the lower 25 µM dose, the majority of this staining is seen with the monomer band, as this dose may not cause higher molecular weight (MW) aggregation. The staining is also dose dependent on

DOPAL concentration (panel B) However, at 500 µM, the monomer band and the high MW region are highly stained. There was no redox cycling/staining in the MOPAL or DHPAN treated GAPDH samples, even at high doses (500 µM).

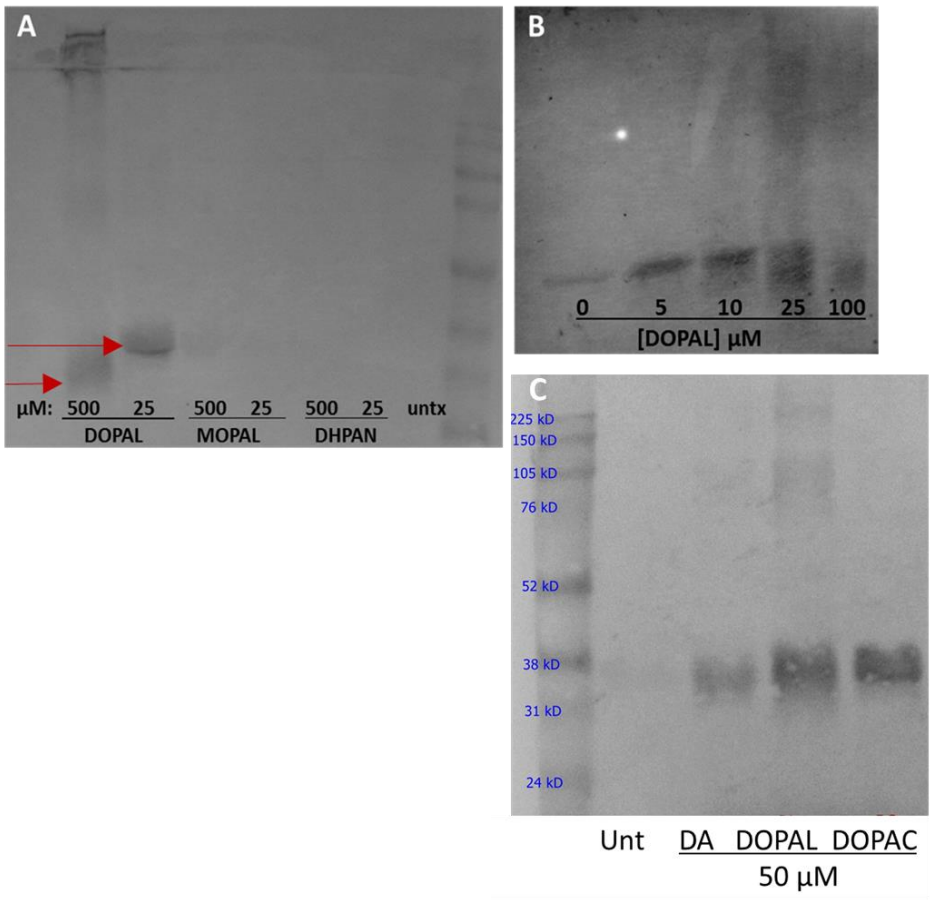


Figure 3.5 GAPDH treatment with DOPAL yields NBT staining of redox-active catechols on GAPDH, 12 µg load per lane. GAPDH (0.3 mg/ml) was incubated with A) 25 or 500 µM DOPAL, MOPAL, DHPAN for 4 h at 37°C. Redox staining is observed with DOPAL, however there is none with either MOPAL or DHPAN. B) Catechol staining increases from 1-100 µM. C) Catechol binding to GAPDH with DA, DOPAL and DOPAC, but catechol binds to aggregated GAPDH, in the presence of DOPAL only.

Evidence for Cys modification by DOPAL

There are 4 Cys residues per GAPDH monomer. With 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) treatment, the control GAPDH sample, has nearly 4 free Cys residues. In the presence of DOPAL, the number of free Cys residues decreased in a dose-dependent manner (Figure 3D). These results indicate that DOPAL does affect thiol status, although it is not known if the loss of Cys is due to covalent modification or oxidation. Slightly higher DOPAL concentrations were used (50, 125 μM) than in other experiments since slightly higher protein concentration (0.5 mg/mL) was needed for sufficient assay sensitivity.

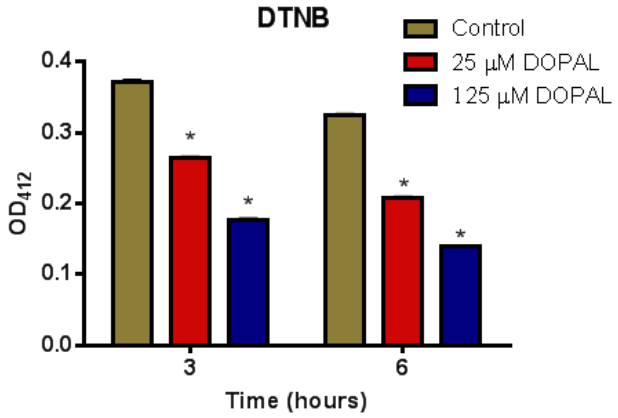


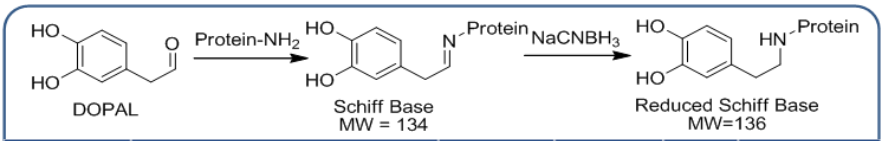
Figure 3.6 Free cysteines on GAPDH were labeled by 1 mM DTNB, absorbance was measured for the reduced 2-nitro-5-benzoic acid product at 412 nm. Loss of free Cys following DOPAL treatment. Significance was determined for each DOPAL concentration at each time point, and is denoted by asterisk (*), n=3.

DOPAL adducts are primarily on Lys and Arg residues

GAPDH and DOPAL-GAPDH were incubated for 4h as described above and digested with trypsin. The sequence coverage for the GAPDH peptides was 81.1% and

DOPAL-GAPDH was slightly lower at 69.1%. The LCMS spectra of DOPAL-GAPDH produced unique m/z peaks not found in the GAPDH spectra. These peaks corresponded to a mass shift of a DOPAL adduct of either 134 or 136 Da and to peptides containing Arg or Lys residues. A few of these peptides also contained numerous missed cleavages such as the 54-70 peptide contained two missed cleavages and the 253-258 peptide contained a missed cleavage.

A

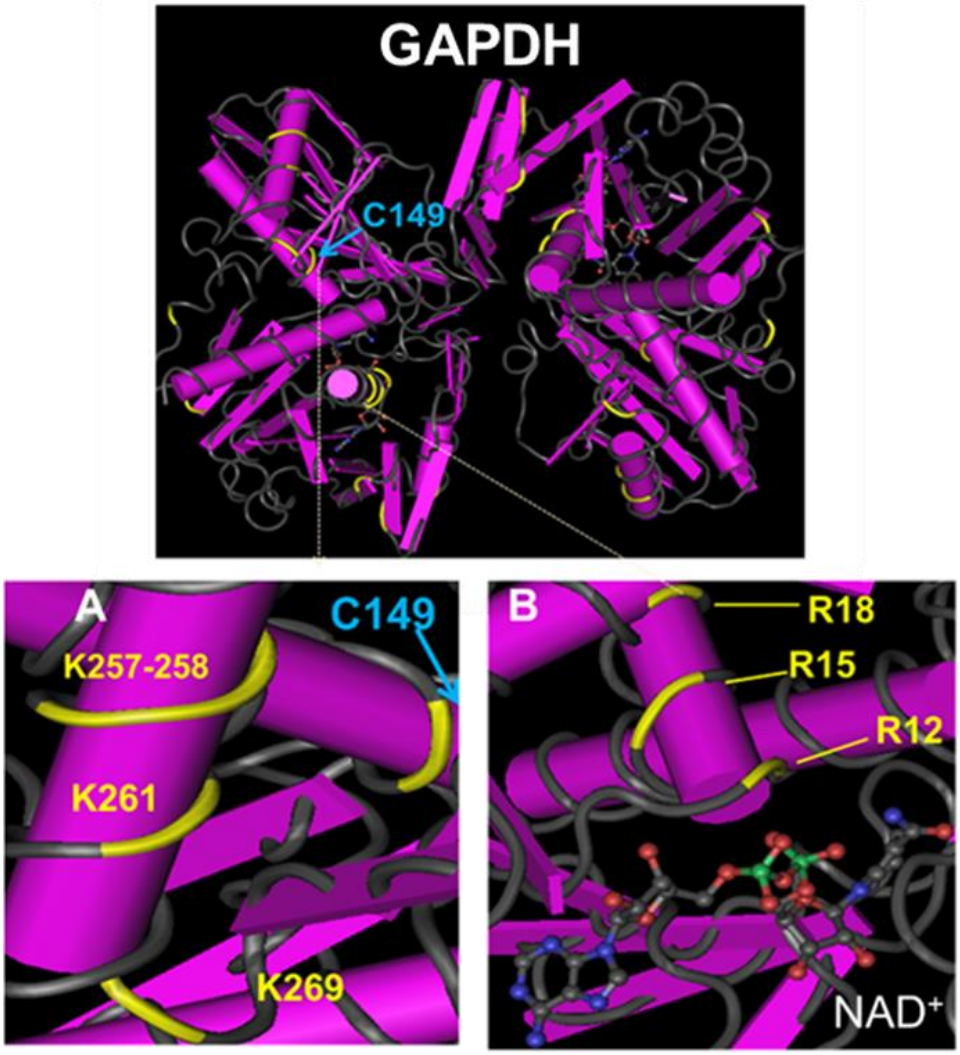


AA SEQUENCE	PEPTIDE	MH+ Control	MH+ DOPAL Treated	Mass Shift	Type of Modification
12-18	(R)IGRLVTR(A)	814.902	948.902	134	Schiff Base
54-70	(K)FHGTVKAENGKLVINGK(A)	1813.72	1947.72	134	Schiff Base
65-70	(K)LVINGK(A)	643.37	779.373	136	Reduced Schiff Base
117-137	(R)VIISAPSADAPMFVMGVNHEK	2229.96	2363.96	134	Schiff Base
138-143	(K)YDNSLK(I)	739.31	873.307	134	Schiff Base
253-258	(K)YDDIKK(V)	781.52	917.52	136	Reduced Schiff Base
253-261	(K)YDDIKKVVK(Q)	1107.11	1243.11	136	Reduced Schiff Base
262-269	(K)QASEGPLK(G)	812.38	947.38	136	Reduced Schiff Base

B

AA SEQUENCE	Cysteine peptides oxidized by DOPAL	M+H	Missed cleavages	Mass shift	Type of Modification
226-232	(K)LTGMAFR(V)	812.21	0	16	Met-oxidation
85-115	(R)WGDAGAEYVVESTGUFTTMEKAGAHLKAGGAK(E)	3210.16	2	16	Met-oxidation
112-136	(K)GGAKRVIISAPSKAPMFVGMNHEK(Y)	2663.15	2	16	Met-oxidation
225-249	(K)LTGMAFRVPTPNVSVVPLTCK(A)	2327.21	2	16	Sulfenic acid or Met-oxidation
232-249	(R)VPTPNVSVVPLTCR(I)	1533.30 1563.16 1597.17	1	16 32 48	Sulfenic acid Sulfinic acid Sulfonic acid
269-306	(K)GILGYTEDQVVSCDFNSATHSSTFDAGAGIALNDHFVK(I)	4273.72	0	48	Sulfonic acid

Table 3.1 Identification of DOPAL adducts on GAPDH trypsin-digested peptides were subject to LC-MS and peptides were mapped according to addition of a A) DOPAL mass shifts of 134 or 136 Da, pertaining to reduced and non-reduced Schiff bases on Lys or Arg. B) Unique mass shift peaks due to DOPAL-induced oxidation of GAPDH pertaining to 16, 32, of 48 kDa. Lower panel lists unique peaks pertaining to mass of dipeptides linked by a DOPAL molecule, all containing Cys residues.



Scheme 3.1 GAPDH dimer is shown. The yellow ribbons denotes the modified residues by DOPAL. A) 3 modified Lys residues are spatially close to the key Cys in active site. B) Modified Arg residues are sequence and spatially near the NAD^+ binding site.

3.4 Discussion

The *in vitro* studies with GAPDH were important in demonstrating protein modification of GAPDH by DOPAL. Given that GAPDH utilizes NAD^+ cofactor, the protein aggregation with DA metabolites was investigated in the presence and the lack of

NAD^+. Data in Figure 3.1 show that DOPAL caused protein aggregation and decreased the parent monomer band, and the presence of NAD^+ did not protect from DOPAL-mediated protein modification. Given that both DA and DOPAC contain catechols that can rearrange to ortho-quinones to modify proteins, it was plausible for DA and DOPAC to modify GAPDH. However, the lack of reactivity of DA and DOPAC illustrates the importance of the aldehyde in DOPAL reactivity with proteins. This is further exemplified by Figure 3.5A, which showed that MOPAL and DHPAN analogs did not produce a significant amount of catechol-adduct staining on GAPDH. Even at high concentrations (500 μM) of MOPAL and DHPAN, there was no binding with protein. DOPAL was the only catechol compound out of the three that produced catechol-GAPDH staining via NBT. Furthermore, Figure 3.5B shows that at 100 μM, the GAPDH parent band is almost completely disappeared, and there is significant catechol-GAPDH smearing in the high MW region for both 25 and 100 μM DOPAL. The reactivity of DOPAL with GAPDH is dependent on the dose and time. Results from Figure 3.2 showed GAPDH staining in the high molecular weight region at 50 and 100 μM, this effect is greatly increased at a later time point- 4 h versus 2 h. The low DOPAL dose of 5 μM also illicited a similar effect, but only at 4 h.

A recent study showed that GAPDH aggregation in SH-SY5Y cells was directly related to increasing concentration of DA, and contributed to DA-induced cell death[86]. However, it was proposed that an electrophilic metabolite downstream of DA, such as DOPAL was a relevant contributing factor in dopaminergic neuron toxicity. The reactivity of DOPAL appears to be protein selective, inducing oligomerization in GAPDH but not in bovine serum albumin (BSA)[113]. This reactivity is dependent on two

functional groups of DOPAL, the catechol and aldehyde groups[60; 113]. More specifically, the present work highlights that both catechol and aldehyde are needed for GAPDH modification. Whereas others report the DA interaction with GAPDH being dependent only on the catechol, and forming a 5-S-cysteinyl DA conjugate[101; 116]. In regards to redox cycling and catechol formation, DOPAL levels are key in producing catechol adducts on proteins. This fact is supported by the finding that cell lysate treated with an ALDH inhibitor produced more catechol staining, however, catechol staining was reversed with the MAO inhibitor, pargyline[117]. This suggests that redox cycling is decreased at lowered DOPAL levels, as the MAO inhibitor will prevent DA to DOPAL conversion.

The co-incubation studies of GAPDH and alpha-syn with DOPAL were key in determining the role of DOPAL in GAPDH and alpha-syn in Lewy bodies[84]. To date, there have been no studies showing the effect of GAPDH and alpha-syn co-aggregation as a function of DOPAL. In our hands, both proteins co-aggregated to a greater extent than either alone. This supports previous reports showing that GAPDH promotes the aggregation of alpha-syn[84]. It has been suggested that the loss of function of GAPDH cues the cell to synthesize more GAPDH mRNA and protein.[80] In this scenario, it is envisioned that more GAPDH is shuttled to the cytoplasm and causes increased aggregation with alpha-syn, catalyzed in the presence DOPAL. Utilizing SH-SY5Y as a dopaminergic cell model, DOPAL treatment increased GAPDH mRNA in a dose-dependent manner, however, only 50 µM DOPAL produced a pronounced change in mRNA~1.5-fold difference versus untreated cells, shown in (Figure 3.4). The alpha-syn mRNA was a more subtle increase than GAPDH mRNA. To date, there are no published

studies showing the change of protein expression in response to DOPAL. The above mentioned PCR data and the co-aggregation results suggest that GAPDH plays an active role in PD-related toxicity. A clinical study sampled cerebral spinal fluid (CSF) from PD patients, and treated the CSF to cultured cells. The PD-CSF resulted in significant cell apoptosis, however, CSF from healthy patients did not produce cell apoptosis. Interestingly, the toxicity observed from the PD CSF was prevented by treatment of GAPDH antisense oligonucleotide, suggesting that synthesis of new GAPDH protein is required for apoptosis caused by PD CSF[118].

A pertinent question has been which amino acids are necessary and participate in the oligomerization induced by DOPAL? Adduct sites on GAPDH were confirmed by trypsin digest and peptides analyzed by ESI LC-MS. The matched DOPAL adducts resulted from a mass shift of a DOPAL Schiff base or reduced Schiff base of 134 and 136 Da, respectively. The adduction would take place at the aldehyde site of DOPAL as shown in Figure 6A. All matched peptides contained Lys or Arg residues, which are likely nucleophilic target sites for DOPAL. This is well supported, given the rapid decrease in DOPAL when a N-acetyl lysine is present[61]. Two of the modified peptides also contained multiple Lys residues or missed cleavage sites by trypsin. The lack of recognition by trypsin serves as an indirect indication for DOPAL adduction, as it has been reported that DOPAL adduct sites interfered with trypsin digest[75]. It was also of interest that the majority of these matched peptides were found in the NAD^+ binding domain. However, the presence of NAD^+ cofactor did not protect or affect GAPDH aggregation in the presence of DOPAL (Figure 3.1).

