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INVESTIGATING NAT PRODUCTS & PHARMACEUTICALS
TARGETING INFLAMMATION IN NEURODEGENERATIVE
DISEASES

BY
SHELBY L. JOHNSON

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
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UNIVERSITY OF RHODE ISLAND

2020

DOCTOR OF PHILOSOPHY DISSERTATION
OF
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ABSTRACT

Alzheimer's and Parkinson's disease (AD and PD, respectively) are the two most common neurodegenerative diseases. Currently, no interventions have been successful in stopping the progression of these diseases. Here, we utilize two independent strategies: the use of natural products and repurposed pharmaceuticals to target neuroinflammation in models of neurodegeneration.

Natural products have been used for their medicinal properties for centuries in traditional eastern medicine practices. *Mucuna pruriens* (Mucuna) is a well-known natural source of levodopa, typically prescribed in Ayurveda for PD. A novel levodopa reduced seed extract exhibited protective effects against oxidative stress and PD specific toxic agents, both *in vitro* and *in vivo*. Further phytochemical investigation of our extract led to the isolation and identification of seven newly reported compounds. Isolates failed to protect against toxin inducers of PD in cellular models. These data support that our novel levodopa reduced seed extract, but not isolated compounds, protect against toxin-induced models of PD.

The Mediterranean diet, which is primarily composed of polyphenols, has gained considerable interest in the management of age-related diseases. Therefore, common polyphenols classes, isoflavones, and lignans with their gut-derived microbial metabolites were evaluated. Polyphenol microbial metabolites generally showed greater blood-brain barrier permeability and protection against oxidative stress, as compared to their parent compounds. Moreover, polyphenol microbial metabolites may heavily contribute to the beneficial effects of polyphenol enriched diets in disease prevention.

Repurposing pharmaceuticals is an approach that allows for expedited drug discovery to fast track pharmaceuticals to a new targeted patient population. Here, we analyze direct thrombin inhibitor, dabigatran etexilate (Pradaxa®), against

neuroinflammation in AD and PD models. In a *Drosophila melanogaster* transgenic model of PD, dabigatran treatment improved locomotor ability and reduced neuroinflammatory markers in males. Further, in a tau-based animal model of AD short-term treatment with dabigatran modulates expression of proteins related to antioxidants, mitogen-activated protein kinases, tau, thrombin, and oxidative stress. Taken together, these data indicate short-term treatment with a direct thrombin inhibitor modulates protein expression in the brains of aged Tg4510 mice. These findings support our hypothesis that targeting thrombin, a key mediator of neuroinflammation and neurotoxicity may be effective in reducing neuroinflammation in neurodegenerative diseases.

Our results indicate two common drug discovery methods, namely, investigation of natural products and repurposing pharmaceuticals, may provide insights for targeting neuroinflammation in neurodegenerative diseases. Further research should focus on moving these therapeutics through the drug discovery pipeline to the patient population.

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DEDICATION

The following dissertation is dedicated to my family. You are and have always been the inspiration in my doctoral pursuit. Your daily reminder of the very real impact of neurodegenerative diseases motivates me to do better.

“You are braver than you believe, stronger than you seem, and smarter than you think” A.A. Milne

PREFACE

The following dissertation is presented in manuscript format which creates four major chapters. The publication status of each manuscript is as follows:

Chapter 1: Generate and evaluate the bioactivity of Levodopa-reduced *Mucuna pruriens* extract and isolated compounds in cellular models of PD

Manuscript 1: Levodopa-reduced *Mucuna pruriens* seed extract shows neuroprotective effects against Parkinson's disease in murine microglia and human neuroblastoma cells, *Caenorhabditis elegans*, and *Drosophila melanogaster*. Published in *Nutrients*, 2018.

Manuscript 2: Isolation and identification of compounds from *Mucuna pruriens* seeds. Under review at *Chemistry of Natural Products*, Nov 2019.

Chapter 2: Examine common polyphenols and corresponding gut-derived microbial metabolites for blood brain barrier permeability, reduction of neuroinflammation and protection in cellular and *Drosophila melanogaster* PD models

Manuscript 3: Polyphenol microbial metabolites exhibit gut and blood-brain barrier permeability and protect microglia against LPS-induced inflammation. Published in *Metabolites*, 2019.

Manuscript 4: Phytoestrogen, Equol, provides protection against toxin-induced models of Parkinson's disease. In preparation.

Chapter 3: Explore the protective ability of direct thrombin inhibitor, Dabigatran, against neuroinflammation and motor impairments in *Drosophila melanogaster* models of PD

Manuscript 5: Direct thrombin inhibitor, Dabigatran, protects against neuroinflammation in LRRK2 transgenic *Drosophila melanogaster* model of Parkinson's disease. In preparation.

Chapter 4: Investigate Dabigatran etexilate influence on oxidative stress and neuroinflammation in aged, transgenic tau AD mouse model

Manuscript 6: Direct thrombin inhibitor, dabigatran etexilate, reduces oxidative stress *in vivo* in a transgenic mouse model of Alzheimer's disease. In preparation.

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JUSTIFICATION OF RESEARCH

Neurodegenerative diseases are broadly defined as age-related disorders where cells of the nervous system gradually lose function. The two most common neurodegenerative diseases are Alzheimer's (AD) and Parkinson's disease (PD). In 2016, nearly 5.4 million Americans were affected by AD; PD is expected to affect nearly 930,000 individuals by 2020^{1,2}. Although these diseases present vastly different symptoms, there are several commonalities. For instance, both diseases are associated with protein aggregations, genetic predispositions, mitochondrial dysfunction and neuroinflammation, all processes which heavily contribute to neuronal death³.

Alzheimer's Disease

AD typically affects the elderly population, characterized by episodic memory deficits later progressing to full-blown dementia with language impairment and severe behavioral changes⁴. Specific causes of AD are not known; however, lifestyle, genetics, and environment are all identified as risk factors^{5,6}. Pathologically, AD is identified postmortem by the presence of β -amyloid ($A\beta$) plaques and neurofibrillary tangles, comprised of $A\beta$ fragments and hyperphosphorylated tau, respectively. The AD brain exhibits extensive neuronal death, believed to originate in the hippocampus and move to adjacent brain regions⁴. For decades, the amyloid hypothesis has dominated AD research. This hypothesis suggests there is a cascading event that begins with $A\beta$ accumulation leading to neurofibrillary tangles, which in turn disrupts synaptic and neuronal function⁷. Pharmaceutical interventions targeting disease pathogenesis have not been successful in stopping the progression of the disease.

It has become more widely accepted that AD is much more complicated than the original amyloid hypothesis. Research has shifted to explore age-related decline

through the influence of tau, inflammation, gut microbiota and vascular dysfunction in AD pathogenesis⁸⁻¹¹. Our previous research has focused heavily on neuroinflammation induced by the activation of the vasculature. Driving this interest is the correlation of cardiovascular disease and vascular risk factors, such as hypertension and diabetes, with AD incidence rates⁶. Further, injury to the cerebrovasculature leads to the activation and dysfunction of brain endothelial cells¹². In the AD brain, vascular activation has detrimental consequences for neuronal viability. Many vascular-derived factors, such as thrombin, are neurotoxic and likely critical in the pathogenesis of AD^{11,13,14}.

