HEALTH RESEARCH ABSTRACT SUBMISSIONS #2

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Title of Research *	3,4-dihydroxyphenylacetaldehyde, an endogenous neurotoxin, inhibits tyrosine hydroxylase and leads to potent toxicity: implications for Parkinson's disease
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Introduction & Purpose *

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting over a million people in the United States. This disease leads to the selective loss of dopaminergic neurons in the substantia nigra, causing a decrease in the important neurotransmitter dopamine (DA), with patients exhibiting a variety of motor and cognitive problems. It was previously shown in our lab that the reactive and toxic metabolite of dopamine, 3,4-dihydroxyphenylacetaldehyde (DOPAL) caused significant mitochondrial dysfunction in dopaminergic cells and potently inhibited the enzyme tyrosine hydroxylase (TH). TH catalyzes the rate-limiting step in DA synthesis, oxidizing tyrosine to L-DOPA, which is further metabolized to DA. This enzyme is potently inhibited by DOPAL in dopaminergic cell lysate, but the mechanism behind inhibition is not fully understood.

Experimental Design *

Materials
DOPAL was biosynthesized as previously described using enzyme-catalyzed conversion of DA to DOPAL by rat liver MAO (Nilsson & Tottmar 1987), and the concentration was determined via an ALDH assay (Ungar et al. 1973) and HPLC analysis as described below. Tyrosine, L-DOPA, DA, DOPAC, phenylacetaldehyde, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Malondialdehyde (MDA) was obtained by heating 1,1,3,3-tetraethoxypropane in an aqueous solution containing HCl (Esterbauer et al. 1991).
2.3 Synthesis of DOPAL Analogs
3,4-Dimethoxyphenylacetaldehyde (DMPAL) and 3-methoxy-4-hydroxyphenylacetaldehyde (MOPAL) were synthesized as previously described (Rees et al. 2009). Briefly, DMPAL was obtained using an alkene oxidation of eugenol methyl ether (0.096 µL, 5.5 mmol) with 3.5% mol $RuCl_3$, and 4 mol equivalents of $NaIO_4$ (0.4798 g, 5 eq) for 3 hrs in 6:1 acetonitrile:water (20% yield). NMR values were as follows: 1H ($CDCl_3$): δ 3.6 (d, 2H), 5.93 (s, 2H), 6.65-6.82 (m, 3H), 9.71 (t, 1H) (Yang & Zhang 2001). MOPAL was biosynthesized using a procedure similar to the production of DOPAL, with rat liver MAO being used to convert 3-methoxytyramine to MOPAL (Rees et al. 2009).
3,4-Dihydroxyphenylacetonitrile (DHPAN) was synthesized via the deprotection of 3,4-(methylenedioxy)-phenylacetonitrile (0.109 g) by 1 equivalent of boron tribromide in methylene chloride at -80°C under N_2. After stirring for 8 minutes, the solution was allowed to slowly warm to room temperature, and it was left to stir overnight at room temperature under N_2 (19% yield). NMR values were as follows: 1H (DMSO-d6): 3.67 (d, 2H), 6.65-6.68 (m, 3H), 8.99 (s, 1H), 9.1 (s, 1H) (Mzengeza 1988, Nguyen et al. 2009).
2.5 Tyrosine Hydroxylase Isolation and Activity in Cell Lysate and PC6-3 Cells

As previously described in Mexas, et al., cell lysate was collected from PC6-3 cells and assayed for TH activity. Lysate was stored at -80 ºC until assays were performed. It is important to note that TH activity is stable up to a year when stored under these conditions (Laschinski et al. 1986). Activity was measured using HPLC to follow the production of L-DOPA over a 2 h time course. Cell lysate experiments were performed for DOPAL analogs in order to directly compare previous data obtained with DOPAL. Furthermore, this was a starting point from which to investigate the effect of the analogs on TH activity without the complication of trafficking or possible metabolism. As previously reported, cell lysate (0.2 mg/mL) was incubated with tyrosine (100 µM), tetrahydrobiopterin (cofactor, 0.25 mM), and the addition of either 10 or 20 µM PAL, MOPAL, DMPAL, or DHPAN. Time points were taken at 0, 30, 60, 90, and 120 min and placed in 5% (v/v) perchloric acid to stop the reaction. All assays were done in 10 mM sodium phosphate buffer (pH 6.8, optimal for TH activity (Gahn & Roskoski 1993)).