It is likely that Lys and Arg are not the only residues modified by DOPAL[113], as sulfhydryl-containing amino acids such as Cys and Met are also possible sites of modification. In fact, Met oxidation is measured by an increase m/z = 16, and is a common fragment generated by DA and DOPAL treatment of peptides.[75] In the case of thiol modification, a DOPAL reactive center is at the catechol site; the catechol can undergo 2 electron oxidation and form the ortho-quinone, and then hypothetically bind to Cys via Michael addition[57]. This DOPAL Michael adduct retains a free aldehyde, in which it can bind to additional amino acids via Schiff base, and this reaction is a reasonable model for the protein crosslinking observed with GAPDH (Figure 3.2). However, it was not possible to detect these cross-linked peptides due to limited tryptic proteolysis and complexity of the MS data. Previous findings showed that DOPAL is not reactive towards N-acetyl cysteine[113]. However, DOPAL binding to Cys had not previously been tested in whole protein. One way to test DOPAL binding to thiols was probing with a thiol-sensitive reagent (DTNB). LC-MS data did not provide peptides containing Cys Michael adducts by DOPAL, so it was surprising that the number of free thiols was inversely related to DOPAL concentration (Figure 3.6), and the number of free Cys decreased significantly at high DOPAL concentration. One possibility is that DOPAL may not be binding to Cys directly, but causing Cys oxidative modification by disulfide or sulfenic acid formation. This may be a likely alternative for a measured decrease in the total free thiols, given that DOPAL is a source of reactive species,[57] undergoing 2 electron oxidation to form the DOPAL ortho-quinone[57]. Additionally, the use of antioxidants inhibit protein binding with DOPAL, suggesting that protein modification is dependent on oxidation of DOPAL[61; 75]. Upon further inspection of the

LC-MS data, Table 3.1B highlights the Cys containing peptides with a mass shift of 16, 32 or 48 kDa, corresponding to the sulfenic, sulfinic or sulfonic acids. The mass peaks pertaining to Met and Cys oxidative modifications were only found in the GAPDH-DOPAL mixture and were not found in the untreated GAPDH mixture. The use of a sulfenic acid probe (e.g. dimedone) could be a useful future step in the identification of Cys modification[119]. Many Cys residues are important redox switches in eukaryotic proteins[120], and it has been reported that when the active site Cys of GAPDH is oxidized, the protein translocates to the nucleus and induces neuronal apoptosis; treatment with selective compounds that effectively prevented this oxidation also protected from apoptosis[121].

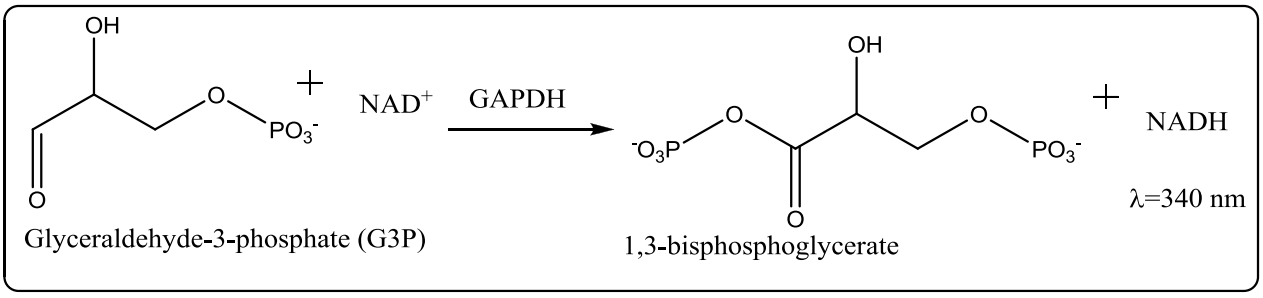
As a multifunctional enzyme, GAPDH has been utilized by a number of research groups as a model of electrophilic modification[86; 122; 123]. GAPDH has a Cys in its active site and has been known to be sensitive to oxidative modification. Previous work has confirmed DOPAL as an endogenous DA metabolite, capable of electrophilic modification[48; 113; 124]. However, this work identifies DOPAL as a novel electrophile for GAPDH, causing protein inhibition and conformational changes, leading to monomer oligomerization. Treatment with ROS donors also caused widespread conformational changes to GAPDH structure, and led to amyloid-like intracellular fibril formation,[100] however, very high concentrations were needed to cause a significant reduction in cell viability. High GAPDH oligomerization by DOPAL is possible at physiological concentrations in relatively short time periods of 2-4 h (Figure 3.2). GAPDH poses as a likely target by DOPAL modification since GAPDH is ubiquitously present in DA neurons and is one of the most plentiful proteins within the cytosol and mitochondria[77]

CHAPTER 4

DOPAL INHIBITS GAPDH ACTIVITY

4.1 Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the reversible conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the presence of nicotinamide adenine dinucleotide (NAD^+) to reduced NADH as part of the glycolytic cycle,[125] outlined in Scheme 1. Due to the presence of several redox-sensitive thiols on GAPDH, the protein has commonly been utilized as a model for cysteine modification by a number of electrophiles including dopamine (DA),[87] DA-quinone (DAQ)[126] and 4-hydroxy-2-nonenal (4-HNE)[122]. GAPDH is a highly abundant and constituitively expressed enzyme and is routinely utilized as an internal standard for quantitative protein comparison. However, recent years have accumulated evidence for the multi-factorial roles of GAPDH and its role as a cell death mediator. As outlined in Chapter 3, GAPDH is highly sensitive to oxidative modification and has been considered a protein sensor of oxidative stress, such as GAPDH exposure to nitric oxide[112; 127] and peroxynitrite.[128] One major consequence of oxidative stress is a cellular decrease in cellular ATP levels and blocked glycolysis[129; 130].



Scheme 4.1 GAPDH is a dehydrogenase converting glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate, reducing the NAD cofactor to NADH, and oxidizing the substrate G3P.

Recent work has shown deficiencies in GAPDH activity as possible factors in several neurodegenerative disorders such as Alzheimer's disease, Huntington's disease, and Parkinson's Disease (PD)[77; 131; 132]. In the well accepted PD model, rotenone-treated mice produced a number of PD like pathological characteristics such as hypokinesia, and Lewy body formation. Interestingly, these animals also displayed a significant decrease in GAPDH activity; brain post mortem tissue revealed that GAPDH was reduced by 34% in the substantia nigra, but only by 20% in the cerebellum, which is a less affected brain region than the substantia nigra in PD etiology[80]. Decreased activity in energy metabolic pathways are side effects of PD[79] and furthermore, inhibition of GAPDH may serve as a marker in PD pathology. Chapter 3 showed that GAPDH was covalently modified by DOPAL in a time and DOPAL-dose dependent manner. Therefore, it was hypothesized that DOPAL would also affect GAPDH enzymatic activity. In this study, the mode of inhibition by DOPAL on GAPDH activity were thoroughly investigated.

4.2 Experimental Procedures

Materials

DOPAL was biosynthesized via rat liver MAO procedure as previously established[51], and DOPAL stock concentrations were determined using an ALDH assay with NAD^+ and HPLC[52]. MOPAL was biosynthesized according to a similar procedure as DOPAL[113]. DA, 3,4-dihydroxyphenylacetic acid (DOPAC), GAPDH from rabbit muscle, glyceraldehyde-3-phosphate (G3P), glutathione (GSH), and 2,2',2",2"'-(ethane-1,2-diyldinitrilo)tetraacetic acid (EDTA) and all other reagents were acquired from Sigma-Aldrich (St. Louis, MO). The DOPAL analog, 3,4 dihydroxyphenylacetonitrile (DHPAN) was synthesized via deprotection of 3,4-(methylenedioxy)-phenylacetonitrile by boron triboromide in methylene chloride in N_2 conditions[60] and 3-methoxy-4-

hydroxy-phenylacetaldehyde (MOPAL) was biosynthesized via MAO-mediated oxidative deamination of methoxytyramine as previously described [113]. Micro bio-spin columns were purchased from Bio-Rad Laboratories.

GAPDH Activity Assay

Enzymatic activity of purified GAPDH was measured by NADH production and absorbance at 340 nm for 3 min[76]. After incubation in 50 mM sodium phosphate pH 7.4 buffer, GAPDH (0.3 mg/ml) with and without DOPAL and DOPAL analogs, 100-µL aliquots at given time points were transferred to a 96-well plate with a final volume of 200 µL, containing: 50 mM sodium phosphate buffer at either pH 7.4 or 8.0, 1 mM NAD^+, 1 mM EDTA. The reaction was initiated by adding 0.8 mM glyceraldehyde-3-phosphate to the reaction mixture. Enzyme activity incubated with DOPAL is expressed as the percentage of the basal activity obtained from samples incubated without DOPAL.

GAPDH Activity Recovery Assay

For reversibility assays, GAPDH was incubated with DOPAL as previously described. Following incubation, excess DOPAL was removed from the mixture by filtration with size–exclusion spin columns (Micro Bio-Spin 6 columns, Bio-Rad laboratories) for 3 mins at 3000 x g. Prior to sample elution, columns were prepped and exchanged with 50 mM sodium phosphate buffer as explained in the product protocol. 100-µl aliquots were transferred from mixtures with and without filtration, and activity was measured as described above in the activity assay. Activity at each DOPAL concentration was measured with and without spin column filtration to control for any binding of enzyme to column resin. To measure the recovery of inhibition as a product of

GAPDH thiols, GSH (0.5 mM) was added to each incubation mixture immediately prior to activity measurement.

4.3 Results

DOPAL Inhibits GAPDH Activity

GAPDH catalyzes the oxidation of G3P and reduction of cofactor NAD^+ to NADH. Therefore, activity can be measured by spectrophotometric absorbance of the reduced NADH at 340 nm. All DOPAL concentrations (5-25 μM) inhibited GAPDH activity, and a loss of ~50% of activity is achieved at 1h with 25 μM DOPAL (Figure 4.1A). However, at 4 h, GAPDH activity with 25 μM DOPAL is nearly zero). Therefore, DOPAL inhibits GAPDH in a time and dose-dependent manner. Additional experiments have shown that complete enzyme inactivation is achieved after 4 h for higher DOPAL doses and 8 h for lower DOPAL doses. The presence of physiological concentration of NAD^+ (100 μM) cofactor in the incubation mixture attenuated enzyme inhibition, and is therefore protective to DOPAL-induced inhibition of GAPDH (Figure 4.1C-D).

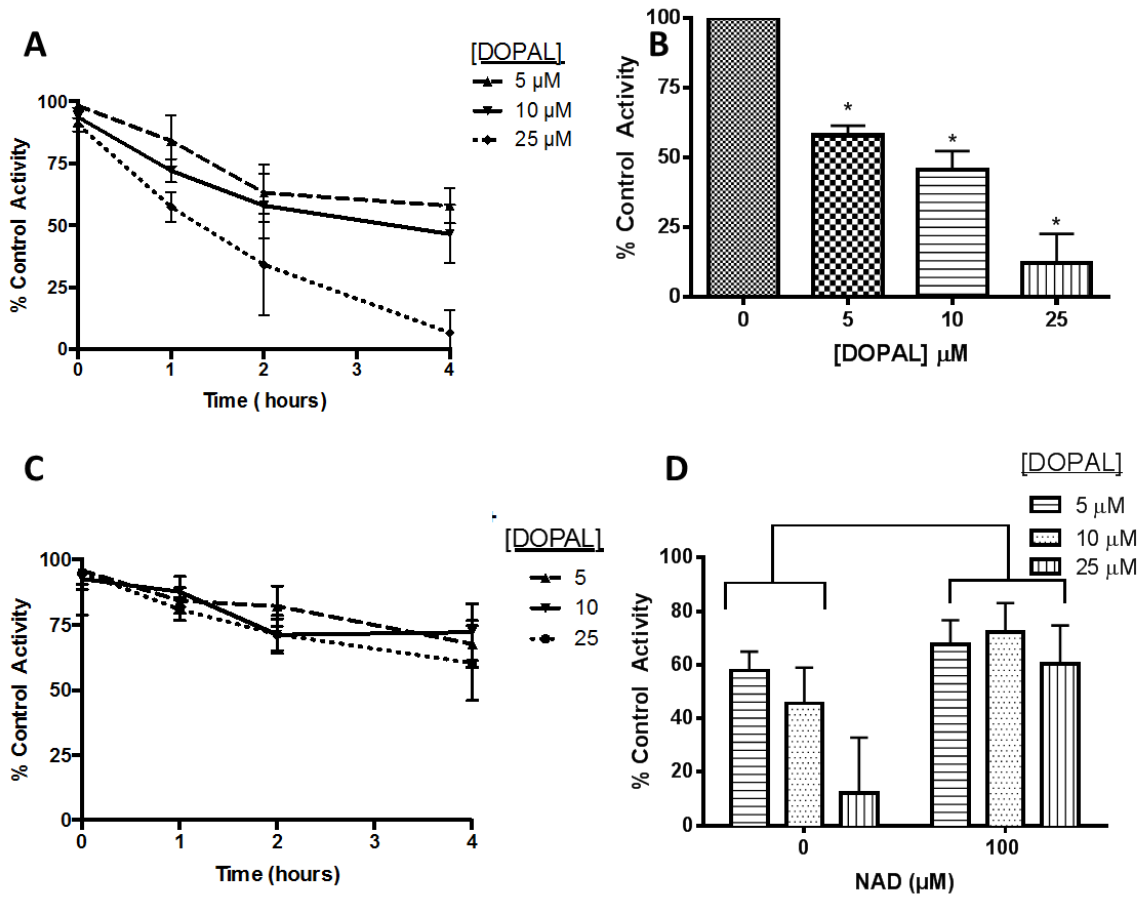


Figure 4.1 DOPAL inhibition of GAPDH activity. DOPAL inhibits GAPDH activity. 0.3 mg/ml GAPDH incubated with DOPAL: 5, 10, 15 µM for 4 h in 50 mM sodium phosphate buffer at 37ºC. All values were calculated as the activity ratio of the control or non DOPAL-treated sample, and enzyme activity of G3P oxidation is measured by NADH absorbance at 340 nm. (A)Time and DOPAL concentration dependence on GAPDH inhibition. (B) The inhibition of GAPDH activity by DOPAL at 4 h. (C) GAPDH activity is assayed as before but containing 100 µM NAD+ in the incubation mixture. (D) Comparison of the attenuated inhibition by DOPAL at 4 h, when NAD+ cofactor is present in the incubation mixture, connecting bars show non-significance. Panel B was analyzed by 1-way ANOVA, and panel C via 2-way ANOVA analysis. For all experiments, n=3, significance is denoted with (*), $p \leq 0.05$.

DOPAL Analogues do not Inhibit GAPDH Activity

DOPAL is a bifunctional electrophile, and it has been previously shown to be dependent on the catechol and aldehyde for protein binding[61]. Therefore, it was of interest to test both DOPAL electrophilic groups (catechol and aldehyde) for GAPDH inhibition. The two analogues were 3,4-dihydroxyphenylacetonitrile or DHPAN, containing a catechol but not the aldehyde, and 3-methoxy,-4-hydroxyacetaldehyde or MOPAL, which retains the aldehyde but not the catechol. As shown in Fig. 4.2A, neither DOPAL analogues affect GAPDH activity.

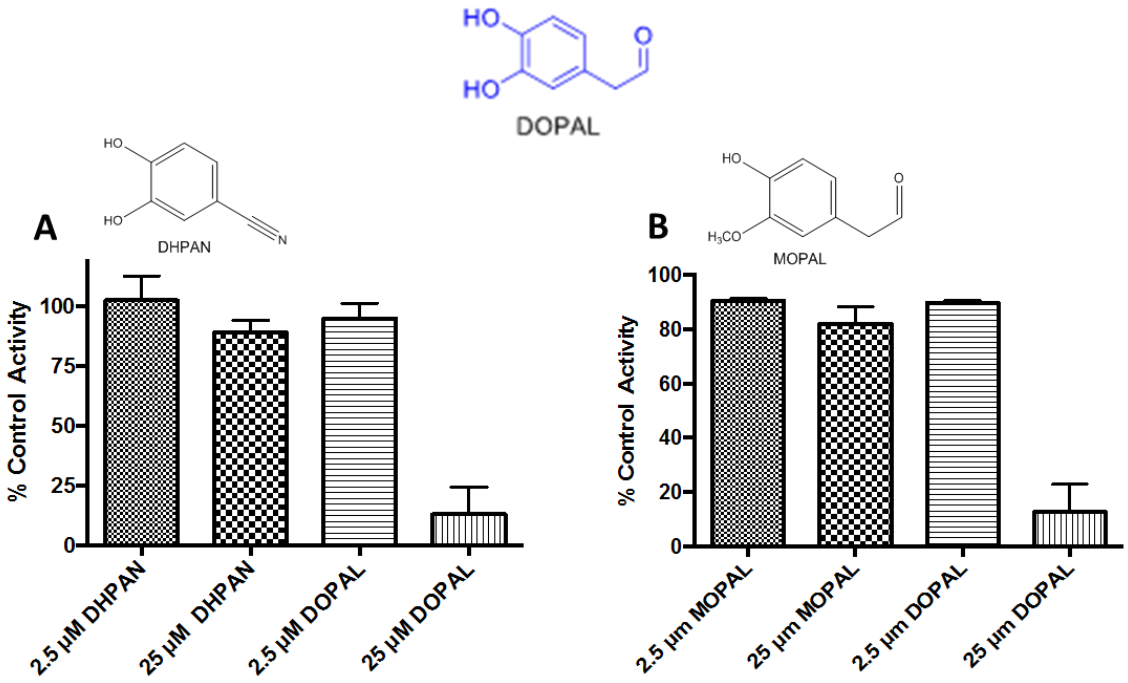


Figure 4.2 GAPDH is not inhibited by DOPAL analogs, lacking aldehyde or catechol. GAPDH activity was measured at 4 h in the presence of 2.5 and 25µM of DOPAL, DHPAN and MOPAL.