Currently, available medications target symptoms, but not disease pathology or progression. The two approved drug treatments for AD are inhibitors of cholinesterase and *N*-methyl-D-aspartate (NMDA) receptors; both exhibit modest beneficial effects on cognition^{5,15}. Significant research has focused on identifying novel disease-modifying drugs, but there have been several failures in the clinic¹⁶. Current trends for AD research are now focusing on identifying biomarkers for earlier diagnosis and identifying interventions that aim to modify disease progression^{4,17}. Continued AD research must identify new ways to modify disease progression and pathogenesis to drive the future of pharmaceutical developments.

Parkinson's Disease

PD is characterized by irregular, uncontrolled movements, frequently presenting as resting tremors or dyskinesias. Pathological hallmarks include the loss of dopaminergic neurons in the substantia nigra and an abundance of α -synuclein aggregates, termed Lewy bodies, throughout the brain¹⁸. Direct causes of Parkinson's disease are not known; however, a variety of genetic predispositions, gender, and environmental factors have been identified as contributors. At least 17 genetic mutations have been identified as contributing factors in PD including α -synuclein,

parkin, DJ-1, and leucine-rich repeat kinase 2 (LRRK2)^{18–20}. Environmental exposure to pesticides, such as paraquat, and similarly structured chemical 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can also result in symptoms indistinguishable from PD^{18–20}. Interestingly, males are 1.5 times more likely to have PD than females, and incident rates in females increase dramatically after menopause, suggesting a protective role of estrogen^{21,22}.

Pharmaceutical interventions for PD have mainly focused on the replenishment of dopamine to reduce motor impairments. These pharmaceutical classes include catechol-O-methyl transferase (COMT) inhibitors, monoamine oxidase type B (MAO-B) inhibitors, dopamine agonists, and levodopa paired with a dopamine decarboxylase inhibitor^{23–25}. Levodopa combination therapy limits the production of dopamine in the periphery and attempts to restore dopamine imbalance in the brain. Levodopa is then converted to dopamine by dopamine decarboxylase, increasing levels of dopamine in the affected brain regions²⁶. Levodopa paired with a dopamine decarboxylase inhibitor is currently the standard of care for PD; however, with prolonged treatment, levodopa becomes less effective, and motor deficits can increase²⁶. The aforementioned therapies are effective in some cases, but new formulations and administration routes are being explored to overcome problems of prolonged efficacy²³. Surgical interventions have also been approved as therapies for PD. Deep brain stimulation using high-frequency stimulation in the subthalamic nucleus and globus pallidus internus exhibits improved motor control^{24,25}. Similar to AD, available interventions cannot stop or prevent disease progression; therefore, it is essential to identify and target disease-modifying mechanisms. Current literature proposes the investigation of genetic mutations, mitochondrial function, neuroinflammation, and oxidative stress, among others^{18,26}.

Neuroinflammation in Alzheimer's and Parkinson's Diseases

Neuroinflammation has been recognized as a major factor in AD and PD pathogenesis^{6,7,17,18,26–28}. While these two diseases present quite differently, the neuroinflammatory response is similar³. Integral to the regulation of inflammation in the brain is the neurovascular unit (NVU). Astrocytes, microglia, neurons, pericytes, and endothelial cells collectively create the NVU that maintains normal cerebral blood flow and blood-brain barrier (BBB) functioning²⁹. Under normal conditions, microglia, the major immune cells of the brain, work to detect pathogens or tissue damage and elicit a response to clear cellular debris and degenerating cells¹⁷. However, when microglia are continuously activated by external stimuli such as aggregated protein or foreign pathogens, this response can then exacerbate any present neuronal damage³⁰. This toxic microglial inflammatory response includes the release of a variety of inflammatory mediators, such as chemokines, cytokines, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and reactive mediators, namely, reactive oxygen species (ROS) and nitric oxide species (NOS)^{17,31,32}. The chronic production of these inflammatory mediators increases oxidative stress and contributes to neuronal death. Although complex, neuroinflammation plays a major role in neurodegenerative diseases and has been identified as a worthy target for pharmaceutical interventions in neurodegenerative diseases.

Therapeutic Strategies

In recent years, big pharmaceutical companies such as Pfizer have shut down their neuroscience research groups. A major part of this is due to the lack of success in developing drugs to treat neurological diseases. Since 1990, there has been a decline in nervous system drugs, with fewer Phase I trial starts, as well as a greater probability of failure in Phase III³³. The investigation of new therapeutics for central nervous system diseases has proven to be extremely cost and time ineffective.

Therefore, it is imperative to acknowledge and utilize methods that will reduce both factors. Herein, we investigate two commonly used drug discovery strategies: natural products and repurposing pharmaceuticals.

Therapeutic Strategy I: Natural Products

Plant-based approaches to medicine have been utilized for centuries. The generational knowledge of traditional Chinese medicine (TCM) and traditional Indian medicine (Ayurveda) have driven the investigation of natural sources to identify biologically active compounds³⁴. Among these discoveries have come major medical advancements for cancer, hypertension, malaria, and pain, among others³⁵. One of the most commonly known natural product derived pharmaceuticals is aspirin. Willow bark as a pain and fever reliever dates back more than 3,500 years. The active compound, salicylic acid, was initially identified in 1838. Scientists at Bayer then acetylated salicylic acid to create a more stable compound, now known as aspirin³⁶. This story is not unique to aspirin, approximately 51% of all newly approved drugs from 1981 to 2014 were derived from or structurally related to a natural product³⁷. Natural product, *Ginkgo biloba*, is an example that has been successful in mitigating the cognitive decline in animal models but has been controversial in AD clinical trials^{38–41}. The success of natural products has been instrumental in the foundations of modern medicine and thus should not be ignored as a viable option for future therapeutic developments^{35,42}.

Mucuna pruriens is a legume common to southern China and eastern India, used heavily as a food source, and for the treatment of tremors⁴³. Ayurveda traditionally used *Mucuna* to treat PD, as the beans are naturally high in levodopa⁴⁴. This use of *Mucuna pruriens* led to further investigation of this medicinal plant. In two clinical trials, *Mucuna pruriens* preparations exhibited more rapid onset and more tolerable profiles as compared to levodopa paired with a dopamine decarboxylase

inhibitor, suggesting potential advantages of the natural product to the common pharmaceutical^{45,46}. Multiple studies suggest that the anti-PD activity of *Mucuna* is entirely due to the presence of levodopa^{46–49}. However, newer evidence also suggests that there may be bioactive compounds other than levodopa could provide neuroprotection⁵⁰.