To study the effect of DOPAL and analogs on TH in a whole cell environment, NGF-differentiated dopaminergic PC6-3 cells were preincubated with HBS for 15 min and then 10 µM tyrosine, 5 µM malondialdehyde (MDA, to inhibit ALDH and ALR, (Jinsmaa et al. 2009)), and 5 µM of DOPAL were added and incubated for 2 hs. In analog studies, 20 µM of PAL, DMPAL, MOPAL, or DHPAN were added in place of DOPAL. At time points of 0, 30, 60, 90, and 120 min, the supernatant was removed and cells were lysed using 300 µL of 0.1% triton-X in potassium phosphate (pH 7.4). Both supernatant and lysis were analyzed using HPLC as described below for L-DOPA production (and the change in analog or DOPAL over time). It is important to note that MDA does not inhibit or alter TH activity (data not shown), which has also been demonstrated in previous studies (Stone et al. 1986).

Mass Spectroscopy Analysis of DOPAL Adducts. A Schimadzu IT-TOF mass spectrometer was employed coupled with a Schimadzu HPLC. A Phenomenex Aeris Widepore XB-C18 column (100 x 2.1 mm), with a particle size of 3.6 µm and a pore size of 200 Å was used to separate peptides. A mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B) was used in gradient with the following time course: 0 min: 5% B, 5-15 min: 5-50% B, 15-30 min: 50-90% B, 31 min: 95% B, 35 min: 50% B, 36 min: 5% B, 40 min: 5% B. 15 µL of sample was injected and analytes were run through the photodiode array detector prior to mass spectrometry analysis. Scanning was performed from m/z 200 to m/z 2000 in 3.3 seconds.

In MS/MS, the same samples were once again subjected to HPLC-MS analysis followed by a second MS in order to determine which amino acid the DOPAL adducts were bound to. 15 µL of sample was injected and MS scanning was performed from m/z 200 to m/z 2000 in 3.3 sec. MS/MS was performed from m/z 300 to m/z 1000 with a repeat value of 3. Ion accumulation was set to 50, with ASC at 70%, and a CID of 50%. The same gradient was used to separate peptides as for LC-MS as described above.

Results *

Previous studies have demonstrated that changes to the structure of DOPAL leads to a decreased ability to interact with and bind model proteins (i.e. bovine serum albumin), and N-Acetyl-Lys residues. To further these results, we studied how the structure-activity profile changes in terms of inhibition of tyrosine hydroxylase, and mitochondrial dysfunction. Results demonstrated that even slight changes to the structure of DOPAL (i.e. masking of the catechol) lead to a significant decrease in the ability to inhibit tyrosine hydroxylase action.

Furthermore, mass spectrometry was used to identify sites of DOPAL modification to tyrosine hydroxylase. For the first time, 5 adducts were found on a known protein target of DOPAL, with results demonstrating how modification leads to the potent inhibition of this enzyme.

Conclusions *

DOPAL inhibition and potent toxicity rely on both the catechol and aldehyde, with small changes to the structure causing significant decreases in the ability to inhibit tyrosine hydroxylase and to cause mitochondrial dysfunction in cells. Furthermore, sites of DOPAL modification were determined by mass spectrometry. These sites are predicted to lead to significant structural changes to the enzyme, which in turn, lead to the potent inhibition observed when in the presence of DOPAL. These results have implications for the onset and progression of Parkinson's disease. MAO activity is known to increase with age, while products of lipid peroxidation inhibit aldehyde dehydrogenase and aldehyde reductase, both important metabolizers of DOPAL. Combined, these would lead to increases in DOPAL levels, and inhibition of important enzymes, such as tyrosine hydroxylase. Furthermore, mitochondrial dysfunction would increase,

causing cells to have altered or decreased energy, and possibly lead to dopaminergic cell death, a hallmark of Parkinson's disease.

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