GAPDH Inhibition by DOPAL is Irreversible

GAPDH and DOPAL (0, 25 μM) were incubated for 1-4 h. At the end of the incubation period, excess DOPAL was removed by micro Bio-Spin columns. The cofactor NAD^+ was added to the GAPDH-DOPAL mixture and kinetic activity recorded shortly after G3P substrate addition. The untreated GAPDH was also filtered as a control for any possible protein loss during filtration. The non-filtered mixture of GAPDH-DOPAL was then compared to the filtered GAPDH-DOPAL, accounting for the reversibility of DOPAL binding to GAPDH. As shown in Figure 4.3A**,** at shorter time points such as 1 h, the filtered GAPDH-DOPAL regained some activity compared to the non-filtered GAPDH-DOPAL. However, at 4 h, there is no recovery in enzyme activity. There was also no difference in activity between the filtered and non-filtered GAPDH mixtures, accounting for negligible enzyme loss during the filtration and centrifugation. In addition to filtration for removal of DOPAL, glutathione (GSH) was added to the incubation mixture immediately prior to enzyme activity measurement (Figure 4.3B). GSH will reduce the Cys, located in the active site, and the reduced thiol is necessary for proper enzyme activity. However, the addition of 1 mM GSH at the end of the incubation period to samples containing 5 or 50 μM DOPAL did not reverse inhibition compared to DOPAL treated GAPDH.

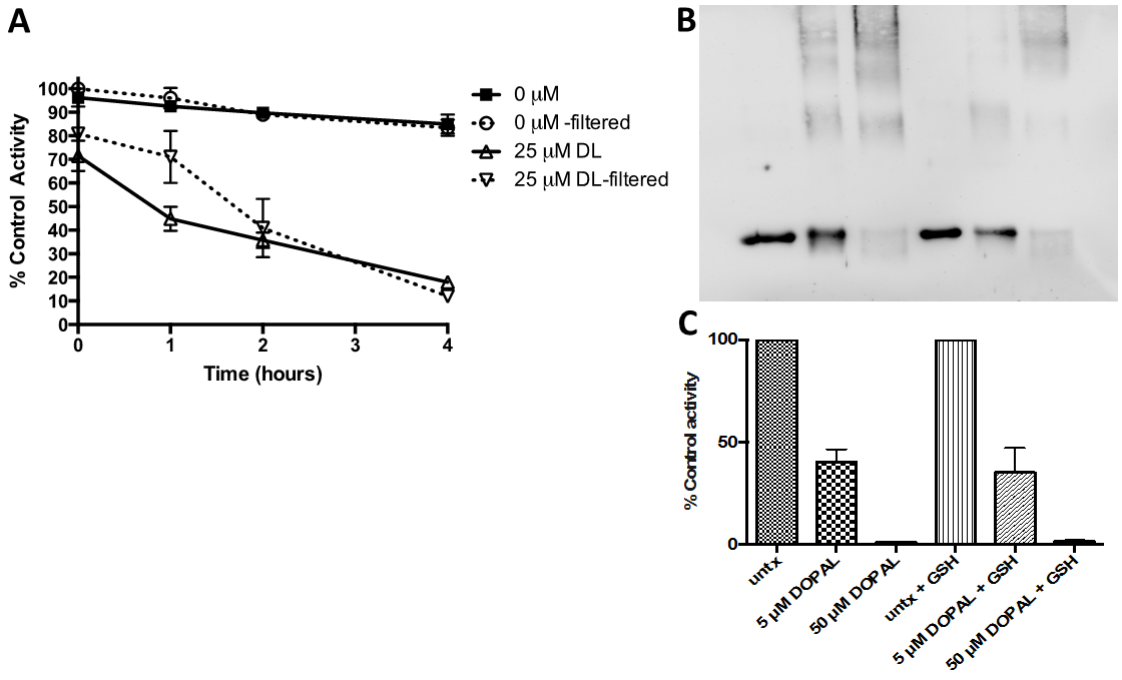


Figure 4.3 Inhibition of GAPDH by DOPAL is mainly irreversible and cannot be reversed by addition of GSH. (A) Aliquots from GAPDH (0.3 mg/ml) and 25 μM DOPAL were taken and subjected to centrifugation filtration. Non-filtered aliquots were also assayed and compared to the filtered aliquots to measure reversibility of DOPAL inhibition and binding or regain in activity. (B) Western blot (1 μg per lane) of GAPDH with DOPAL (5, 25 μM), with and without 0.5 mM GSH (C) To the GAPDH-DOPAL incubation mixture, at 4h, with and without 0.5 mM GSH added prior to NADH measurement.

4.4 Discussion

It has long been proposed that DA is a contributing factor for the heightened and selective vulnerability of dopaminergic neurons. [133; 134]. A recent study showed that GAPDH aggregation in SH-SY5Y cells was directly related to increasing concentration of DA, and that GAPDH directly contributed to DA-induced cell death[86]. However, in the current study, it was proposed that a highly reactive metabolite downstream of DA, i.e. DOPAL may also be a contributing factor of the selective neurotoxicity. Data in Chapter 3 demonstrated that GAPDH modification by DOPAL is dependent on two functional

groups, the catechol and aldehyde. More specifically, this work highlights that both catechol and aldehyde are needed for GAPDH inhibition (Figure 4.2) as neither MOPAL or DHPAN significantly inhibited GAPDH activity. Such a finding correlates well with previous work that neither of these analogs reacted covalently with GAPDH, as shown in Figure 3.5B. Additionally, it had been previously shown that MOPAL and DHPAN did not inhibit tyrosine hydroxlase, an enzyme intrinsic to DA biosynthesis[60].

Since it was previously proven that DOPAL covalently modified GAPDH and created adducts on specific residues, it was hypothesized that DOPAL could potentially also inhibit GAPDH activity. The results from Figure 4.1 showed that DOPAL is a potent inhibitor of GAPDH at low micromolar levels (5-25 μM). This is contrasted by previously tested electrophiles by other groups, which demonstrated GAPDH inhibition at much higher electrophile concentrations. The inhibitory concentrations for these electrophiles (acrylamide, acrolein and 4-HNE) were in the mid to high millimolar range (250-1000 μM)[122; 123], and the mechanism of binding for these electrophiles was proposed to be via Michael addition to nucleophilic Cys residues. A separate study found that inhibition of GAPDH by acrylamide was responsible for peripheral nerve degeneration[135].

The nucleophilic reactivity of Cys is highly selective according to the microenvironment and varies according to the pH environment. Cys residues are generally unreactive at physiological pH 7.4 and have pK_a 8.3. Thiol reactivity and GAPDH inhibition were greater at higher pH, and formed more Cys adducts with nucleophiles[123]. This is due to the ionization of the sulfhydryl, yielding a sulfhydryl anion., and this ionization occurs more readily at higher pH. Such a finding suggests that

DOPAL may also form Michael adducts with GAPDH at a higher pH, but the relevancy of this finding should be questioned since cytosolic pH is ~7.4, and GAPHD is primarily located within the cytosol[111]. However, the pH environment within certain subcellular structures such as the inner and outer mitochondria is considerably higher, between pH 8.0-9.2[136]. This elevation in pH is relevant to DOPAL-mediated inhibition of GAPDH for two reasons: the site of DOPAL production takes place by the outer mitochondria membrane MAO, and GAPDH is located in the mitochondria, albeit levels are low at basal conditions. GAPDH is elevated during apoptosis and cell stressed conditions[137]. For enzyme assays, fresh buffer was routinely prepared, and pH was carefully monitored, as the basal activity and inhibition by DOPAL were greatly influenced by any pH deviation.

Given the fact that GAPDH is a dehydrogenase and the native substrate is an aldehyde (G3P), it was hypothesized an aldehyde of similar size such as DOPAL would also interact at the GAPDH active site. However, it was unknown whether this would be a reversible or irreversible reaction. When excess DOPAL from the GAPDH-DOPAL mixture was removed by filtration, the subsequent activity assay confirmed irreversibility since there was no regain of activity with DOPAL removal (Figure 4.3). Other protein targets, such as tyrosine hydroxylase were inhibited by DOPAL, and the mode of inhibition was concluded to be slowly irreversible[138]. The irreversibility of GAPDH inhibition by DOPAL demonstrates the potential adverse effects in dopaminergic neurons, given that neurons have very high energy requirements[139]. A study utilizing a PD mouse model, showed that inhibition of GAPDH caused a feedback loop of increased GAPDH expression, which led to GAPDH transport to the cytosol and increased protein aggregation and cell death[80]. This finding is in agreement with our studies that showed an

increase in GAPDH mRNA after treatment with DOPAL in SH-SY5Y cells (Figure 3.4). Increased levels of cytosolic GAPDH can be detrimental as GAPDH caused *in vivo* toxic protein aggregates by itself[87], and also promote formation of Lewy bodies with alpha-syn[84].

According to standard GAPDH activity assays[128; 140], the activity of GAPDH-DOPAL mixture was initially measured with addition of NAD^+ cofactor directly before the spectrophotometric measurement. However, it was decided to add NAD^+ simultaneous with DOPAL into the incubation mixture, in order to mimic the cellular endogenous environment in which NAD^+ is already bound to GAPDH and to test for possible protection by NAD^+ in DOPAL-mediated inhibition of GAPDH. The presence of NAD^+ resulted in considerable protection from inhibition by DOPAL (Figure 4.1C-D), and these results suggests that DOPAL competitively binds with NAD^+, and it is likely that potential DOPAL binding sites are blocked when NAD^+ is present. This protection of inhibition correlated well with previous LC-MS data (Table 3.1) showing that the majority of DOPAL-modified peptides were within the NAD^+ binding site. In particular, one modified peptide (AA 12-19) contained multiple modified Arg residues and was spatially close to a NAD^+ cofactor binding site shown in Scheme 3.1B.

Although LC-MS results showed DOPAL adducts on Lys and Arg, but not Cys; other assays such as titration with DTNB proved that thiols were also modified by DOPAL. In Scheme 3.1, the modified Lys residues (K261, K269 and K257-258) were spatially close to the active site Cys 149. This was an important finding, given that Cys149 in the GAPDH active site is crucial for activity. However, others have previously shown that the active site Cys149 and Cys152 (human) GAPDH modification could be

reversed via a transarylation reaction that occurs with 1,2-napthoquinone[140]. 1,2-Napthoquinone is a Michael acceptor to the Cys, binding with Cys requires catechol oxidation to the quinone. The addition of GSH to the reaction mixture reversed the 1,2-napthoquinone adduct binding to GAPDH, producing a GSH-conjugate of the electrophile and reduced Cys152-GAPDH. Therefore, the GSH displaced the Cys152-1,2-napthoquinone adduct by directly binding to1,2-napthoquinone. Such a reaction seemed like a relevant possibility to DOPAL, given the ability of DOPAL to re-arrange and oxidize to a reactive quinone[57]. However, the addition of GSH into our samples at the end of the DOPAL incubation period did not affect GAPDH activity or rescue from enzyme inhibition (Figure 4.3C). These findings agreed with Western blot results, illustrating that GSH also did not reverse or alter GAPDH oligomerization (Figure 4.3B).

GAPDH is a central enzyme in energy metabolism and neuronal homeostasis. It has been shown that GAPDH and other energy metabolism enzymes are inhibited via oxidation in Lewy bodies of the frontal cortex[79]. This highlights the importance of proper GAPDH activity in Parkinsonian models. This work demonstrates GAPDH as a novel enzyme irreversibly inhibited by DOPAL.

CHAPTER 5

DOPAL AND INHIBITION OF ALDH BY ENVIRONMENTAL AGENTS: BENOMYL AND CHLORPYRIFOS-OXON

5.1 Introduction

Parkinson's disease (PD) is a neurodegenerative disorder which affects over a million people in the United States alone. The pathogenesis of PD has not been clearly elucidated and is attributed to several genetic and environmental factors[23; 90; 141-143]. Although there is no current cure or preventative treatment for PD, there are exogenous substances such as tobacco[19; 144; 145] and caffeine[146] which have consistently showed a lowered risk for PD development. The protective effects of tobacco and caffeine against PD are not fully understood, however it is speculated that tobacco leaves contain compounds that are MAO activity modulators, and this was initially supported by studies documenting that MAO-B activity was dramatically lowered in blood platelets from smokers compared to non-smokers[147]. More recent studies have shown decreased MAO-B activity in smoker's peripheral organs versus non-smokers, through PET imaging[148]. In 2006, a research group fractionated tobacco extract, and isolated *trans,trans-farnesol,* with MAO-B inhibition properties[149]. Subsequent SAR studies have produced an increased potency of *trans,trans-farnesol,* and provides an neuroprotection explanation to tobacco exposure[150]. An alternative theory is that since dopamine pathways are related to reward mechanisms[151], PD patients are less prone to addictive behaviors and are less likely to be smokers[145]. Epidemiological studies showed that both current and past smokers have a significant inverse association for developing PD.

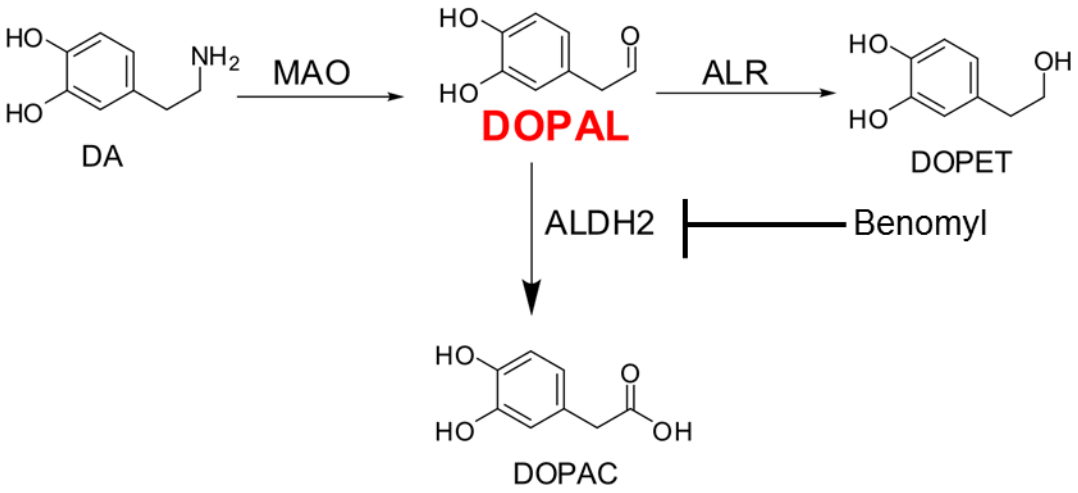
Genetics contributes to PD pathology. However, despite the elucidation of 18 genes in familial PD and a wide array of multiple risk factor genes using genome studies from thousands of PD patients, familial linked cases encompass less than 10% of the total PD cases[152]. This highlights the necessity fn the identification of environmental risk factors that predispose individuals to developing PD. The past few decades have produced a wealth of evidence attributing an increased PD risk due to exposure to environmental agents, such as pesticides and fungicides[23; 141-143]. These studies strive to report the implied PD risk in a given population from exposure to only one type of pesticide, however, this has its challenges due to the likelihood that the population is exposed to multiple types of pesticides. The association between pesticides and PD has been firmly established, but the question of direct causality has not been clear. However, subsequent studies addressing recall bias and pesticide dose-effect relationships have strengthened the causal role of pesticides for PD. Some of the more hazardous pesticides are those with long half-lives and poor solubility, allowing for accumulation in well-water and bio-accumulation in soil[153]. This work will focus on pesticides which have these bioaccumulative characteristics and are related to disruption of DA metabolism and regulation.