Another interesting class of natural products, namely, polyphenols, exhibits a wide range of bioactive properties including antioxidant, anti-inflammatory, anti-apoptotic and lipid-lowering properties^{51,52}. Interestingly, the Mediterranean diet is characterized by a high intake of polyphenols and has been identified as a beneficial intervention for the management of cardiovascular disease, obesity and neurodegenerative diseases^{53–55}. While polyphenols themselves may exhibit biological effects, it is generally considered that the consumed compounds get metabolized in the colon by microbiota to produce bioactive metabolites^{56–60}. Two common classes of polyphenols: lignans and isoflavones are found primarily in flaxseed and soy, respectively. The common and prototypical dietary lignan, secoisolariciresinol (SECO), is known to be converted to the polyphenol microbial metabolites (PMM) enterodiol (ED) and enterolactone (EL)⁶¹. The isoflavones, genistein (GEN) and daidzein (DAI), are metabolized in the gut by intestinal microflora to produce equol (EQ)⁶². Isoflavones are structurally similar to estrogen, containing 3-phenylchromen-4-one backbone, making them a curious class of compounds for PD research⁶³. Additionally, GEN was identified as a potential nutraceutical for AD due to the ability to inhibit mitochondria-dependent apoptosis, and alleviate β -amyloid neurotoxicity, but failed in clinical trials^{64–66}. Failing in the clinic may have been due to rapid metabolism into metabolites, therefore it would be of interest to examine gut microbial-derived metabolites.

Therapeutic Strategy II: Repurposing Pharmaceuticals

Another promising strategy for new therapeutics is to repurpose “old” drugs. Repurposing pharmaceuticals is defined as determining alternative uses beyond the scope of the original indication⁶⁷. This approach is advantageous as these therapeutics have been extensively studied for safety, pharmacokinetic profiles, bulk manufacturing, and *in vitro* and *in vivo* screening⁶⁷. This strategy reduces risk, development time and costs, and provides a fast-track to the patient population⁶⁸. Repurposing pharmaceuticals has been utilized to identify new therapies for cancer, depression, and schizophrenia, among others^{68–70}. Aspirin is again a good example drug, as it has been re-purposed for several alternative therapeutic indications. Aspirin was initially FDA-approved to reduce pain and fevers. Recently, aspirin has been extensively studied for antiproliferative and anticancer activities⁷¹. Aspirin is effective in reducing cell viability in multiple subtypes of breast cancer cells⁷². In colorectal cancer patients, aspirin has shown improved survival rates⁷³. In addition to cancer, aspirin has also been analyzed in combating fungal infections⁷⁴. Moreover, recent studies have looked at repurposing approved antibiotics to reduce protein aggregation in neurodegenerative diseases^{75–77}. Using pharmaceuticals to target diseases alternative to initial indications has proven a worthy mechanism to identify new therapies and should be continuously explored.

Dabigatran etexilate (Pradaxa®, Boehringer Ingelheim) is a commonly prescribed anticoagulant that directly inhibits thrombin. Vascular activation results in injured endothelial cells which upregulate the production of thrombin both *in vivo* and *in vitro*^{13,78,79}. Elevated levels of thrombin can be neurotoxic through the interaction with protease-activated receptor 1 (PAR-1), leading to the release of reactive oxygen species (ROS) and increases in a large array of inflammatory proteins^{80,81}. Increased levels of both thrombin and PAR-1 have been identified in both AD and PD⁸². Previous studies have identified that dabigatran treatment mitigates the neurotoxic

effects of thrombin^{13,83,84}. Dabigatran etexilate also provides neuroprotection in a rotenone-induced rat model of PD through nuclear receptor-related 1⁸⁵.

Dissertation Objective

The following dissertation will propose new therapeutic strategies from natural products and pharmaceuticals to reduce neuroinflammation in AD and PD models. Outcomes will provide new insight into the current state for the protection against neuroinflammation.

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CHAPTER 1

MANUSCRIPT 1

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Levodopa-Reduced *Mucuna pruriens* Seed Extract Shows Neuroprotective Effects against Parkinson's Disease in Murine Microglia and Human Neuroblastoma Cells, *Caenorhabditis elegans*, and *Drosophila melanogaster*

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Abstract

Mucuna pruriens (Mucuna) has been prescribed in Ayurveda for various brain ailments including 'kampavata' (tremors) or Parkinson's disease (PD). While Mucuna is a well-known natural source of levodopa (L-dopa), published studies suggest that other bioactive compounds may also be responsible for its anti-PD effects. To investigate this hypothesis, a L-dopa reduced (<0.1%) *M. pruriens* seeds extract (MPE) was prepared and evaluated for its anti-PD effects in cellular (murine BV-2 microglia and human SH-SY5Y neuroblastoma cells), *Caenorhabditis elegans*, and *Drosophila melanogaster* models. In BV-2 cells, MPE (12.5–50 µg/mL) reduced hydrogen peroxide-induced cytotoxicity (15.7–18.6%), decreased reactive oxygen species production (29.1–61.6%), and lowered lipopolysaccharide (LPS)-induced nitric oxide species release by 8.9–60%. MPE (12.5–50 µg/mL) mitigated SH-SY5Y cell apoptosis by 6.9–40.0% in a non-contact co-culture assay with cell-free supernatants from LPS-treated BV-2 cells. MPE (12.5–50 µg/mL) reduced 6-hydroxydopamine (6-OHDA)-induced cell death of SH-SY5Y cells by 11.85–38.5%. Furthermore, MPE (12.5–50 µg/mL) increased median (25%) and maximum survival (47.8%) of *C. elegans* exposed to the dopaminergic neurotoxin, methyl-4-phenylpyridinium. MPE (40 µg/mL) ameliorated dopaminergic neurotoxin (6-OHDA and rotenone) induced precipitation of innate negative geotaxis behavior of *D. melanogaster* by 35.3 and 32.8%, respectively. Therefore, MPE contains bioactive compounds, beyond L-dopa, which may impart neuroprotective effects against PD.

Keywords: *Mucuna pruriens*; levodopa; Parkinson's disease; neuroprotection; *Caenorhabditis elegans*; *Drosophila melanogaster*

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease that leads to impaired motor function and is characterized by a loss of dopaminergic neurons in the substantia nigra and is second only to Alzheimer's disease in its prevalence [1]. The etiology and pathophysiology of PD are not very well understood and have consequently stifled the development of effective therapeutic interventions for PD. Accumulating evidence suggests that elevated oxidative stress and neuroinflammation associated with microgliosis and intracellular aggregation of α -synuclein molecules may be responsible for dopaminergic neuronal atrophy and ultimately the clinical manifestation of PD [2–4].

Mucuna pruriens, commonly known as Mucuna or velvet bean, is native to eastern India and western regions of China. Mucuna seeds, a rich source of naturally occurring levodopa (L-dopa; 4–7% in Mucuna seeds) [5], have been used traditionally as an effective remedy for several brain related maladies, including reducing tremors (as seen in PD), as documented in the ancient treatise of Ayurveda, the Indian traditional system of medicine [6]. The lack of effective pharmaceutical treatments has stimulated research interest in Mucuna as a PD therapeutic agent in several animal studies and a limited number of human clinical trials [7–9]. For example, Mucuna, at a dosage of 17.5 mg/kg, improved motor function and reduced dyskinesia in patients with advanced PD with fewer adverse effects as compared with the conventional treatment of L-dopa paired with a dopamine decarboxylase inhibitor, namely Carbidopa [9]. Mucuna has also been reported to show protective effects against PD in rodent models by increasing the activity of brain mitochondrial complex-I [10] and reducing motor dysfunction [11,12]. While several studies have attributed the anti-PD activities to naturally occurring high levels of L-dopa in Mucuna, emerging evidence suggests that other bioactive compounds besides L-dopa may also have

neuroprotective effects. For example, a *Mucuna* methanolic extract (0.1% dosage) containing low levels of L-dopa (0.01%) showed anti-PD effects including improvements of motor function and olfactory response in a *Drosophila melanogaster* genetic model of PD [13]. The anti-PD effects of the *Mucuna* methanolic extract were superior to that of the treatment of L-dopa (0.01%) alone in the aforementioned *D. melanogaster* model, suggesting that the overall anti-PD effects of *Mucuna* were a result of other compounds beyond L-dopa alone [13].