PD leads to the selective loss of dopaminergic neurons in the substantia nigra, causing a decrease in the important neurotransmitter DA.[154] DA is metabolized by monoamine oxidase (MAO) to the reactive and toxic intermediate 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is further metabolized by aldehyde dehydrogenase (ALDH2) and aldehyde reductase (ALR) to 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenylethanol (DOPET), respectively[48]. These latter

metabolites are not toxic,[48; 155]DOPAL can also undergo 2e$^-$ oxidation to an *o*-quinone, forming superoxide anion, promoting aggregation in a number of pertinent proteins related to PD such as alpha-synuclein[156; 157] and glyceraldehyde-3-phosphate dehydrogenase[113]. DOPAL has demonstrated high toxicity[98; 158] and the ability to interact with and modify both model peptides[61], and proteins.[55]

Reactive dopamine metabolites and pesticide exposure are two major factors in PD etiology, however, the link between them was fairly unexplored and unknown until the past couple of years. As mentioned before, ALDH2 is primarily responsible for detoxification of reactive aldehydes such as DOPAL and 4-hydroxy-2-nonenal (4-HNE). In 2014, Fitzmaurice et al. concluded that individuals who expressed a variant of the ALDH2 and ALDH2 genes[159] were associated with exacerbated PD risk when exposed to ALDH-inhibiting compounds,[67] and pesticides that inhibited ALDH activity were associated with a 2 to 6-fold increase in PD risk. The most potent ALDH inhibitor from this group of compounds was a benzimidazole-containing molecule called methyl N-[1-(butylcarbamoyl)benzimidazol-2-yl]carbamate or benomyl (Scheme 5.1). Benomyl was banned in 2001 due to accumulation in animal livers and teratogenic properties[68]. However, benomyl bioaccumulates within tissues, and workers exposed to benomyl at multiple work and home locations had an increased PD risk than exposure at just one location. The link between benomyl exposure and PD was attributed to alteration of DA homeostasis and ALDH inhibition[70] (Scheme 5.1). ALDH inhibition is detrimental as it inhibits proper detoxification of DOPAL and 4-HNE,and this is highly relevant in PD as increased DOPAL and 4-HNE has been reported in post mortem PD brains as adducts[160]. A separate study from the Fitzmaurice group also discovered that ALDH inhibition was toxic to primary mesencephalic cultures and zebrafish. Although DOPAL levels were not directly measured, the toxicity is attributed to a rise in endogenous DOPAL levels, as the toxicity was rescued by treatment with MAO inhibitors[89]. Knockout and overexpressing

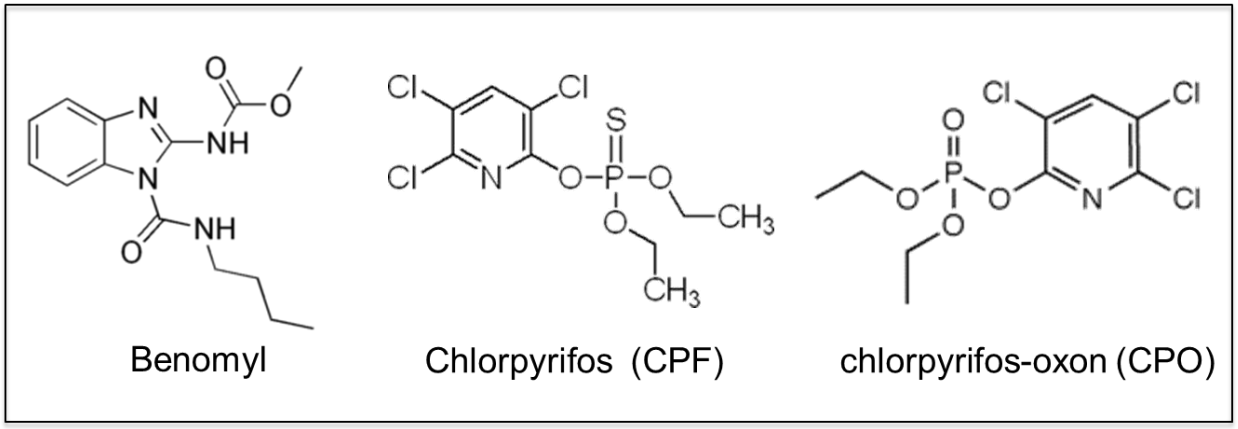
mice have been generated for both ALDH1 and ALDH2 as models for a number of diseases. It was determined that while ALDH2 knockouts had a decreased metabolism of ethanol, no significant neurodegeneration was ellicited[161; 162]. However, ALDH1 and ALDH2 double knockout mice exhibit significant motor dysfunction and neurodegeneration that shared many PD characteristics[163]. Therefore, inhibition of various ALDH isoforms may be needed to produce PD pathology in humans. In summary, the decreased activity or expression of key enzymes, such as ALDH, responsible for detoxifying xenobiotics, has served as a link between pesticide exposure, DOPAL levels and PD risk.



Scheme 5.2 DA undergoes oxidative deamination via monoamine oxidase (MAO) to form DOPAL, the aldehyde metabolite. DOPAL is reduced by cytosolic aldehyde reductase and form the alcohol product or DOPET. However, DOPAL is primarily metabolized by mitochondrial aldehyde dehydrogenase 2 to form the acid product, DOPAC. Environmental agents such as benomyl inhibit ALDH and prevent DOPAL to DOPAC conversion, causing an accumulation of DOPAL levels.

Another key enzyme in metabolizing pesticide xenobiotics is paraoxonase, the product of the PON1 gene, which metabolizes many organophosphates (OPs)[164]. PON1

variants or poor metabolizers of OPs exposed to chlorpyrifos (CPF) and chlorpyrifos-oxon (CPO) compounds at the workplace were found to be at an increased risk for PD[38; 165]. Additionally, a California central valley study revealed that PON1 gene variants who used CPF-containing personal household pesticides were also at an increased risk of PD[166]. The pesticide, CPF belongs to the organophosphate class of pesticides, and is metabolized to CPO by cytochrome P450[42]. OPs are one of the most highly used class of pesticides and are known to primarily target the central nervous system,[167] as Parkinsonian brains contain significantly elevated levels of organophosphates compared to healthy brains[168]. The toxicity of CPF and CPO has been mainly attributed to acetylcholinesterase (AChE) inhibition[141], however, the correlation between AChE inhibition by OP's and PD pathology is unclear. There is growing evidence that alternative non-cholinergic toxic mechanisms are associated with OP exposure: OP treatment caused significant toxicity in AChE knockout -/- mice[169], and clinical symptoms after exposure include impaired attention and memory[170]. The parent compound, CPF inhibits AChE activity, however, the "bioactivated" CPO metabolite is nearly three order of magnitude more potent an AChE inhibitor than CPF[171]. However, it is interesting that CPO is only slightly more toxic than CPF in primary culture neurons[172], and therefore it is implied that alternate mechanisms of toxicity are likely occuring. In addition, CPO was 1000-times more potent than CPF in reducing total axonal length per neuron in differentiating cells of primary sympathetic neurons from embryonic rat superior cervical ganglia, which has implications for developmental neurotoxicity[173]. Both of these compounds have been banned for private use, however, they are still widely used commercially. Clinical studies have shown that OP exposure caused distal degeneration of the motor neurons[39] and is suggested to cause dysregulation of dopaminergic transmission[40]. In addition, exposed acute doses of CPF caused severe transient parkinsonism in patients, and produced clinical motor symptoms highly similar to PD such as bradykinesia and muscle rigidity[43].



Scheme 5.3 Putative ALDH inhibitors include methyl N-[1-(butylcarbamoyl)benzimidazol-2-yl]carbamate or benomyl and two organophosphates: chlorpyrifos and chlorpyrifos-oxon.

Although OPs are the class of pesticides most associated with PD, there are few studies measuring the effects of CPF and CPO on dopaminergic neurons[44]. Benomyl treatment in mesencephalic neurons caused a decrease in ALDH activity and moderate toxicity to these neurons. However, we are interested on measuring its toxicity effect in dopamine neurons and accurate measurement of DOPAL and DOPAC levels *in vitro.* These data support the findings that the fungicide benomyl and CPF and CPO modulate ALDH activity by accumulating DOPAL levels, and causes toxicity to dopaminergic neurons.

5.2 Experimental Procedures

Materials

DOPAL was biosynthesized via a rat liver MAO procedure as previously established[51], and DOPAL stock concentrations were determined using an ALDH assay with nicotinamide adenine dinucleotide (NAD^+) and high-performance liquid chromatography (HPLC)[52]. (12-O)tetradecanoylphorbol-13-acetate (TPA) was purchased from Santa Cruz Biotechnology, chlorpyrifos (CPF) and clorpyrifos-oxon (CPO)

pesticides were purchased from Chem Service Inc. (West Chester, PA). DA and 3,4-dihydroxyphenylacetic acid (DOPAC), standards are commercially available (Sigma), all trans-retinoic acid, and methyl 1(-butylcarbamoyl)-2-benzimidazolecarbamate (benomyl), 2'7'-dihydrofluoroscein diacetate (H_2DCFDA), and MTT reagent were all purchased from Sigma Aldrich. CPF and CPO were purchased from Chem Service (West Chester, PA). Opti-MEM, RPMI 1640 media, heat-inactivated fetal bovine serum, horse serum, Dulbecco's phosphate buffered saline (DPBS), Hanks balanced salt solution (HBSS) containing calcium, magnesium and no phenol red buffer were purchased from Life Technologies (Carlsbad, CA). All other cell reagents and media were purchased from Life Technologies as well. Aldehyde dehydrogenase1 and 2 (ALDH) enzymes were a generous gift from Thomas Hurley (Indiana University). Rat pheochromocytoma PC6-3 cells and human derived SH-SY5Y neuroblastoma cells were a gift from Stephan Strack (University of Iowa).

HPLC Analysis of DA Metabolites

HPLC analysis of cellular DA metabolites as performed by sampling extracellular volumes from 6-well plates (5×10^5) density, and combining this volume with 5% (v/v) perchloric acid. The samples were briefly vortexed, centrifuged at 9800 RPM for 3m, and the supernatant was transferred and injected (40-µL) directly onto HPLC. The injection sample was separated using a Phenomenex Luna C18 column. The mobile phase contained 97% 0.1% trifluoroacetic acid (v/v) in HPLC-grade water (A) and 3% acetonitrile (B). The gradient was as follows: 0-12 min: 3% B, 12-13 min: 15% B, 15-20 min: 15%B, 20-35 min: 3% B. The flow rate was 50 µL/min and retention times for the metabolites were: 4.5 (DA), 7.5 (DOPAL), 8.9 (DOPET) and 12.5 min (DOPAC). Calibration curves were generated using standards for DA, DOPAL and DOPAC. An Agilent 1200 Series Capillary HPLC system equipped with a photodiode array detector

measures absorbance of catechols at 280 nm. Preparation of the extracellular aliquots was performed as previously described[138].

SH-SY5Y Neuroblastoma Cell Culture

SH-SY5Y human-derived neuroblastoma cells were cultured in Opti-MEM supplemented with heat-inactivated fetal bovine serum (10%), penicillin (10 IU/mL), streptomycin (10mg/mL), 2mM glutamine, 1mM sodium pyruvate and 0.1 mM nonessential amino acids. Cells were grown in a 100 mm^2 tissue culture dish at 37°C in a humid atmosphere containing 5% CO_2. SH-SY5Y cells that were selected for differentiation were seeded into 24-well plates at (1×10^5) density, and undifferentiated cells were seeded at (5×10^4), and grown in normal cell media for 3 days. Undifferentiated cells were grown an extra 6 days with media change every 3 days. On the 4th day, differentiated cells were treated with 20 μM retinoic acid (RA) for 3 days, media was changed and treated with 80 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for an additional 3 days. On the 10th day, media was changed for undifferentiated and differentiated cells, and 24 h after, the cells are treated for test compounds[114]. Differentiation with RA a lone produced a cholinergic-type cell phenotype and TPA alone produced an adrenaline phenotype[174]. However, the combination of RA and TPA differentiation protocol produced a dopaminergic phenotype[114].

PC6-3 Cell Culture

PC6-3 cells were cultured in RPMI 1640 medium supplemented with heat inactivated 10% horse serum, 5% bovine serum, penicillin (10 IU/mL) and streptomycin (10mg/mL). Briefly, cells were seeded in 24-well plates at (5×10^4) for 24 h, and differentiated with 50ng/mL nerve growth factor (NGF) for 3d prior to cytotoxicity experiments.

Cell Treatment with Compounds

SH-SY5Y and PC6-3 cells were incubated with compounds in HBSS buffer (calcium, magnesium, no phenol red) and supplemented with 5.5 mM glucose (Fisher Scientific). SH-SY5Y neurons were treated with benomyl and DOPAL for 24 h, at the end of the incubation, cells were thoroughly washed with DPBS and shortly assayed for toxicity with MTT reagent. SH-SY5Y neurons treated with DA and benomyl, were pre-treated with DA for 1 h, washed, and benomyl added to the cells for 24 h. All benomyl doses were at 2 μM, as this was non-toxic to the cells. PC63 cells were treated with CPF or CPO and assayed for toxicity after 24 h.

Cytotoxicity Assay with MTT

Cell viability was assessed by MTT reduction assay as previously described[175]. Viable cells with active mitochondria reduce the colorless tetrazolium salt MTT, to produce dark blue water-insoluble formazan crystals. MTT was dissolved at 5 mg/ml in DBPS, for a final concentration of 0.5 mg/ml per well. MTT solution is added to cells and incubated for 1h at 37 C. At the end of the incubation period, the aqueous solution was aspirated and DMSO was added to each well to solubilize the formazan crystals. An aliquot (100 μL) was removed from each well to a 96-well plate and optical density was measured at 570 nm, and subtracted from the reference wavelength at 650 nm using a Molecular Devices Spectra-Max plate reader. The absorbance of treated wells was expressed as a percentage of the untreated wells.

Cytotoxicity Assay with Trypan Blue

Viable cells were counted as described before[176]. Cells were briefly lifted with 0.25% trypsin (Gibco) for 3 min. The cells were resuspended in DPBS buffer. An aliquot of the suspended cells was mixed with an equal volume of 0.4% trypan blue (Invitrogen) for microscopic cell count. All cells were enumerated (viable+dead), the viable cells had intact cell membranes and cells were clearly visible, dead cells had compromised cell

membranes, and therefore the trypan blue dye infiltrated the cells, and produced a darker cell body. Cell viability in each sample was calculated as the percentage of unstained cells in the culture buffer.

Reactive Oxygen Species Detection in Cells by 2'7'-dihydrofluoroscein

Intracellular ROS were detected by cellular treatment with 2'7'-dihydrofluoroscein or H_2DCFDA as outlined in a previous study[177]. H_2DCFDA is a fluorogenic dye that non-selectively measured a number of reactive oxygen species (ROS), including hydroxyl, peroxyl and superoxide anion[178]. It is a cell-permeable probe that once enters the cell, becomes hydrolyzed to a carboxylate anion, and is then retained within the cell. PC6-3 cells were briefly washed with DPBS and loaded with 25 µM H_2DCFDA for 45 mins. Additionally, certain cells were pre-incubated with 50 µM DA for 30 mins prior to addition of H_2DCFDA, and this served as a control test for the effect of DA on ROS. After the incubation with H_2DCFDA, cells were washed once with DPBS, and treated with various concentrations of CPO and CPF. At various time points (1-24 h), the fluorescence was recorded at 485 nm excitation and 535 nm emission wavelengths.

ALDH Activity Assay

CPF and CPO were incubated with human recombinant ALDH (1 µM) and 2 mM NAD^+ for 10-120 mins in a standard 30 mM N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer at pH 7.5. Reaction was initiated by addition of 1 mM propionaldehyde and activity is determined by NADH formation, measured spectrophotometrically at 340 nm for 3 mins.

AChE Activity Assay

For cell assays, SH-SY5Y cells were utilized for their robust AChE activity and expression[179]. SH-SY5Y cells were seeded at 37,500 cells/cm^2 for 3 days, and

differentiated with 20 μM RA for 3 days, followed by 80 nM treatment for 3 days. Cells were washed and treated with 1 and 5 μM CPF and CPO in 5.6 mM glucose-supplemented HBSS buffer. At 4h, the buffer was aspirated and cells were collected via trypsin dissociation, briefly sonicated, and centrifuged at 3200 RPM. The supernatant was collected and 5 μg of protein was transferred to a 96-well plate, containing 50 mM sodium phosphate buffer pH 7.4[180]. The AChE reaction was initiated by addition of 40-μL of the following final concentrations: 0.32 mM DNTB and 1 mM of acetylthiocholine iodide as substrate for 60m. This assay is based on the change in absorbance at 410 nm and is fully described by Ellman et al[181]. AChE hydrolyzes acetylthiocholine and produces thiocholine and acetate. The thiocholine product reduces the DTNB substrate, liberating nitrobenzoate, which absorbs at 410 nm. For cell-free assays, 5 μg SH-SY5Y cell lysate was incubated with 1 and 5 μM CPF and CPO for 15 mins, and reaction was initiated with addition of 40-μL of 0.32 mM DNTB and 1 mM of acetylthiocholine iodide. An additional control of activity, without cells was also measured to correct for background control.

5.3 Results

DA and Benomyl Combined Treatment is Preferentially Toxic to Differentiated SH-SY5Y Neuroblastoma

The toxic effect of benomyl was tested in undifferentiated, adrenergic (TPA/TPA) and dopaminergic (RA/TPA) SH-SY5Y neuroblastoma cells. Cells were treated similarly as outlined in a previous protocol,[66] the cells were briefly treated with 2 μM benomyl and 10, 100 or 1000 μM DA for 2 h, buffer is aspirated, and fresh buffer was added for 24 h (Figure 5.1A). The concentration of 2 μM for benomyl was chosen as it was not toxic at this concentration. As noted before, SH-SY5Y has low DA synthesis and DOPAL production. To circumvent this, DA was added to the cells to stimulate endogenous DOPAL production *in situ*[182] and test for DOPAL synergistic toxicity with benomyl, a

putative ALDH inhibitor. Using this protocol, toxicity was negligent for undifferentiated and adrenergic SH-SY5Y cell models, however, there was a DA dose-dependent effect on toxicity in the dopaminergic SH-SY5Y assay. This protocol utilized a short exposure time of 2 h, and any long-term toxic effects were measured 24 h later, it also allowed usage of a high DA concentration (1 mM) without dark staining or DA auto-oxidation, and therefore, producing false positive MTT values. Results from Figure 5.2B utilized a more common protocol, in which benomyl and DA were incubated for 24 h, and neither benomyl or 100 µM DA a lone produced toxicity, however, the combination of both produced a synergistic toxic effect. To test the specific toxic effect of DOPAL, an MAO inhibitor, pargyline (Sigma Alrich) was co-treated with benomyl and DA (Figure 5.1B), and cell viability was restored compared to benomyl and DA only. The toxic effects of benomyl and DA also appeared to have a time-dependence, and produced a delayed-toxic effect as suggested by toxicity of other ALDH inhibitors and DA,[66] as the 4 h exposure did not produce toxicity in dopaminergic SH-SY5Y.

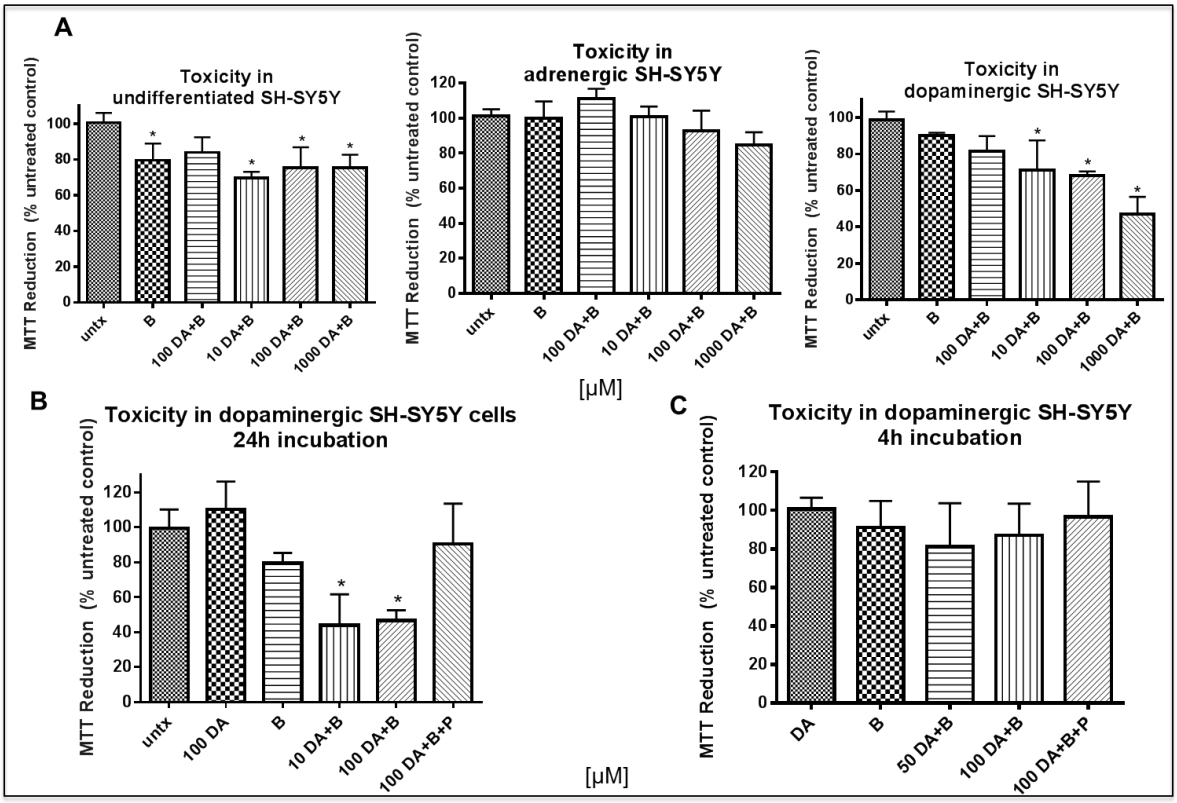


Figure 5.1 Benomyl and DA toxicity on SH-SY5Y neuroblastoma cells was dependent on the dopaminergic phenotype. All cells were treated with 2 μM benomyl and 10-1000 μM DA and 200 μM P or Pargyline (MAO inhibitor) A) for 2 h in HBSS buffer, buffer is aspirated and fresh buffer is added to cells for 24 h, MTT measurements were taken 24 h after in undifferentiated, TPA/TPA or adrenergic, and RA/TPA or dopaminergic SH-SY5Y cells. B) benomyl and DA, and P were incubated for 24h, and MTT taken 24 h after. C) benomyl and DA incubated briefly for 4h. For each experiment, n=3, significance is denoted by (*) $p \leq 0.05$, significance was analyzed via 1-way ANOVA.

Co-treatment of Benomyl and DA Changes SH-SY5Y Morphology

When SH-SY5Y cells were treated with DA and benomyl as outlined before in the toxicity experiments, images in Figure 5.2 show the significant changes that occurred to the cell morphology. At 4 h, untreated cells have long neurite extensions and display a healthy neuronal-like morphology. DA and benomyl alone start to show a loss of the neurite extensions, and more rounded cell bodies appear. However, the DA and benomyl co-treatment showed significant change to the cell size and shape; cells started to ebb and

appeared to shrink, which is characteristic of apoptosis formation. The drastic effect of the combined treatment shows the synergistic toxicity of DA or DOPAL levels and DOPAL inhibition by benomyl.

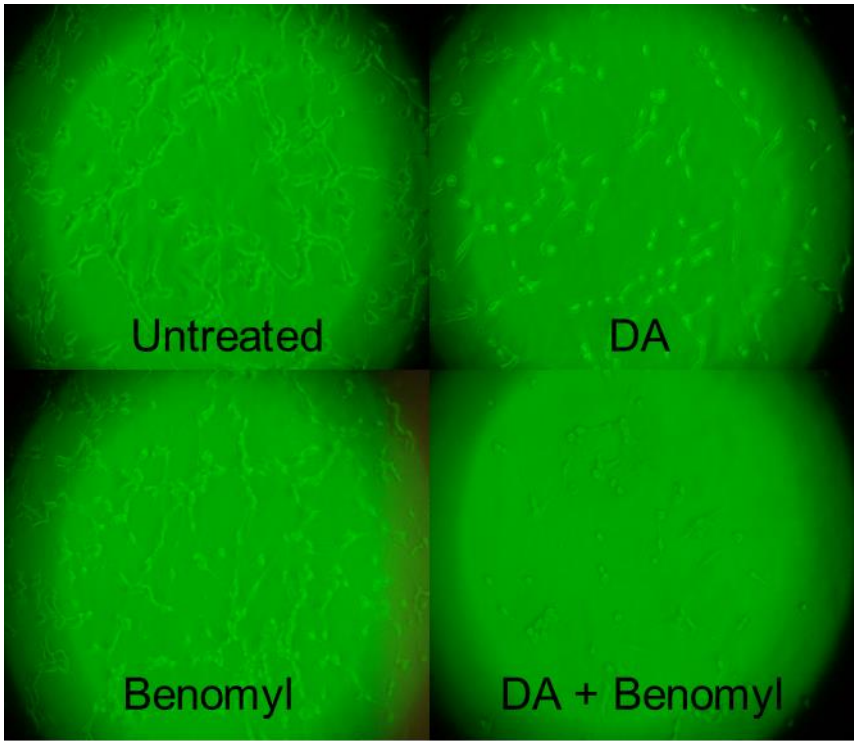


Figure 5.2 Images of SH-SY5Y cells after 4h incubation with 100 µM DA, 2 µM benomyl and 100 µM DA + 2 µM benomyl. The combined treatment of benomyl and DA causes significant changes to cell morphology shown by the shrinking of cell size and ebbing or rounding of the cells, a sign of apoptosis.

DOPAL and Benomyl Treatment is Toxic to SH-SY5Y Neuroblastoma Cells

The toxic effect of DOPAL and benomyl on undifferentiated and differentiated/dopaminergic SH-SY5Y cells was tested for 24 h in HBSS buffer. Higher DOPAL concentrations stained the wells dark blue and interfered with MTT measurements. The effect of benomyl alone produced little to no toxicity (~90%) viable cells, but the combined treatment of benomyl and DOPAL caused toxicity in a DOPAL

dose dependent manner. However, DOPAL alone was also toxic to the cells at 100 µM toxicity was very similar for both undifferentiated and dopaminergic cells, and there was no heightened sensitivity to DOPAL in the dopaminergic cells. Interestingly, there was a lack of synergistic toxicity of co-treatment of benomyl and DOPAL, the toxicity appears to be an additive effect. Since there were issues with DOPAL auto-oxidation and staining of the wells, possibly producing a false positive MTT result, therefore showing a lack of toxicity, a secondary toxicity assay trypan blue exclusion assay was utilized to confirm the results. Measurement with trypan blue dye shown in Figure 5.2B shows a more dramatic toxicity difference between 100 µM DOPAL (~35% viability) versus 100 µM DOPAL and benomyl (15% viability). A positive control, 400 µM paraquat (PQ) is a known toxin of SH-SY5Y and dopamine neurons, however, 100 µM DOPAL was more toxic than at the given paraquat concentration.

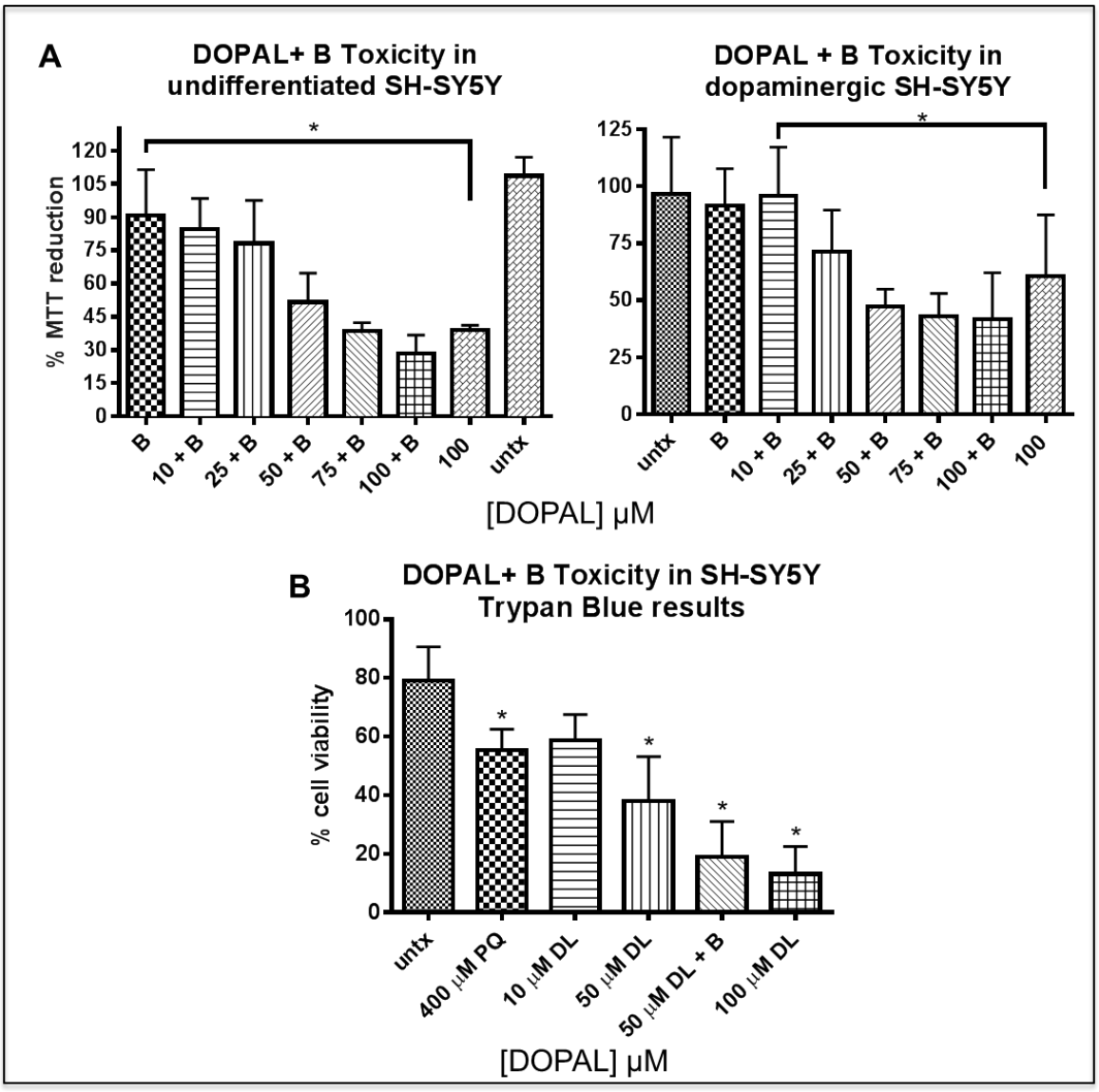


Figure 5.3 DOPAL and benomyl were toxic to SH-SY5Y. Benomyl (2 µM) and 10-100 µM DOPAL were co-incubated in A) undifferentiated and dopaminergic SH-SY5Y cells were assayed 24 h later by MTT assay and B) measured by trypan blue exclusion assay. Paraquat (PQ) was a positive control for toxicity at 400 µM. For each experiment, n=3, significance is denoted by (*) $p \leq 0.05$, significance was analyzed via 1-way ANOVA**.**

Benomyl Inhibits ALDH and Accumulates DOPAL

To study the effect of benomyl on DA metabolite levels in SH-SY5Y cells, the cells were differentiated with RA and TPA as listed before and incubated with 10-100

μM DOPAL and 2 μM benomyl for 4 h, and extracellular aliquots were taken from the incubation buffer. HPLC analysis of aliquots demonstrate that benomyl crosses the cell membrane and inhibits ALDH, as shown by the increase in DOPAL (5.4A) with 50 μM DOPAL+B versus 50 μM DOPAL alone. Both DOPET and DOPAC levels were slightly lowered with benomyl treatment. Of interest are the DOPAC levels, as a decrease in DOPAC formation reflects inhibition of turnover of DOPAL to DOPAC by ALDH. The measurement of ALDH enzyme inhibition is the ratio of the product metabolite (ALDH) to previous metabolites (DOPET and DOPAL). Panel D shows that the ratio is lowered with benomyl, showing adequate ALDH inhibition by benomyl. When SH-SY5Y neurons are treated with DA and benomyl, the concentration of DOPAL increases over time, and panel E shows the steady increase of DOPAL with and without benomyl. However, the DOPAL levels are higher in the presence of benomyl.

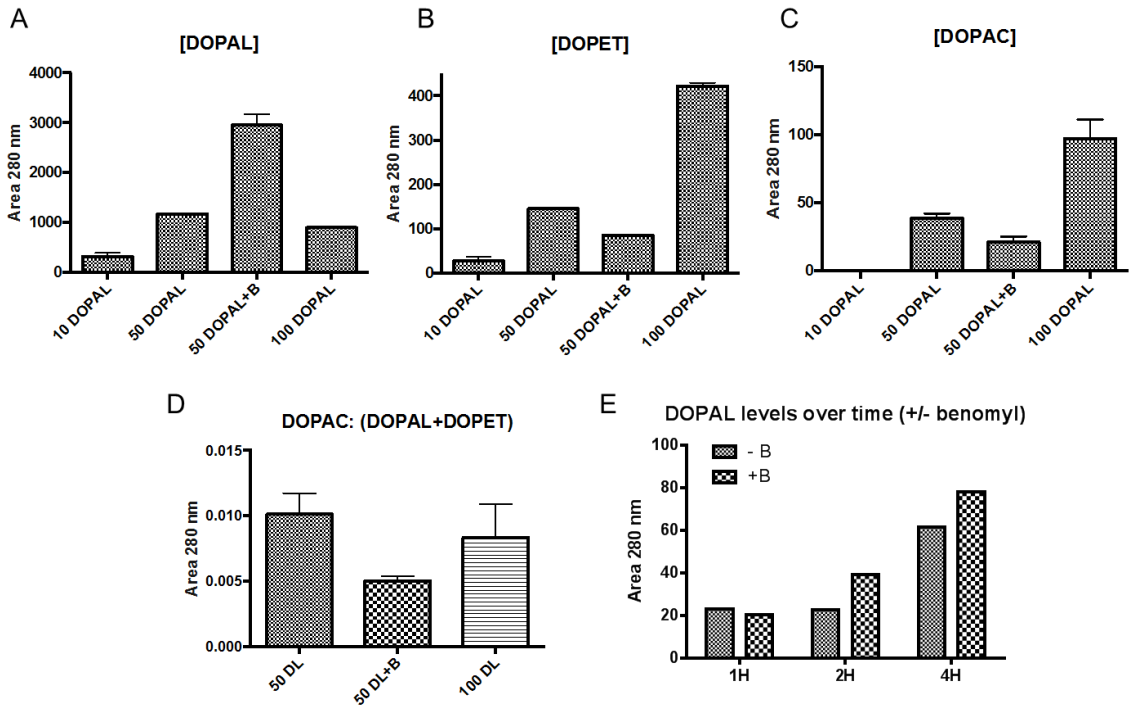


Figure 5.4 Benomyl causes ALDH inhibition and accumulates DOPAL over time. Extracellular aliquots were removed from incubation buffer and analyzed by HPLC-DA. SH-SY5Y neurons were treated with 2 µM benomyl and 10-100 µM DOPAL. UV detection of catechols at 280 nm of A) DOPAL B) DOPET C) DOPAC and the ratio of the metabolites D) DOPAC/(DOPAL+DOPET) displaying ALDH inhibition. E) SH-SY5Y neurons were treated with 2 µM benomyl and 100 µM DA for 4 h, and aliquots were removed at 1, 2, and 4 h. DOPAL levels increased over time, however more so when benomyl was present.