Our group has previously reported on the development of a neuroprotective potential algorithm for several Ayurvedic botanical extracts, among which *M. pruriens* ranked in the top four [14]. Given our group's research interest in this medicinal plant, and to explore the role of its 'non-L-dopa' bioactives against PD, we prepared a *M. pruriens* seed extract (MPE) containing low amounts of L-dopa (<0.1%) with the following objectives: (1) to evaluate the antioxidant and anti-inflammatory effects of MPE in murine microglia (BV-2) and human neuroblastoma (SH-SY5Y) cells; (2) to assess the neuroprotective effects of MPE against neurotoxin-induced cytotoxicity in cellular PD models; and (3) to evaluate the neuroprotective effects of MPE using *Caenorhabditis elegans* and *D. melanogaster* models of chemically induced PD.

Materials and Methods

Chemicals

Dimethylsulfoxide (DMSO), levodopa (L-dopa), Resveratrol (Resv), lipopolysaccharide (LPS), 2',7'-dichlorofluorescein diacetate (DCF-DA), hydrogen peroxide (H₂O₂), 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenylpyridinium (MPP⁺), and rotenone were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) /F-12, phenol red-free DMEM medium and trypsin-versene were purchased from Life Technologies (Grand Island, NY, USA).

Preparation of *Mucuna pruriens* Seeds Extract (MPE)

Mucuna pruriens seeds (3–7% L-dopa) were botanically authenticated and generously provided by Verdure Sciences (Noblesville, IN, USA). *Mucuna pruriens* seeds were authenticated by Dr V. Singh (Pharmanza, Gujarat, India) with voucher specimen (No. PHPL/HB/013) deposited in the Heber-Youngken Garden and Greenhouse at the College of Pharmacy, the University of Rhode Island, RI, USA. Briefly, the ground *M. pruriens* seeds (150 g) were extracted with sonication in methanol (1000 mL) in an ultrasonic bath (Bransonic 8510; Branson Ultrasonics Corp. Danbury, CT, USA) for 0.5 h and macerated in methanol at room temperature for 24 h to afford a crude methanol extract (6.5 g), which was dried *in vacuo* (in a water bath at 35 °C); reconstituted in water; and then partitioned sequentially in *n*-hexanes, ethyl acetate, and butanol with details as follows. The dried crude extract (6.5 g) was reconstituted in distilled water (250 mL) and sequentially partitioned with *n*-hexanes, ethyl acetate, and butanol (250 mL × 3 for each solvent). Each of these fractions, namely, hexanes (0.2 g), ethyl acetate (0.3 g), butanol (2.9 g), and the

remaining water portion (3.0 g), were dried in vacuo (in a water bath at 35 °C) to afford respective extracts. The levels of L-dopa were quantified in each dried extract (described below) and the extract with the lowest level of L-dopa, namely, MPE (the *M. pruriens* ethyl acetate extract; see Table 1), was selected for further biological evaluation.

Quantification of L-dopa by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

L-dopa was quantified by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) using methods and parameters published by our group and others with some modifications [15–19]. L-dopa quantifications were performed on a prominence ultra-fast liquid chromatography (UFLC) system (Shimadzu, Marlborough, MA, USA) coupled with a QTRAP 4500 system (Applied Biosystems/MDS Sciex, Framingham, MA, USA) with data acquired using Analyst 1.6.3 software and processed using MultiQuant 3.0.1 software (Sciex, Framingham, MA, USA). The UFLC system consisted of three LC-20AD pumps, a DGU-20A degassing unit, SIL-20AC auto sampler, CTO-20AC column oven, and CBM-20A communication bus module. Chromatographic separation was performed using a 100 mm × 4.6 mm i.d., 5 µm, XBridge C18 column (Waters, Milford, MA, USA). The mobile phase consisted of A (water containing 0.1% (v/v) formic acid) and B (methanol containing 0.1% (v/v) formic acid) with a gradient elution of 1% B from 0 to 10 min, and 1–4% B from 10 to 20 min. The flow rate was 0.5 mL/min and the injection volume was 10 µL. The column temperature was maintained at 40 °C. The MS operated in electrospray ionization (ESI) in positive mode with multiple reaction monitoring (MRM). Nitrogen was used as the source gas in all cases. Parameters were optimized as follows: IonSpray voltage, 4500 V; nebulizer gas, 40 psi; auxiliary

heater gas, 45 psi; curtain gas, 20 psi; turbo gas temperature, 300 °C. Using an authentic L-dopa standard (purchased from Sigma-Aldrich Chemical Co.; St. Louis, MO, USA), L-dopa was analyzed by the multiple reaction monitor (MRM) mode using ion transition at m/z values of 198/152. All of the analyses of the standard and extracts were performed in triplicates (see LC-ESI-MS/MS spectra in the Supplementary Materials; Figure S1). The calibration curve ($y = 5006.29x - 13189.13$; $R = 0.99825$) was acquired by plotting the peak area against the nominal concentrations of L-dopa. The linearity was in the range of 10–1000 ng/mL. The presence of L-dopa in Mucuna extracts was identified as a peak with a retention time of 3.95 min under the ion transition 198/152. The percentage of L-dopa in the different Mucuna extracts was calculated as follows: (ng/mL of L-dopa in extract)/(μg/mL of extract injected) × 100%.

Cell Culture

Murine microglia (BV-2) cells were kindly provided by Dr. Grace Y. Sun (University of Missouri at Columbia, MO, USA) and human neuroblastoma (SH-SY5Y) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained at 37 °C in 5% CO₂ with high glucose (4.5 g/L) DMEM/F-12 accompanied with 10% heat inactivated fetal bovine serum, and 1% P/S (100 U/mL penicillin, 100 mg/mL streptomycin) (Life Technologies, Gaithersburg, MD, USA). MPE was dissolved in distilled water to obtain a 10 mg/mL stock solution and further diluted in serum free media for treatments. Resv (used as a positive control for the cellular based assays) was dissolved in DMSO (10 mM) and diluted in media to the desired concentration. Control cells were treated with 0.1% DMSO in serum free media.

Cell Viability

BV-2 and SH-SY5Y cells were seeded in white walled 96-well plates at 1×10^5 cells/mL in serum free media. MPE (12.5, 25, and 50 $\mu\text{g/mL}$) were evaluated for cytotoxicity effects in BV-2 and SH-SY5Y cells. After 24 h, cell viability was determined using Cell Titer Glo 2.0 (CTG; Promega, Madison, WI, USA) according to methods previously reported by our group [14,20]. MPE was then evaluated for its cellular protective effects against several oxidative insults as follows. Cells were pretreated with MPE (12.5, 25, and 50 $\mu\text{g/mL}$), Resv (20 μM), or solvent control (0.1% DMSO) for either 1 h (in BV-2 cells) or 2 h (in SH-SY5Y cells). Cellular oxidative stress was induced in BV-2 and SH-SY5Y with H_2O_2 (100 μM), SH-SY5Y with 6-OHDA (25 μM), and MPP^+ (2 mM). Cellular viability of BV-2 and SH-SY5Y cells after treatment were determined at 6 and 24 h, respectively, by the aforementioned CTG assay.