CPF and CPO Organophosphate Compounds Modulate DA Metabolites

To determine the effect of CPF and CPO on dopamine metabolism, PC6-3 cells were utilized given their robust DA metabolism and sensitivity against organophosphates[177]. All cells were simultaneously treated with CPF or CPO (1- 100 µM) and 50 µM DA to stimulate metabolism to DA metabolites. Extracellular aliquots were removed from treated wells and analyzed by HPLC and UV detection of DOPAL, DOPAC and DOPET. At 4h, DOPAL levels were elevated in a dose-dependent manner, and DOPAC was greatly decreased, and DOPET levels did not change much at the

different doses (data not shown). However, CPO was more significant than CPF in elevating DOPAL and decreasing DOPAC levels. Since we are proposing that these compounds may be ALDH inhibitors, the ratio of ALDH product, DOPAC was graphed as a ratio of reactant or DOPAL and the alternative product of DOPAL to DOPET by aldose reductase (ALR). Figure 5.5C shows that the ratio decreased as a function of CPF/CPO concentration, however, CPO produced a more marked decrease in the DOPAC/(DOPAL+DOPET) ratio. Since CPO was more effective as a putative ALDH inhibitor, CPO was subsequently used for cell assays.

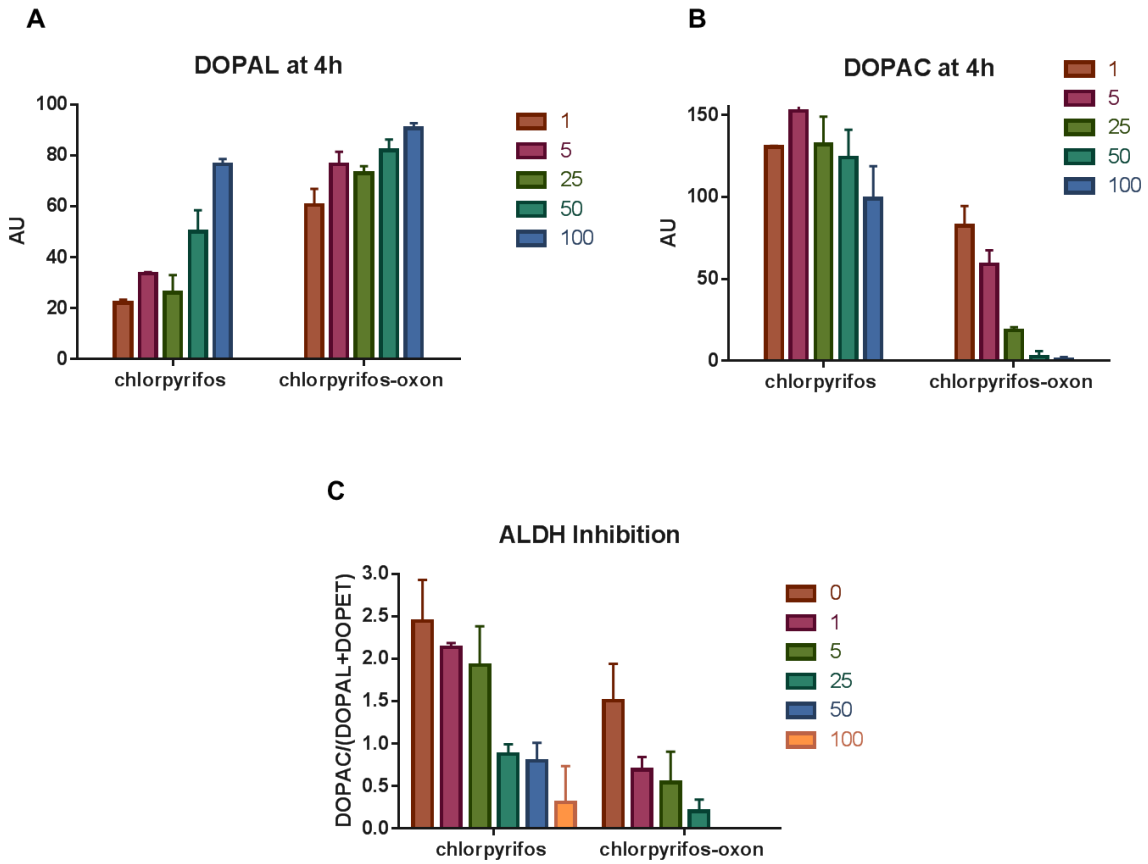


Figure 5.5 PC6-3 treatment of 50 µM DA and CPF or CPO in HBSS buffer at 4 h modulated DA metabolite levels in a dose-dependent manner. However, CPO induced more significant changes in DA metabolites. Extracellular aliquots were removed at 4 h. All concentrations listed are in µM. A) DOPAL increased, B) DOPAC decreased, and the C) ratio of DOPAC/(DOPAL+DOPET) was a measurement of ALDH inhibition greatly decreased.

CPO is Toxic to PC6-3 Cells

To assess the toxicity of CPO in PC6-3 cells, viability was monitored at 4 and 24 h after CPO treatment in HBSS buffer. Immediately prior to viability testing with MTT assay, aliquots were removed from each well, and DOPAL and DOPAC levels were assayed by HPLC. CPO caused minor toxicity at 4 h, even at high concentration of 100 µM, as there were still approximately ~80% viable cells. However, aliquots removed had high extracellular levels of DOPAL and DOPAL increased in a CPO dose-dependent

manner as revealed before in Figure 5.5, and DOPAC decreased similarly. Following a similar trend as at 4 h, DOPAL increased and DOPAC metabolites decreased at 24 h. However, the area under the curve measurements for DOPAL were very high at 24 h, corresponding to a DOPAL concentration of 23-100 µM versus 13-26 µM range at 4 h. Interestingly, CPO was extremely toxic to PC6-3 cells at 24 h, as at only 5 µM, there were only ~65% viable cells, compared to ~100% at 4 h. The cell viability at 24 h also reached less than 20% at only 10 µM CPO.

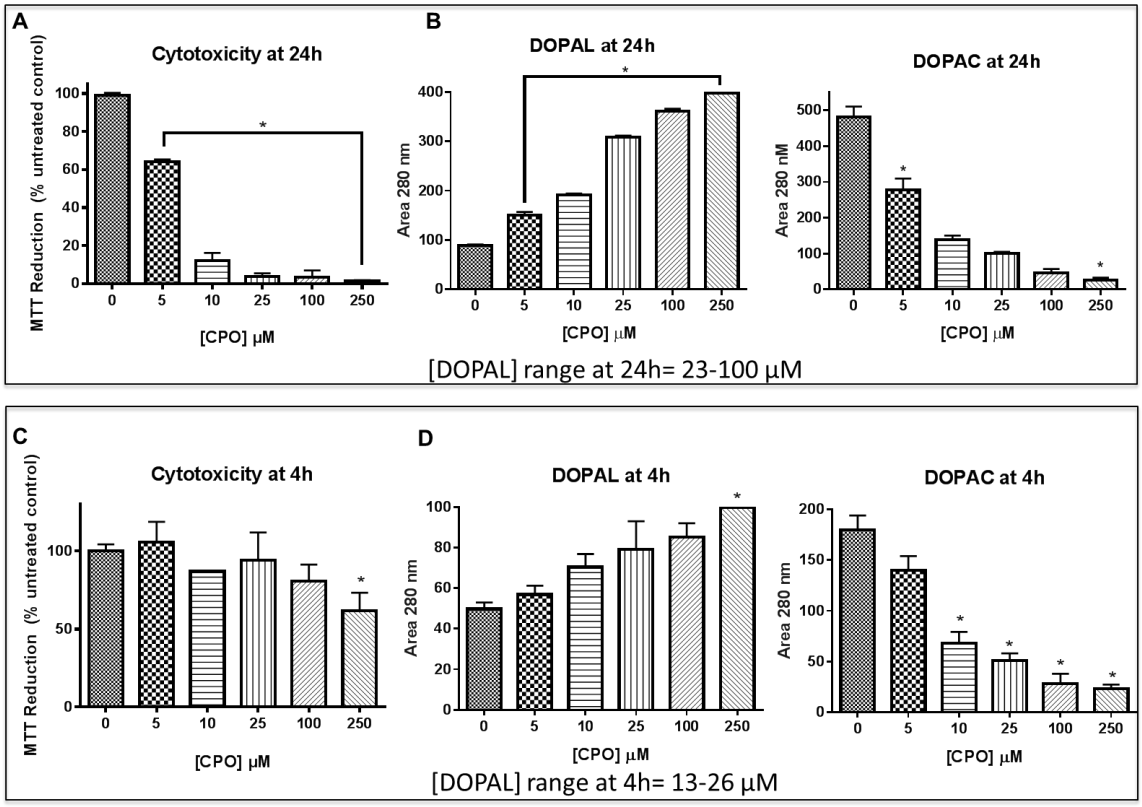


Figure 5.6 PC6-3 cells were sensitive to CPO and is directly related to pathological increases in DOPAL. All cells were treated with 50 µM DA, and 1-250 µM CPO. A) CPO is toxic at 24 h. B) Extracellular aliquots were taken at 24 h. C) CPO was non-toxic at >100 µM at 4 h. D) Extracellular DOPAL levels increased and DOPAC decreased in a similar trend seen as in 24 h. For each experiment, n=3, significance is denoted by (*) $p \leq 0.05$, significance was analyzed via 1-way ANOVA.

CPO and CPF Increases ROS in PC6-3 Cells

The fluorogenic dye, 2'7'-dihydrofluorescein or H_2DCFDA was used to measure intracellular ROS formation caused by CPO (5, 25, 100 µM) and CPF (5, 100 µM) treatment in PC6-3 cells. At 1-2 h, the concentration of ROS rose with low CPO and CPF showed no obvious increase in ROS production with CPO/CPF dose. Interestingly, the ROS greatly increased at 4 h and 24 h, and ROS production displayed a dose-dependent trend with increasing CPO and CPF concentration. However, at 24 h, the ROS signal was the greatest with 100 µM CPF treatment.

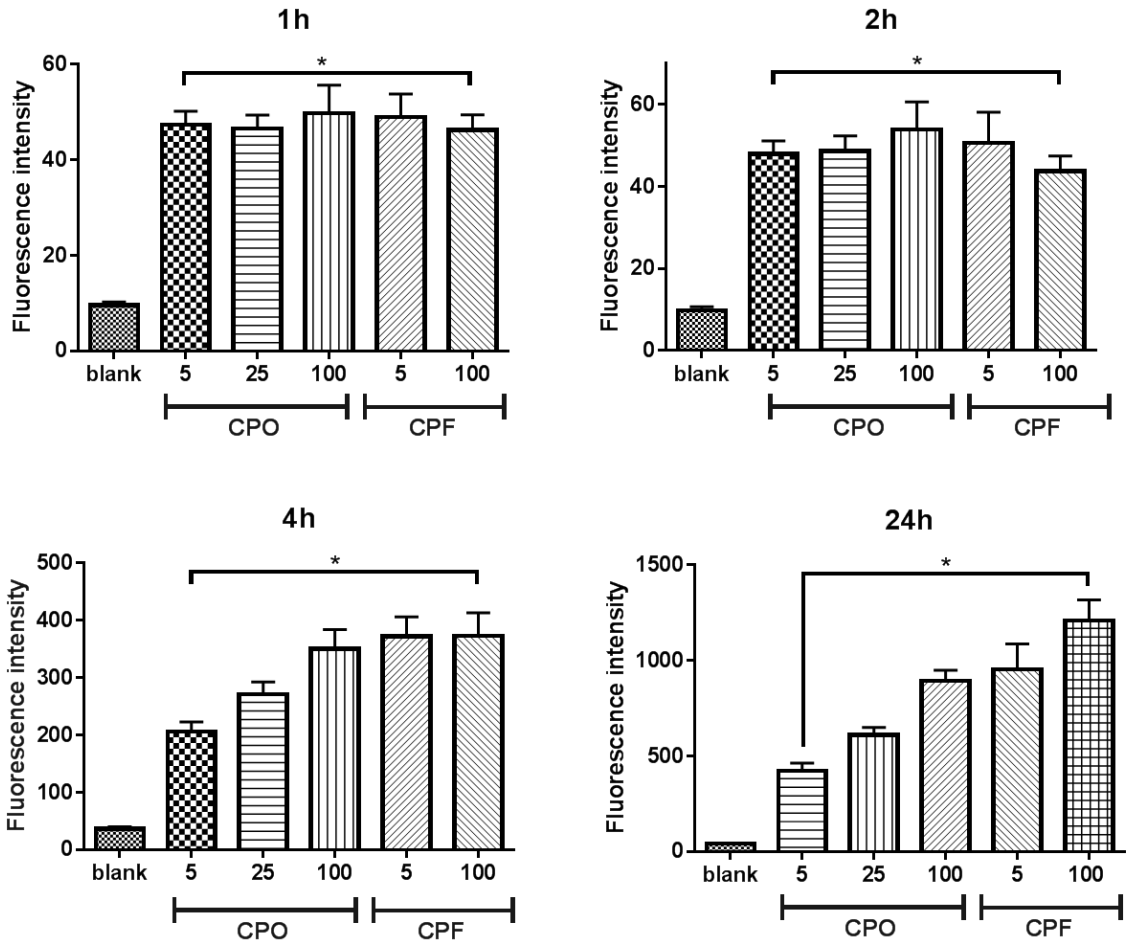


Figure 5.7 CPO and CPF generated ROS in PC6-3 cells. PC6-3 cells were pre-loaded with 25 µM H_2DCFDA dye and treated with 5-100 µM CPO and 5, 100 µM CPF. Fluorescence measurements were taken at 1-24 h after treatment with CPF or CPO. Significance was analyzed via 1-way ANOVA, n=3, , significance is denoted by (*) $p \leq 0.05$.

In vitro ALDH Inhibition with CPF and CPO

The inhibitory effects of CPF and CPO in *in vitro* ALDH enzyme assays were done in collaboration with the Thomas Hurley lab (Indiana University). For ALDH2 assays in BES buffer, only 100 µM CPF inhibited ALDH2 activity in a time dependent manner. 10 µM CPF and 10, 100 µM CPO had minor effects on ALDH activity. However, ALDH1 was inhibited by both CPF and CPO (100 µM) in a time dependent

manner. Rather high concentrations were needed to inhibit ALDH1 and ALDH2 activity. Once the assays were repeated in phosphate containing buffer (DPBS or sodium phosphate buffer), neither CPF or CPO inhibited either enzyme. The strong buffer effect cannot be explained, however it may be due to a competitive binding effect with the OP compounds.

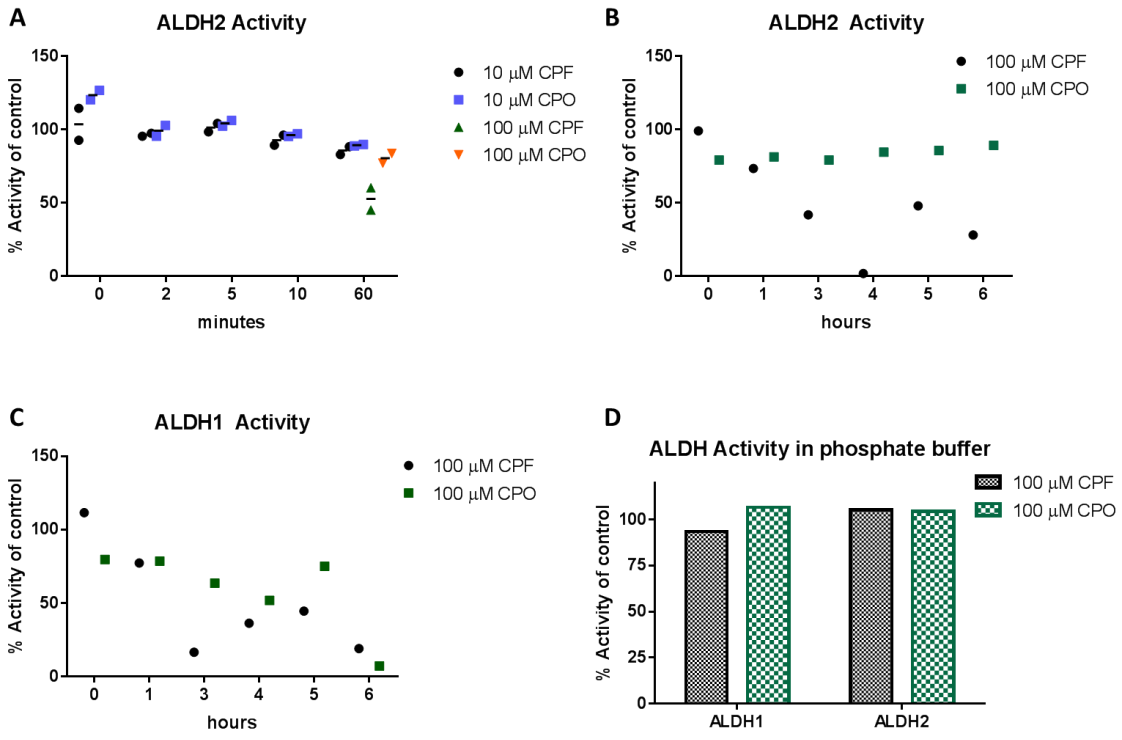


Figure 5.8 CPO and CPF incubation with purified ALDH. A) At 60 min, only 100 µM CPF inhibited ALDH2 activity. B) CPF inhibited ALDH in a time dependent manner. C) Both CPF and CPO inhibited ALDH1 activity in a time dependent manner. A-C assays were incubated in BES buffer. D) In phosphate buffer, CPF and CPO did not inhibit ALDH1 or ALDH2.