Determination of Hydrogen Peroxide (H_2O_2)-Induced Reactive Oxygen Species (ROS) in Murine Microglia BV-2 Cells

The production of H_2O_2 -induced reactive oxygen species (ROS) in BV-2 cells was determined by a fluorescent probe (DCF-DA) using previously reported method with modifications [22]. BV-2 microglial cells were seeded in a black 96-well plate at 1×10^5 cells/mL in serum free media. Cells were allowed to attach for 24 h and pretreated with MPE (12.5, 25, and 50 $\mu\text{g/mL}$), Resv (20 μM), or solvent control (0.1% DMSO) for 1 h. Next, DCF-DA (20 μM) was added to each well and incubated for 25 min. Cells were then washed with PBS and incubated with H_2O_2 (100 μM) for 6 h. The fluorescence signal of each cell was measured at excitation and emission wavelengths of 495 nm and 529 nm, respectively, using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Measurement of Lipopolysaccharide (LPS)-Induced Nitric Oxide Species (NOS) in Murine Microglia BV-2 Cells

The production of total nitric oxide species (NOS) was determined using the Griess reagent as previously reported by our group [14,20]. BV-2 cells were seeded in clear 24-well plates at 1×10^5 cells/mL in serum free media. Cells were treated with MPE (12.5, 25, and 50 $\mu\text{g/mL}$), Resv (20 μM), or solvent control (0.1% DMSO) for 1 h. The cells were exposed to inflammatory stress induced by treating with LPS (1 $\mu\text{g/mL}$) for 24 h. Next, culture media from each well were transferred to a 96-well plate and measured for total NOS using the Griess reagent kit (Promega, Fitchburg, WI, USA). Absorbance values were recorded using the SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA) at 535 nm.

Non-Contact Co-Culture Assay with BV-2 and SH-SY5Y Cells

The non-contact co-culture assay was performed according to protocols previously reported by our group [21]. Briefly, SH-SY5Y cells were seeded in white wall and clear bottom 96-well plates and allowed to adhere for 24 h. BV-2 cells were plated in 24 well plates and treated with MPE (12.5, 25, and 50 $\mu\text{g/mL}$), Resv (20 μM), or solvent control (0.1% DMSO), followed by LPS (1 $\mu\text{g/mL}$) treatment for 24 h. Media from each treatment was collected and centrifuged at 15,000 rpm for 10 min. After centrifugation, BV-2 cell supernatant was used to treat SH-SY5Y cells for 24 h. Cellular viability of SH-SY5Y cells was determined using the CTG assay.

1-Methyl-4-Phenylpyridinium (MPP⁺) Induced Dopaminergic Neurotoxicity in C. elegans

Wild type *C. elegans* (N2) were maintained on nematode growth media culture plates at 20 °C and age synchronized as previously reported by our group [21]. Then,

40 μ L of age synchronized L1 worms washed in S-complete were transferred to a 96-well microplate (approximately 20 worms/well) with *Escherichia coli* OP50 (5 mg/mL), MPP⁺ (750 μ M), and MPE (20 or 40 μ g/mL) to a final volume of 50 μ L. S-complete media was used for control groups. Live worms were counted every 12 h post treatment until no live worms remained.

D. melanogaster Strains and Maintenance

Wild type STR-5 flies were obtained from the Bloomington Stock Center (Department of Biology, Indiana University, Bloomington, IN, USA). Strains were reared on Formula 4-24[®] Instant *Drosophila* Medium (Carolina Biological Supply, Burlington, NC, USA) and reared on Bloomington Formulation (Genesee Scientific, San Diego, CA, USA) at 25 °C with 75% humidity and a 12-hour light/dark cycle [23]. Approximately 40–50 mating pairs were transferred into flasks and allowed to lay eggs. After nine days, newly eclosed male flies were collected over a period of three days and used in further experiments.

Negative Geotaxis (Climbing) Assay in D. melanogaster

Newly eclosed wild type (STR-5) male flies were randomly separated into 10 groups of 50 flies each and transferred to control flasks (media only) or in treatment flasks (media + 40 μ g/mL MPE). To induce neurotoxicity, every four days, flies were starved in empty vials for 24 h and transferred into vials containing a filter paper saturated with 1 mL of 10% sucrose (blank), 6-OHDA (1 mM), or rotenone (500 μ M). After 24 h, the flies were transferred into vials with a fresh supply of their respective diets and used for climbing assay on day 10 post-eclosion. Flies from control and treatment groups were then tapped into the bottom of graduated cylinder (diameter: 2.7 cm, height: 25 cm) superimposed with a ruler and allowed to climb for 10 s. Flies

were photographed (Canon, Inc, Tokyo, JP, EOS 50D; 15.1 MP Digital SLR) at t_0 and t_{10} seconds to calculate the climbing distance [24].

Statistical Analyses

All data are presented as mean $\pm$ standard errors of three separate biological samples. Analyses of cellular data were conducted by analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons of group means. The Kaplan–Meier method was used to compare the survival curves of *C. elegans* and the survival differences were tested for statistical significance using the log rank test (Mantel Cox). For the *D. melanogaster* climbing assay, Welch's *t*-test was used to compare the different treatment groups and generate *p* values (alpha = 0.05). Significance compared with control group is presented as $p \leq 0.05$ (#), $p \leq 0.001$ (###), and $p \leq 0.0001$ (####). Significance for all tests compared with toxic treatment was defined as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****). GraphPad Prism software 6.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to calculate statistics for both the in vitro and in vivo analyses.

Results and Discussion

Preparation of Levodopa (L-dopa)-Reduced Mucuna pruriens Extract (MPE)

Mucuna pruriens is a medicinal plant that is well known to naturally contain L-dopa (4–7%) [5], which might be attributed to its neuroprotective effects against PD [6]. However, the presence of other phytochemicals in *M. pruriens*, including polyphenols (tannins, flavonoids, gallic acid, phenolic acids), saponins, terpenoids, alkaloids, and fatty acids, have been reported with various pharmacological activities (see Supplementary Materials; Figure S2 and Table S1) [6,25–29]. Recent studies also suggest that phytochemicals apart from L-dopa may also contribute to the overall neuroprotective activities of *M. pruriens* [13,30]. Therefore, in this study, we prepared a *M. pruriens* seed extract (MPE) containing reduced L-dopa levels (<0.1%), which was subsequently evaluated for its neuroprotective effects using a panel of in vitro and in vivo assays. The seeds of *M. pruriens* were extracted/solvent-solvent partitioned in varying solvents to yield extracts, which were evaluated for L-dopa content by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). As shown in Table 1, the L-dopa levels in the initial methanol *M. pruriens* seeds extract was 28.0%, which was significantly reduced to 0.03% in the ethyl acetate *M. pruriens* extract (MPE). As even this low level (0.03%) of L-dopa could impart biological effects, we evaluated a pure L-dopa solution (<0.1%) in several of the in vitro assays. Our preliminary data showed that the MPE, but not this pure L-dopa was active in these assays (data shown in Supplementary Materials Figures S5 and S6). Therefore, this MPE extract was selected for further evaluation of its neuroprotective effects in a panel of cell-based and in vivo bioassays as described below.