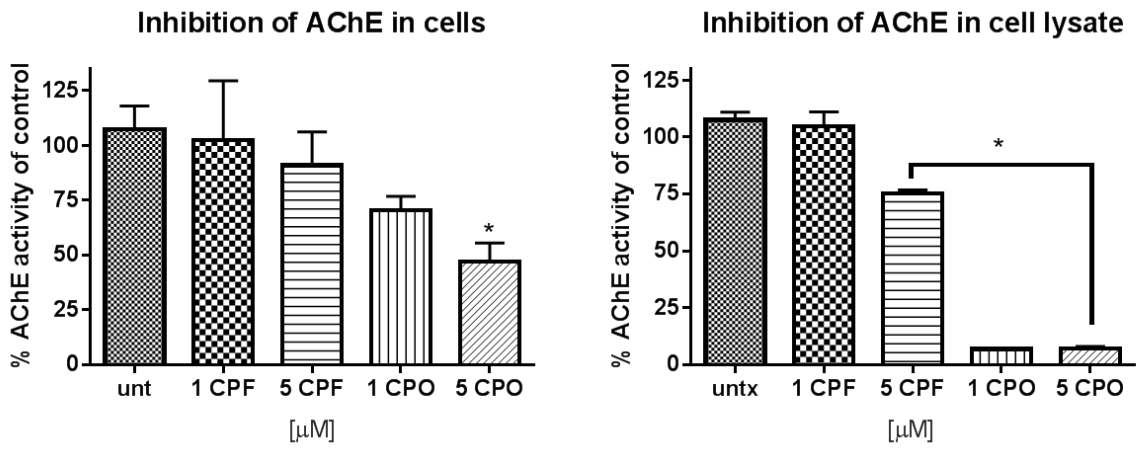


Figure 5.9 AChE activity was measured with CPF and CPO in SH-SY5Y A) cells for 4h, B) cell lysate for 15 min. Activity was determined by liberation of the nitrothiobenzoate at 410 nm.

Low CPF Dose Did Not Inhibit AChE in SH-SY5Y Cells

There is growing concern for long term CNS damage due to low level or sub-clinical OP exposures. When SH-SY5Y cells were treated with CPF and CPO for 4h, the AChE activity was inhibited with CPO, but not with CPF at the designated doses (1 and 5 µM). However, when the AChE activity was measured in a cell free system utilizing cell lysate, 5 µM was sufficient to cause 75% activity, or ~ 25% reduction in activity. The 1 µM CPF did not produce a significant inhibition of AChE. Following the same trend as with the CPO-treated cells, CPO inhibited AChE at both 1 and 5 µM. However, CPO inhibited AChE in lysate to a greater extent than in cells, likely due to the more direct access of enzyme.

5.4 Discussion

The *in vitro* cell models for this work were carefully chosen as an accurate model for dopaminergic neurons. Finding the correct cell model for PD-related experiments has been a great challenge in the neurotoxicology field. The neuroblastoma SH-SY5Y is commonly used as a model to test parkinsonian neurotoxicants[183-186]. Utilization of SH-

SY5Y neuroblastoma cells has major advantages: they are human-derived and express many of the human-specific protein isoforms, they express DA-specific proteins such as dopamine transporter (DAT) vesicular monoamine transporter (VMAT2) and monoamine oxidase (MAO). However, the major disadvantage is that these cells have low basal DA synthesis enzymes, including L-DOPA decarboxylase, the enzyme precursor of DA, yielding low concentrations of DA and metabolites. Undifferentiated SH-SY5Y cells resemble immature chatecholaminergic neurons and are also prone to rapid proliferation, which is uncharacteristic for neurons. To overcome this problem, SH-SY5Y can be differentiated into dopaminergic phenotype by treatment with retinoic acid (RA) and TPA[114], and this differentiation protocol elevates DA synthetic enzymes and metabolites[187]and significantly slows down proliferation[188]. In addition, differentiated cells are more sensitive to the PD-associated neurotoxin, MPTP than are undifferentiated cells. Similarly, since benomyl is a PD-associated fungicide, its toxicity and metabolism effects were investigated here utilizing the RA/TPA differentiated or dopaminergic phenotype SH-SY5Y cells.

The heightened vulnerability of dopamine neurons and cell models such as SH-SY5Y to ALDH inhibitors and DOPAL levels had been previously suggested by Legros et al.[66] Similar to the protocol outlined in this work, dopamine was exogenously added to SH-SY5Y cells to stimulate intracellular DOPAL synthesis and treated with disulfiram, a specific ALDH inhibitor. It was found that treatment of disulfiram and DA produced a time and concentration dependent loss of SH-SY5Y viability, albeit exhibiting minor toxicity at 1 h, but significant toxicity when cells were treated for 1h, and then fresh media was added and the toxicity measurement was taken 24 h later. Disulfiram itself is non-toxic, and it is suggested that DOPAL is responsible for this delayed toxicity, as toxicity by disulfiram treatment was enhanced when MTT measurements were taken 24 h after in drug-free medium, versus immediately after cell treatment. This protocol was adapted by incubating with DA and benomyl for 2 h, removing the incubation buffer, and

adding fresh buffer to the cells to test for a delayed toxic effect. It was interesting that this treatment was non-toxic to undifferentiated and adrenergic cells, however, the combined treatment was toxic to the dopaminergic phenotype in a DA dose-dependent manner (Figure 5.1A). Another reason that this protocol was preferred was that it decreased excess DA in buffer and circumvented the technical issue of buffer staining with a dark blue color, due to catechol auto-oxidation, which produces a false positive MTT signal. Both DA and DOPAL auto-oxidize and produces this dark staining. Interestingly, the DA and benomyl-treated cells produced the darkest staining. The 24 h treatments of DA and benomyl produced variable results due to staining, however, washing alleviated this effect, and the greatest toxicity was measured with the 24 h incubation in differentiated (Figure 5.1B) but little to no toxicity was observed in undifferentiated cells. This supports the preferential toxicity of benomyl to dopaminergic neurons. However, the short incubation time of 4 h was non-toxic to both undifferentiated (not shown) and differentiated cells (Figure 5.1C). The proposed delayed toxic effect of DOPAL determined in these studies could be considered analogous to the steady degeneration of dopaminergic neurons observed in PD etiology.

Cellular morphology during DA and benomyl treatment was significantly altered, and showed neurite extension loss and shrinkage of cell walls. In contrast, the cell morphology was only slightly altered in DA and benomyl alone treatments, and the cells resembled healthy neuronal bodies. Therefore, elevation in DOPAL is likely to account for the change in cell morphology and apoptotic bodies shown in Figure 5.3 with co-treatment of DA and benomyl. A previous study showed that low levels of DOPAL (5 μM) induced cell ebbing and apoptotic bodies in PC6-3 cells as early as 1h, and cells deteriorated in a time and DOPAL dose-dependent manner[55]. The HPLC data also revealed that benomyl increased DOPAL levels over time in SH-SY5Y neurons.

Benomyl toxicity was previously described in mesencephalic neurons,[89] and the

toxicity was attributed to an increase in DOPAL levels, however, the authors did not directly measure DOPAL in the assays. Therefore, one goal of this work was to measure DOPAL after benomyl treatment, and HPLC data confirmed that benomyl inhibited ALDH in a cellular model, and that DOPAL increased as a function of time (Figure 5.4E). The results of this work supports the previous findings that toxic levels of DOPAL are responsible for benomyl-mediated toxicity in dopaminergic neurons[26; 69; 89]. It was also of interest to observe preferential toxicity in undifferentiated, adrenergic and dopaminergic neurons, and utilizing the same cell type confirmed the selectivity of the benomyl toxicity profile towards dopaminergic neurons. However, it was interesting that DOPAL and benomyl co-treatments were not preferentially toxic to dopaminergic neurons, and were equally toxic to undifferentiated cells. Both undifferentiated and differentiated cell types were subject to DOPAL-mediated death, especially at 100 µM DOPAL (Figure 5.3A).

The toxic profile may be explained by differences of cell entrance mechanisms of DA and DOPAL. DA mainly enters the cell via DAT, however, it is unknown how DOPAL crosses the cell membrane. Differentiated SH-SY5Y cells have higher levels of TH and DAT, and increased DAT levels could lead to an increase in DA entrance into the cell, which may explain for the DA and benomyl co-treatment preferential toxicity in dopaminergic neurons. However, measurement via trypan blue assay resulted in greater cell death with DOPAL and benomyl co-treatment, compared to DOPAL alone (Figure 5.3B). This finding suggests that the trypan blue assay is more sensitive in determining toxicity for this specific treatment involving oxidative compounds for long incubation times.

The second type of cells studied was the PC6-3 cell line, which is a rat pheochromocytoma subline of PC-12 cells and is considered to be an accepted model of dopaminergic neurons for their ability to synthesize and release DA[189]. The greatest

advantage of these cells is the robust DA metabolism and the high expression of enzymatically active tyrosine hydroxylase[190]. In addition, there is a vast literature base for PC-12 cells, which allows for a well characterized cell model and convenient comparison of results with other groups utilizing PC-12 cells.

The OP compounds: CPF and CPO are potent inhibitors of various esterases, and since ALDH has an alternative esterase activity[191], we presumed that these OP compounds may also have an inhibitory effect on ALDH esterase activity. Currently, there are no other studies showing the effect of OP compounds on ALDH activity, and only one study showing CPF effect on DA metabolism pathways. Karen et al group[192] chronically dosed mice with CPF at 200 mg/kg and measured a decrease in DA uptake by mouse dissected ex vivo striatal synaptosomes. Treatment with CPF decreased synaptosomal DA uptake, and the group also observed a slight increase of DOPAC levels, which contrasts to the reduced DOPAC levels observed in this work with the PC6-3 cells. However, the decrease in DOPAC, agrees with studies showing that even after 69% of dopaminergic neurons are killed naturally or via toxins in aging mice, it is accompanied by a 103% increase in DA synthesis as a compensatory action by existing DA neurons[193; 194]. It is well known that DA synthesis and turnover increases in aged neurons, however, it is less straight forward to recognize that neuronal loss also causes an increase in the DOPAC:DA ratio in the surviving neurons, and it linearly increases with neuronal loss within the substantia nigra[194]. Therefore, the insult by CPF presumably kills neurons, causing an initial systemic increase of DA, which eventually leads to an increase in DOPAC. However, the Karen group did not measure the intermediate metabolites; DOPAL and DOPET. Therefore, a determination of the relative DOPAL to DOPAC ratios would be a more accurate measure of CPF effects on DA metabolism in systemic or *in vivo* models. It is also interesting that in this model, CPF only affected the DA system, and did not affect other neurotransmitter systems including serotoninergic or glutamatergic, highlighting the selectivity for the DA neurotransmitter system.

Furthermore, CPF (100 mg/kg) treatment caused a significant impaired movement in mice. The movement deficit phenotype, along with changes in striatal DA uptake imply specific effects of CPF on the nigrostriatal pathway and may have implications for PD.

The cellular change in DOPAL concentration was measured at various CPF and CPO doses, and similar to benomyl, both OPs caused a major increase of DOPAL that was strictly dose dependent. However, CPO produced a more obvious trend in increasing DOPAL with increasing dose and incubation time, and subsequent metabolism cell studies used CPO. Inhibition of ALDH was a likely mechanism of CPO since DOPAL increased, and a concomitant decrease in DOPAC was also evident, in a dose and time dependent manner. However, ALDH inhibition often increased DOPET levels as well, since there is more DOPAL to be converted by aldose reductase to form DOPET. However, in these studies, although DOPET was greater in CPO-treated versus untreated cells, DOPET levels were largely unaffected as a function of CPO dose. The presumed ALDH inhibition by CPO (Figure 5.6) and CPF did not cause toxicity at 4 h, but toxicity was observed on continuous exposure at 24 h. The total DOPAL produced at 24 h was far greater than DOPAL produced at 4 h, and concentrations above 6 µM DOPAL are considered pathological or toxic in human brains.

To confirm that CPF and CPO were causing direct ALDH inhibition, purified ALDH2 and ALDH1A1 were incubated at various drug concentrations. The results showed a time-dependent inhibition of ALDH1A1 to a greater extent than with ALDH2 at higher doses of CPF, but not with CPO. These results were conflicting and did not agree well with cell studies showing greater inhibition of ALDH with CPO than CPF. Additionally, the assay results were highly dependent on the buffer system used. For example, 100 µM CPF inhibited ALDH ~ 50% at 1 h, and ~80-90% inhibition was observed at 8 h in N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer, a routinely used buffer in ALDH enzyme assays. However, there was no inhibition with CPF in PBS or phosphate-containing buffers. Though this strong buffer effect could not

easily be explained, one possibility is that the phosphate is inhibiting the binding interaction of OPs to ALDH, which is feasible since the phosphate concentration would be ~3 orders of magnitude greater than the OPs in the enzyme assay. Additionally, when NAD cofactor was not co-incubated with ALDH and OP compounds, both CPF and CPO (100 µM) inhibited ALDH.

Based on the inconclusive *in vitro* ALDH assays, it was assumed that the OPs may be inhibiting ALDH indirectly via either inhibition of cofactor NAD^+ synthesis or formation of reactive oxygen species (ROS) and lipid peroxidation products, such as 4-hydroxy-2-nonenal and malondialdehyde, which both inhibit ALDH activity[56]. Although the increase in DOPAL and ALDH inhibition causes toxicity, as has been shown by others in various cell lines[66; 89; 195], the cause of toxicity for CPF and CPO may be multi-factorial. Formation of ROS is a likely mechanism for the observed CPO toxicity at 24 h. Lee et al. generated ROS-mediated apoptosis and measured mitochondrial dysfunction with CPF treatment in PC-12 cells[44]. They found that cell viability at 24 h decreased from 25-200 µM, but lower than >25 µM was well tolerated by the cells. In addition, 100 µM CPF was toxic after 6h, and caused ~60% cell death at 24 h. Similar with start time of toxicity, ROS production was measured to significantly increase ~2-fold at 100 µM CPF, therefore, toxicity was mainly attributed to ROS production. There was a dramatic increase in ROS with CPO and CPF from 1-24 h, however, low (5 µM) and high (100 µM) doses of each compound, produced similar amounts of ROS. It was not until 4 h, that there is a dose –dependent increase in ROS formed. It is also interesting that at all time points, CPF produced more ROS than at any dose with CPO. The fluorescent assay results confirmed that CPF and CPO caused an elevation in ROS, however to determine if ROS production was also causing ALDH inhibition, PC6-3 cells were pre-loaded with an antioxidant to determine if there was a change in DOPAL and DOPAC levels. Cells were pre-treated with 5 mM N-acetyl-cysteine (NAC), and also with a pro-oxidant, such as buthionine sulfoximine (BSO) for 24 h before CPO and CPF exposure. NAC is a cell

permeable antioxidant, which is converted to glutathione (GSH) within the cells, and BSO significantly decreases GSH levels by inhibiting γ-glutamylcysteine synthetase, an enzyme involved in GSH synthesis[16; 196].

Others have reported that CPF significantly alters the antioxidative defense mechanism by producing lipid peroxidation products in liver,[197] and rat neuronal cells[198]. CPF and CPO did not alter GSH levels but significantly increased oxidized glutathione (GSSG), and the OPs increased toxicity in a mouse model of glutathione deficiency[199]. The above reports make it clear that production of intracellular ROS is partially responsible for CPF toxicity.

There is growing concern for long term CNS damage due to low level or sub-clinical OP exposures. Acute OP poisoning is a well-established clinical feature, but low chronic exposure may lead to mechanisms affecting enzymes other than AChE[200]. The AChE assays proved that CPF is exerting toxicity via alternative routes and possibly, through other enzymes. At low CPF doses (1 and 5 µM), AChE was not inhibited in cells. However, 5 µM CPO did cause potent AChE inhibition (Figure 5.9). It was hypothesized that since CPF effectively lowered DOPAC and increased DOPAL, CPF modulates toxicity, DOPAC and DOPAL levels via ALDH. However, whether CPF and CPO inhibit ALDH by direct binding or inhibit ALDH via production of secondary species, (ie. Reactive oxygen species, lipid peroxidation products) is unknown.

Inhibition of ALDH is a relatively new link to PD pathology[69; 89], and discovering environmental ALDH inhibitors will be useful in determining those at risk for PD. In addition, small molecule activators for ALDH are a promising form of neuroprotection and alternative PD therapy. This work proposes that toxicity conferred by CPF and CPO is partially related to ALDH activity. Considering the epidemiological studies relating OP exposure and PD risk, these new data serves as a novel mechanistic link to impaired DA metabolites and PD risk.