MPE Reduces Hydrogen Peroxide (H₂O₂)-Induced Toxicity and Reactive Oxygen Species (ROS) Production in Microglia BV-2 Cells

Microglia are the native immune cells of the central nervous system (CNS) that undergo activation and proliferation to carry out phagocytosis, release inflammatory cytokines, and produce ROS and reactive nitrogen species (RNS) in response to injury and/or infection. Unresolved inflammation and excessive oxidant production by microglia are lethal to both neuronal and non-neuronal cells in the CNS and have been associated with PD. All of the Mucuna extracts including the crude methanol, hexanes, ethyl acetate (MPE), butanol, and water extracts (at 25 µg/mL) were evaluated for their protective effects against H₂O₂-induced toxicity in BV-2 cells. Our data showed that among the extracts, only the MPE significantly increased the viability of BV-2 cells exposed to H₂O₂ (see Supplementary Materials Figure S3A). Therefore, we evaluated the effects of MPE on oxidative stress induced by H₂O₂ in microglia BV-2 cells. MPE (12.5, 25, and 50 µg/mL) was non-toxic to BV-2 cells with cell viability greater than 90.3% at 24 h (Figure 1A). As shown in Figure 1B, the cell viability of H₂O₂-treated BV-2 cells decreased by 39.2%, as compared with the control group. Although MPE, at concentrations of 12.5, 25, and 50 µg/mL, showed a trend to ameliorate the H₂O₂-induced cytotoxicity in BV-2 cells, only MPE at a concentration of 25 µg/mL significantly increased the cell viability of H₂O₂-treated BV-2 cells, by 18.6%. The protective effects of MPE against the production of ROS by H₂O₂ in BV-2 cells were then evaluated. As shown in Figure 1C, the production of ROS in H₂O₂-treated BV-2 cells was elevated by 3.29-fold as compared with the control cells. MPE (12.5, 25, and 50 µg/mL) reduced the H₂O₂-induced production of ROS by 35.5, 29.1, and 61.6%, respectively, compared with the H₂O₂-treated BV-2 cells. Resveratrol (Resv; 20 µM), used as the positive control, reduced the H₂O₂-induced production of

ROS by 44.52%. These results are in agreement with our previous observation, wherein an *M. pruriens* water extract increased viabilities of murine BV-2 microglia and differentiated human SH-SY5Y neuronal cells that exposed to H₂O₂ [14]. Moreover, studies from other research groups also reported that *Mucuna* seeds powder (300 mg/kg/BW in diet) reduced oxidative stress in rodent sperm cells [31].

MPE Reduces Lipopolysaccharide (LPS)-Induced Nitric Oxide Species (NOS)

Production in Microglia BV-2 Cells and Protects SH-SY5Y Cells in a Co-Culture Model

Elevated production of NOS leading to massive neuronal death has been implicated in PD [32]. All of the aforementioned *Mucuna* extracts (at 25 µg/mL) were evaluated for their protective effects against LPS-induced NO production in BV-2 cells. Among the extracts, MPE showed the highest ability to reduce NO production in BV-2 cells exposed to LPS (see Supplementary Materials Figure S3B). Therefore, MPE was evaluated for its protective effects against neuroinflammation induced by LPS in BV-2 cells and in a non-contact co-culture model with SH-SY5Y neuroblastoma cells [21]. As shown in Figure 2A, LPS increased the NOS production in BV-2 cells by 4.46-fold as compared with the control group (control 8.987 µM vs. LPS 40.06 µM). MPE (12.5, 25, and 50 µg/mL) reduced the NOS production in LPS-stimulated BV-2 cells by 8.9, 37.8, and 60.1%, respectively, as compared with the cells treated with LPS alone. Resv (positive control; 20 µM), also reduced the NOS production by 43.2% in the LPS-treated BV-2 cells and was similar to our previous observation [21]. In the non-contact co-culture model (Figure 2B), conditioned media collected from BV-2 cells treated with LPS alone reduced the cell viability of SH-SY5Y cells by 43.1%. The conditioned media from treatment of LPS and MPE (12.5, 25 and 50 µg/mL) significantly increased the cellular viability of SH-SY5Y cells by 19.4,

23.2%, and 40.1%, respectively, as compared with the cells treated with media from LPS-treated BV-2 cells. The positive control, Resv (20 μ M), also increased the cell viability of SH-SY5Y cells by 29.3%. Our results support other studies on *Mucuna* reporting a reduction in nitrite levels induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the nigrostriatal region of Parkinsonian mice brain [33].

MPE Reduces Oxidative Stress Induced Cytotoxicity in SH-SY5Y Cells

Several neurotoxins, including 6-OHDA and MPP, induce oxidative cytotoxicity in dopaminergic neurons by multiple mechanisms and thus are used to model PD [2,34,35]. The protective effects of MPE were evaluated in SH-SY5Y neuroblastoma cells against oxidative stress induced neurotoxicity. MPE (12.5, 25, and 50 μ g/mL) did not induce cytotoxicity of SH-SY5Y cells after 24 h incubation (viability >90%) in the CTG assay (Figure 3A). Toxicity was induced in SH-SY5Y neuroblastoma by treatment with 6-OHDA, H₂O₂, and MPP⁺ (25 μ M, 100 μ M, and 2 mM, respectively). Treatment of 6-OHDA significantly reduced the viability of SH-SY5Y cells by 71.9% as compared with the control group, while MPE (12.5, 25, and 50 μ g/mL) reduced 6-OHDA-induced cell death of SH-SY5Y cells by increasing cell viability compared with the 6-OHDA treated group by 11.9%, 38.5%, and 23.9%, respectively (Figure 3B). Treatment of H₂O₂ reduced SH-SY5Y cells viability by 65.1% as compared with control, while MPE (at higher concentrations of 25 and 50 μ g/mL) showed moderate protective effects by increasing SH-SY5Y cell viability compared with cell viability of the H₂O₂ treated group by 19.5% and 16.3%, respectively (Figure 3C). MPP⁺ treatment significantly reduced the viability of SH-SY5Y cells by 46.7% as compared with control (Figure 3D); however, MPE showed no protective effects. Our findings obtained from these cellular PD models are in agreement with previously reported neuroprotective effects of *Mucuna* in neurotoxins-induced PD animal models. For

example, *Mucuna* treatment reduced 6-OHDA-induced L-dopa depletion in nigrostriatal tract of rats with PD symptoms [10,33].

MPE Reduces Lethality of MPP⁺ Induced Dopaminergic Neurotoxicity in C. elegans

The neurotoxin, MPTP, is metabolized to MPP⁺ by monoamine oxidase-B and is subsequently taken up by dopaminergic neurons, where it inhibits mitochondrial complex I, resulting in ATP depletion to induce neuronal death [35]. Therefore, we evaluated the effects of MPE against MPP⁺ dopaminergic neurotoxicity in wild type *C. elegans*. The effects of MPE in MPP⁺ induced neurotoxic paralysis and lethality in *C. elegans* were evaluated at concentrations of 20 and 40 µg/mL (IC₁₀ = 42.1 µg/mL). The median and maximum survival of worms after exposure to 750 µM MPP⁺ was 72 h (Table 2).

Treatment of MPP⁺ significantly reduced the median and maximum survival by 3.2-fold (72 h) and 3.5-fold (72 h), respectively, compared with worms in the control group (Figure 4A). MPE at 20 µg/mL significantly increased ($p < 0.001$) the median and maximum survival by 1.3-fold (96 h) and 1.9-fold (138 h), respectively, compared with worms treated with MPP⁺ alone (Figure 4B). MPE at 40 µg/mL significantly increased the mean and maximum survival in *C. elegans* by 1.8-fold (132 h) and 2.25-fold (162 h) respectively, compared with worms treated with MPP⁺ alone (Figure 4C and Table 2).

MPE Abrogates Chemically Induced Neurotoxicity in D. melanogaster

Changes in several behavioral phenotypes of *D. melanogaster* in response to genetically or chemically induced neurotoxicity have been exploited extensively to evaluate potential neuroprotective effects of therapeutics [36]. As MPE was significantly more neuroprotective at 40 µg/mL in reducing MPP⁺ induced

dopaminergic neurotoxicity in *C. elegans* (Figure 4), we used this dosage to determine its effect on climbing behavior (negative geotaxis) in *D. melanogaster* neurotoxin induced PD model. The two neurotoxins (6-OHDA and rotenone) used in our study induce a PD-like phenotype in *D. melanogaster* characterized by several behavioral changes including a muted innate negative geotaxis response due to locomotor defects. The aforementioned toxins generally injure dopamine neurons and cause behavioral defects including climbing, which can be measured by negative geotaxis assay. Similar to MPP⁺, rotenone is another mitochondrial complex I inhibitor that causes ATP impairment and ROS production and induces neuronal death [35]. In our study, *D. melanogaster* were exposed to 6-OHDA and rotenone to induce PD like phenotype. After 10 days, flies exposed to neurotoxins showed a highly muted climbing ability compared with control group. This loss of negative geotaxis ability was significantly ameliorated when flies were pre-treated with MPE.

The median climbing distance in 6-OHDA treated flies and rotenone treated flies was 18.6% (8.7 cm; $p \leq 0.05$) and 37.8 % (6.2 cm, $p \leq 0.001$) lower than in control flies (10.95 cm), respectively (Figure 5). Treatment of MPE alone significantly increased the climbing distance in flies by 42.5% (15.9 cm) compared with the control group (Figure 5). Pre-treatment with MPE abrogated the effect of neurotoxins on climbing behavior. In the MPE + 6-OHDA treated flies, the median climbing distance was 54.5% (13.95 cm) higher as compared with flies that were treated with 6-OHDA alone (Figure 5). In the MPE + rotenone treated flies, this was 48.7% (9.9 cm) higher than in flies that were exposed to rotenone only without any MPE pre-treatment (Figure 5). Our results on the neuroprotective effects of MPE on neurotoxin induced PD models using *C. elegans* and *D. melanogaster* support previous studies with Mucuna in rodent models of PD using MPTP [33] and 6-OHDA [25] and provide further evidence on the neuroprotective effects of non-L-dopa bioactives in MPE.

Conclusions

In summary, we developed a L-dopa reduced *Mucuna pruriens* extract (MPE) and evaluated its neuroprotective effects in murine microglia BV-2 and neuroblastoma SH-SY5Y cells. MPE treatment decreased BV-2 and SH-SY5Y cytotoxicity induced by oxidative stress and inflammation. In addition, MPE ameliorated dopaminergic neurotoxin-induced lethality in SH-SY5Y (6-OHDA), *C. elegans*, and recovered climbing ability *D. melanogaster* models for PD. Taken the data from the in vitro and in vivo experiments together, MPE showed neuroprotective effects in our PD models. Studies on the anti-PD effects of purified compounds isolated from MPE and their potential mechanism/s of action will be pursued by our group in the future.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6643/10/9/1139/s1>, Figure S1: LC-ESI-MS/MS spectra for quantifications of L-dopa in *Mucuna pruriens* extracts, Figure S2: HPLC-DAD chromatograms of *Mucuna pruriens* extracts, Figure S3: Effects of *Mucuna pruriens* extracts on the cell viability and LPS-induced NO production in murine BV-2 microglia, Figure S4: Morphology of BV-2 murine microglia treated with H₂O₂+MPE, H₂O₂+0.07% L-dopa, LPS+MPE, and LPS+0.07% L-dopa, Figure S5: Effects of MPE and 0.07% L-dopa on H₂O₂-induced toxicity in murine BV-2 microglia, Figure S6: Effects of MPE and 0.07% L-dopa on LPS-induced NO production in murine BV-2 microglia, Table S1: Chemical constituents of *Mucuna pruriens*.

Author Contributions: H.M., N.P.S., and D.A.V., conceived and designed the experiments; S.L.J., H.Y.P., and N.A.D., performed the experiments; S.L.J., H.Y.P., H.M., D.A.V., and N.P.S. analyzed the data. D.A.V. and N.P.S. contributed reagents and materials; S.L.J., H.M., H.Y.P., D.A.V., and N.P.S. wrote the paper. All authors read and approved the final manuscript.

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Table 1. Levodopa (L-dopa) content for each *Mucuna pruriens* seed extracts as determined by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

Extract	Yield (%; w/w)	L-dopa Content (%; w/w)
methanol	100	28.0
hexanes	3.1	0.54
ethyl acetate	4.7	0.03
butanol	45.3	10.05
water	46.9	21.39

Figure 1. Effects of *Mucuna Pruriens* Seeds Extract (MPE) (12.5, 25, 50 μg/mL) on cellular viability and reactive oxygen species (ROS) levels in BV-2 cells. Effects on BV-2 cellular viability by MPE alone (**A**); by MPE after H₂O₂-induced BV-2 cell toxicity (**B**); and on ROS levels after BV-2 cell exposure to H₂O₂ (**C**). All data expressed as mean ± standard error ($n = 3$), significance was reported by analysis of variance (ANOVA) followed with Dunnett multiple comparison testing, as compared with control $p \leq 0.001$ (###), and $p \leq 0.0001$ (####); as compared with toxic agent, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****). Resv—Resveratrol.

Figure 2. Effects of MPE (12.5, 25, 50 μg/mL) on production of nitric oxide in BV-2 microglia and resulting influence in non-contact co-culture in SH-SY5Y neuroblastoma. Effects on levels of nitric oxide produced in BV-2 induced with lipopolysaccharide (LPS) (**A**), and on SH-SY5Y cell viability after co-culture with BV-2 LPS-induced media (**B**). All data expressed as mean ± standard error ($n = 3$), significance was reported by ANOVA followed with Dunnett multiple comparison testing, as compared with control $p \leq 0.0001$ (####); as compared with toxic agent, $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****).