CHAPTER 6

RESEARCH SUMMARY

Restatement of Hypothesis

Parkinson's disease (PD) is a progressive neurodegenerative disorder which is characterized by the production of toxic protein aggregate bodies (Lewy bodies) and the selective loss of dopaminergic neurons. Dopamine (DA) neurons only account for less than 1%. Of the total neuronal population of the brain, however, the loss of these neurons produces a profound effect on systemic function. Although the pathogenesis is currently unknown, there is evidence that the presence of DA and a reactive downstream metabolite within DA neurons is posed to play an important role in pathogenesis. 3,4-Dihydroxyphenylacetaldehyde (DOPAL) is the aldehyde metabolite of DA, and is orders of magnitude more toxic than DA itself [59; 61; 88]. Much of the toxicity of DOPAL is attributed to the electrophilic nature of DOPAL, leading to modification of proteins. Multiple proteins targeted by DOPAL have been positively identified, including aggregation of alpha-synuclein.[54; 60] Given that alpha synuclein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) co-aggregate in toxic Lewy bodies, it was of interest to identify the interaction between GAPDH and DOPAL. Furthermore, the identification of additional protein targets is needed to show the overall picture of DOPAL in PD pathogenesis. GAPDH is an essential protein for neuronal homeostasis, and has been shown to be modified and participate in DA-induced cell death[86]. Therefore, it was hypothesized that DOPAL would covalently modify and inhibit GAPDH.

DOPAL levels have been linked to inhibition of aldehyde dehydrogenase (ALDH) and pesticide exposure, which are two major factors in PD pathology[69; 89; 90]. Therefore, the link between these three independent factors were examined. The effects of fungicide, benomyl and chlorpyrifos (CPF) and chlorpyrifos-oxon (CPO) on ALDH activity, and neuronal viability were investigated. The goal of this study was to identify GAPDH as a

protein target of DOPAL. Additionally, the effects of certain pesticides such as benomyl, CPO and CPF on ALDH activity were determined for neuronal viability.

Discussion of Specific Aims

Specific Aim 1: Investigate GAPDH modification by DOPAL

DOPAL is a highly reactive aldehyde species which interacts with nucleophilic sites of proteins. Given the fact that DOPAL is central to various impaired DA metabolism pathways and that DOPAL causes protein aggregation, it was of interest to determine the interaction of GAPDH and DOPAL. GAPDH was initially utilized in these studies as a model protein for Cys modification by DOPAL. GAPDH monomers oligomerized in the presence of DOPAL, the oligomerization was time and DOPAL dose-dependent (Table 3.2). Formation of GAPDH high molecular weight oligomers were concomitant with a decrease in the parent band. In addition, it was demonstrated that GAPDH co-aggregated with alpha-synuclein, and aggregation increased with DOPAL dose. This finding provides additional information on the role of DOPAL during Lewy body formation. Little to no change in the parent band and high molecular weight bands were detected in the presence of NAD^+ cofactor. Therefore, NAD^+ offers no protection from GAPDH oligomerization when co-treated with DOPAL (Figure 3.1). In addition, oligomerization and catechol adducts were not found with DOPAL analogs; MOPAL and DHPAN, highlighting the importance of both the catechol and aldehyde for DOPAL binding and reactivity with GAPDH (Table 3.4). Several DOPAL adducts were determined on GAPDH residues, including: Lys, Arg, and modification of Cys and Met amino acids. There were 8 Lys containing peptides with DOPAL Schiff base and reduced Schiff base adducts. There were 3 Met containing peptides with a Met oxidation and 3 other Cys peptides containing sulfenic acid modifications (Table 3.1). Global GAPDH thiol modification was also supported by the loss of free thiols in the presence of DOPAL

(Table 3.5). These results support the hypothesis that DOPAL covalently modifies GAPDH.

Specific Aim 2: Determine the effect of DOPAL on GAPDH activity

Since previous findings showed that DOPAL modifies specific residues on GAPDH and causes global protein aggregation, it was hypothesized that DOPAL would cause a change in GAPDH enzyme activity. Figure 4.1A-B demonstrates the decrease in GAPDH activity with increasing DOPAL concentration, and enzyme activity also decreased over time, reaching nearly 0% activity at 4h with 25 µM DOPAL. However, the result of NAD^+ in activity assays contrasted to the null effect of NAD^+ in DOPAL-caused aggregation of GAPDH. The presence of NAD^+ greatly protected enzyme inhibition by DOPAL (Figure 4.1C-D). This finding is supported by the fact that DOPAL-modified peptides are spatially near the NAD^+ binding site, shown in Scheme 3.1B. Inhibition by DOPAL was concluded to be irreversible, since the removal of DOPAL from the enzyme mixture did not reverse enzyme inhibition. DOPAL binding to GAPDH appears to be covalent and form stable GAPDH high molecular weight oligomers. The oligomerization and GAPDH aggregation were not reversed by glutathione treatment (Figure 4.3B). Lastly, neither MOPAL or DHPAN affected GAPDH activity, which is in agreement with the notion that both catechol and aldehyde are responsible for DOPAL reactivity with GAPDH.

Specific Aim 3: Determine the changes in DA metabolism and cell viability as a function of benomyl in SH-SY5Y neuroblastoma

The fungicide, benomyl has recently been implicated in PD pathology and affecting DOPAL levels by inhibition of ALDH, however, DOPAL had previously not been measured. Treatment of benomyl to SH-SY5Y neurons produced an increase of DOPAL and a slight decrease of DOPAC, and DOPAL levels also increased over time from 1-4 h (Figure 5.4). These findings support the inhibition of ALDH by benomyl. It

was also of interest to determine toxicity of benomyl in dopaminergic versus non-dopaminergic neurons. Figure 5.1 shows that benomyl toxicity was greatest in SH-SY5Y neurons that were differentiated to a dopaminergic phenotype, since adrenergic and undifferentiated neurons showed negligible cytotoxicity. Toxicity was time delayed, and was not evident before 4 h. Dopaminergic SH-SY5Y underwent significant morphological changes with DA and benomyl co-treatment. Co-treated cells had a loss of neurite extensions and displayed cell ebbing and shrinkage to small round apoptotic bodies (Figure 5.2). The preferential toxicity of DA and benomyl to dopaminergic cells was not observed with DOPAL and benomyl co-treatment. Although DOPAL was effectively toxic to the cells at 24 h, the toxicity did not vary between undifferentiated and differentiated SH-SY5Y (Figure 5.3). These results show that DOPAL levels increased and DOPAC decreased with treatment of benomyl, which further proves that benomyl is an effective ALDH inhibitor. In addition, the synergistic toxic effect of DA and benomyl is attributed to the *in situ* accumulation of DOPAL.

Specific Aim 4: Determine the changes in DA metabolism, formation of reactive oxygen species and cell viability as a function of CPF and CPO in PC6-3 cells.

Previous to this work, DA metabolites had not been measured in a cell model after exposure to the OPs: CPF and CPO. In cell studies, DOPAL levels greatly increased when treated with CPF and CPO. The ratio of DOPAC/DOPAL+DOPET was the decreased the most with CPO treatment, and the ALDH inhibitory effect of CPO and CPF occurred at early (4 h) and later (24 h) time points. However, cytotoxicity was only observed at 24 h, corresponding to pathological levels of DOPAL (23- 100 µM). This concentration of DOPAL is likely lethal to cells, given that concentrations above 6 µM are toxic[59]. Incubation of CPF with purified ALDH was time-dependent, however, CPO did not reproducibly inhibit the ALDH enzyme, and neither compound inhibited ALDH in the presence of phosphate (Figure5.8). An alternative mechanism of ALDH toxicity

included ROS and lipid peroxidation products, which are known to potently inhibit ALDH[56]. The CPF and CPO compounds rapidly produced ROS as early as 1 h and increased ROS production as a function of CPF/CPO dose (Figure 5.7). However, the greatest ROS formation was observed with CPF.

Conclusions and Implications for Parkinson's Disease

The neurotransmitter, DA was once thought to be an insignificant intermediate of noradrenaline[201]. The discovery that the DA pathway is intrinsically related to PD pathology took place only 50 years ago[202]. Subsequent discoveries of reactive DA metabolites, such as DOPAL, have greatly changed the view of PD pathology and the selective degeneration that occurs within DA neurons. The existence of DA and endogenous metabolite, DOPAL is paradoxical in PD pathology, as clinical manifestations are due to the loss of DA and it's neurons, the unregulated levels of DOPAL can also be detrimental to neuronal health. Impaired DA metabolism and accumulation of DOPAL is important PD pathological features. Regulation of DOPAL levels is crucial in the aging and PD-prone population due to: the age-related increase of MAO activity,[62] and inhibition of ALDH by lipid peroxidation products,[56] pesticides and fungicides[69; 89]. It is of great importance to elucidate toxicity mechanisms related to DOPAL, and protein targets of DOPAL. This work has shown that DOPAL modifies, aggregates, and inhibits GAPDH. The role of GAPDH in neuronal protein aggregation may be utilized as a biomarker for PD diagnosis. However, GAPDH aggregation may not be specific to only PD, since GAPDH is also over expressed in amyloid plaques in Alzheimer's brains[203] and a reduction in activity is observed in Huntington brains[78]. Therefore, GAPDH may serve as a redox switch and a global marker for oxidative stress[112]. It will be highly useful to optimize compounds that deter GAPDH oxidative modification and aggregation[204; 205], and hence, offer protection from cell death. In addition, this work supports the hypothesis that DOPAL is the mechanistic link between

pesticide/fungicide exposure and ALDH inhibition observed in PD-related model systems. Future PD preventative therapies may include ALDH activators.

Future Directions

Dimedone for Probing Sulfenic Acids on GAPDH

It is hypothesized that the DOPAL quinone structure could be a Michael acceptor and bind to nucleophilic Cys. However, there were no mass shifts corresponding to a Michael adduct in GAPDH-DOPAL mixtures. Although DOPAL Michael adducts with Lys have been discovered[75], there is no empirical evidence of a Michael Cys adduct. However, thiol modification was implied via titration with DTNB reagent and a loss of free thiols in the presence of DOPAL (Figure 3.5). The use of a dimedone probe would be useful in confirming the evidence for sulfenic acid formation on Cys residues. LC-MS data revealed mass shifts of 16 Da pertaining to Cys and Met amino acids.

Detection of DOPAL on HPLC-ECD

Previous authors were unable to measure intracellular DOPAL due to low levels and poor sensitivity. The measurement of DOPAL to DOPAC and DOPET ratio is necessary to determine ALDH inhibition in cells. It would be useful to measure DOPAL in benomyl treated cells. However, at baseline and with benomyl treatment, DOPAL levels were virtually undetectable in SH-SY5Y neurons utilizing an HPLC-UV detector. However, an electrochemical detector (ECD) has heightened sensitivity and can measure metabolites in the picomole range[206].

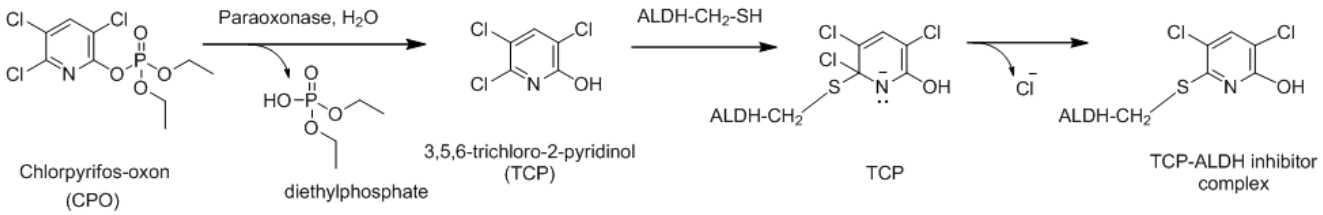
Determine ALDH Activity with Antioxidant/Pro-oxidants PC6-3 Treatment

To test whether ROS formation is responsible for ALDH inhibition with CPF/CPO treatment, PC6-3 cells could be pre-treated with either 5 mM N-acetyl-cysteine (NAC), and with the pro-oxidant buthionine sulfoximine (BSO) for 24 h before CPO and CPF exposure. NAC is a cell permeable antioxidant, which is converted to glutathione

(GSH) within the cells, and BSO significantly decreases GSH levels by inhibiting γ-glutamylcysteine synthetase, an enzyme involved in GSH synthesis[16; 196]. If ROS is causing ALDH inhibition, it is expected that NAC would reduce ALDH inhibition, and BSO would increase ALDH inhibition with CPF and CPO.

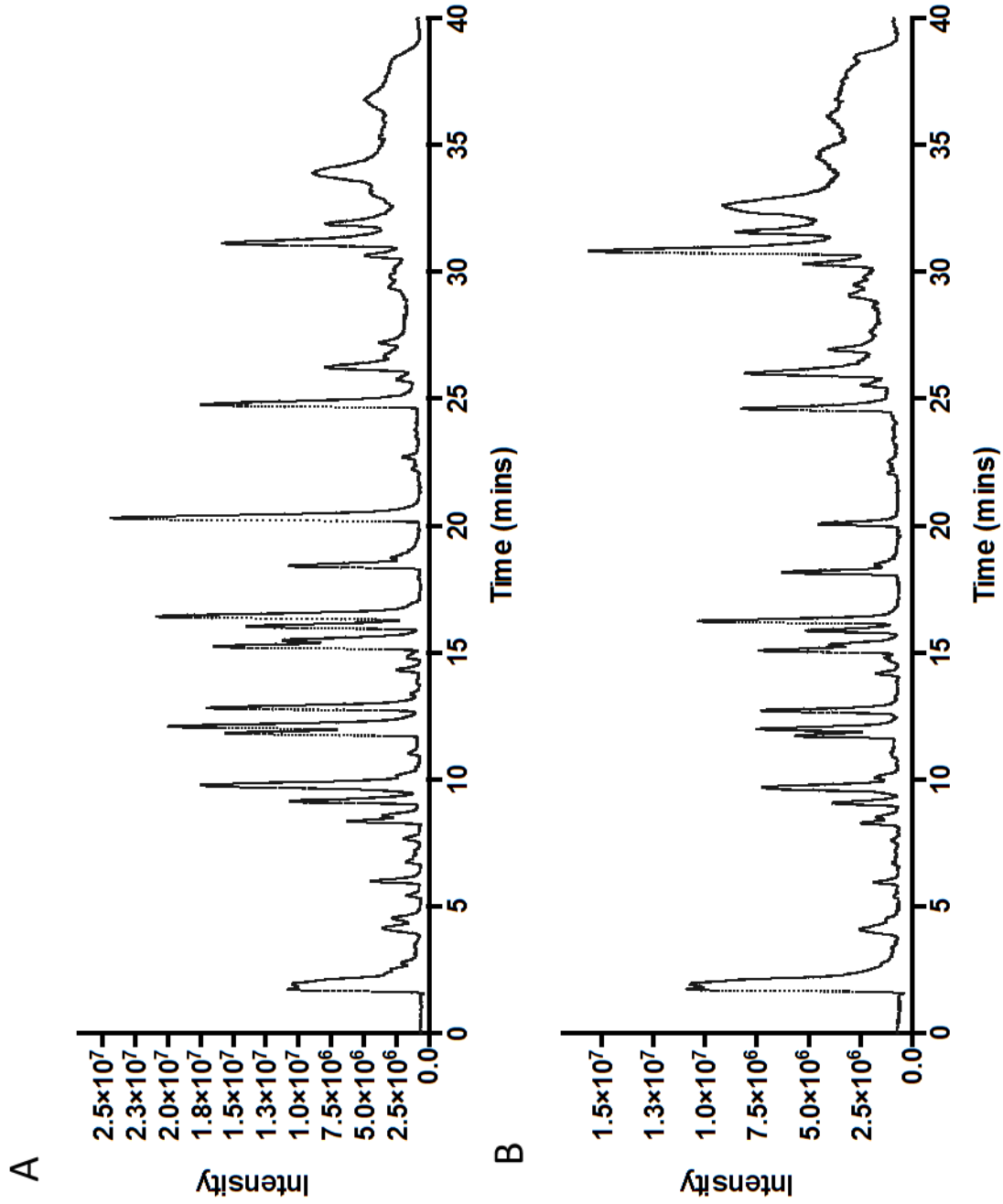
Determine the Mechanism of ALDH Inhibition by CPF and CPO

The ALDH inhibition observed in cell studies.by CPF and CPO did not correlate well with the *in vitro* ALDH1 and ALDH2 assays, in which inhibition was dependent on buffer and at high concentrations > 100 µM. Therefore, ALDH inhibition by the OP compounds may be dependent on intracellular events. Cellular metabolism of CPO includes hydrolysis by paraoxonase to form 3,5,6-trichloro-2-pyridinol (TCP) and diethylphosphate[207]. CPO is a more likely substrate to paraoxonase than CPF, therefore there more hydrolysis products and TCP than would be observed with CPF hydrolysis. It is proposed below that TCP may be the inhibitory compound for ALDH. This hypothesis would agree well with the finding that CPO was a more potent ALDH inhibitor in cell assays. Therefore, the inhibitory compound would be dependent on hydrolysis of CPO, and hydrolysis was not observed in the *in vitro* cell-free ALDH assay. A future step would be to incubate the TCP compound with purified ALDH or induce hydrolysis of CPO and measure activity.



APPENDIX

SPECTRA



The TIC for GAPDH (0.5 mg/mL) trypsin digest peptides. A) GAPDH without DOPAL. B) Mass chromatogram of GAPDH treated with 50 µM DOPAL for 4h.

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