Figure 3. Effects of MPE (12.5, 25, and 50 $\mu\text{g/mL}$) on cellular viability of SH-SY5Y human neuroblastoma cells against toxic models of Parkinson's disease. Effects of MPE (12.5, 25, and 50 $\mu\text{g/mL}$) alone on SH-SY5Y cell viability (**A**), of MPE after 6-OHDA-induced toxicity (**B**), of MPE against H_2O_2 -induced toxicity (**C**), and of MPE against MPP^+ induced toxicity (**D**). Data shown as mean $\pm$ standard error ($n = 3$), significance was reported by ANOVA and subsequent Dunnett multiple as compared with control $p \leq 0.001$ (###), and $p \leq 0.0001$ (####); as compared with toxic agent, $p \leq 0.05$ (*), and $p \leq 0.01$ (**).

Table 2. Survival (median and maximum) of *C. elegans* (N2), as compared with 1-methyl-4-phenylpyridinium (MPP⁺) treatment (750 μ M). MPE treatment at both 20 and 40 μ g/mL significantly increased survival, as determined by log rank test (Mantel Cox), $n > 100$, $p \leq 0.05$ (*), $p \leq 0.001$ (***).

Survival (h)	MPP ⁺	MPP ⁺ + MPE (20 μ g/mL)	MPP ⁺ + MPE (40 μ g/mL)
Median	72	96 *	138 ***
Maximum	72	132 *	162 ***

Figure 4. Effects of MPE (20 and 40 µg/mL) on lifespan of *C. elegans* after MPP⁺ exposure. MPP⁺ at concentration of 750 µM reduces *C. elegans* lifespan, as compared with control group (**A**). MPE at concentrations 20 µg/mL (**B**) and 40 µg/mL (**C**) increase *C. elegans* lifespan, as compared with toxic MPP⁺ exposure (750 µM). Survival curves of *C. elegans* were statistically analyzed by log rank test (Mantel Cox), as compared with MPP⁺ treatment ($n > 100$).

Figure 5. Effects of MPE on negative geotaxis in *Drosophila melanogaster*. Effects of MPE (40 µg/mL) alone on climbing ability, by MPE after 6-OHDA (1 mM) exposure, and on MPE climbing ability after rotenone exposure (500 µM). Significance was determined as compared with control and $p \leq 0.05$ (#) and $p \leq 0.0001$ (#####); compared with toxic treatment using Welch's t-test with three replicates of $n > 40$, $p \leq 0.0001$ (****).

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CHAPTER 1

MANUSCRIPT 2

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Chemistry of Natural Products

Isolation and identification of compounds from *Mucuna pruriens* seeds

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Abstract

Mucuna pruriens, known to naturally contain levodopa, is used as a traditional medicine in Ayurveda to treat symptoms of Parkinson's disease (PD). Our group has previously reported that a levodopa-reduced *M. pruriens* ethyl acetate (MPEA) seed extract showed neuroprotective effects against PD in several *in vitro* and *in vivo* models but its chemical constituents remain unknown. Herein, a phytochemical investigation of MPEA led to the isolation and identification of phaseic acid (**1**), cis- and trans-dihydrophaseic acid (**2** and **3**), gancidin W (**4**), maculosin (**5**), levodopa (**6**), 2,6-dihydroxybenzoic acid (**7**), and protocatechuic acid (**8**). All of the compounds, apart from levodopa, are being reported from *Mucuna pruriens* for the first time. Compounds **3-8** were evaluated for their neuroprotective effects in human SH-SY5Y neuroblastoma cells.

Keywords: *Mucuna pruriens*, Parkinson's disease, neuroprotection, levodopa

Body

Mucuna pruriens L., belonging to the family Fabaceae, and commonly known as Mucuna or velvet bean, is a medicinal plant which naturally contains levodopa [1]. The seeds of this medicinal plant have been used traditionally to treat brain disorders associated with Parkinson's disease (PD) in the Indian traditional system of medicine, Ayurveda. Although levodopa is believed to be the major constituent in Mucuna responsible for its neuroprotective effects against PD symptoms [1,2], increasing evidence suggests that other phytochemicals may also contribute to the anti-PD effects of this natural product [3–5].

Our group has previously reported that a levodopa-reduced *M. pruriens* ethyl acetate (MPEA) seeds extract showed neuroprotective effects against PD in several *in vitro* and *in vivo* models including murine BV-2 microglia and human SH-SY5Y neuroblastoma cells, *Caenorhabditis elegans*, and *Drosophila melanogaster* [6]. However, the phytochemical constituents of this MPEA remain unknown. Herein, we report the isolation and identification of eight compounds from this MPEA including phaseic acid (**1**) [7], cis-dihydrophaseic acid (**2**) [7], trans-dihydrophaseic acid (**3**) [7], gancidin W (**4**) [8], maculosin (**5**) [9], levodopa (**6**) [10], 2,6-dihydroxybenzoic acid (**7**) [11], and protocathechuic acid (**8**) [11]. While levodopa (**6**) has been previously reported from *Mucuna pruriens* [12], this is the first report of the isolation and identification of compounds **1-5**, **7**, and **8** from *M. pruriens*.

Due to limited quantities, only isolates **3-8** (at non-toxic concentrations of 10 and 20 μ M) were evaluated for their neuroprotective effects in human neuroblastoma SH-SY5Y cells. The SH-SY5Y cells were pretreated with compounds **3-8** for 1 h after which neurotoxicity was induced with known neurotoxic agents, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP⁺). Although MPEA was previously

reported to show anti-PD effects in several *in vitro* and *in vivo* PD models [6], the purified isolates alone (at these test concentrations) were not effective in the SH-SY5Y cells. It is possible that the complex matrix of constituents present *in toto* in MPEA may exert their neuroprotective effects in an additive, synergistic and/or complementary manner. However, further studies would be required to confirm this which will be pursued by our group in the future. In summary, the isolation and identification of eight phytochemicals from a *M. pruriens* ethyl acetate seeds extract are reported here. Also, this is the first report of compounds **1-5**, **7**, and **8** from *M. pruriens*.

Experimental

General Experimental

The ^1H and ^{13}C nuclear magnetic resonance spectroscopic experiments were conducted on a Varian 400 MHz instrument. Deuterated methanol (CD_3OD) was used as solvent for the NMR experiments. Solvents were either ACS or HPLC grade purchased from Tansoole (Shanghai, China) or Sigma-Aldrich (St. Louis, MO, USA). Column chromatographic experiments were conducted on a C18 column with reverse-phased silica gel (12 nm, S-50 μm , YMC) and pre-coated silica gel GF254 plates (Qingdao Marine Chemical Plant, Qingdao, People's Republic of China).

Plant Material

Mucuna pruriens seeds were kindly provided by Verdure Sciences (Noblesville, IN, USA) and voucher specimen (No. PHPL/HB/013) are deposited in the Heber Youngken Herbarium and Greenhouse at the College of Pharmacy, the University of Rhode Island, RI, USA.

Extraction and Isolation

M. pruriens seeds were extracted with methanol to obtain a crude extract. The crude extract was suspended in distilled water and successively partitioned with petroleum ether, ethyl acetate, and *n*-butanol for 3 times to afford corresponding extract after solvent removal. The ethyl acetate extract was subjected to a silica gel column and eluted with dichloromethane and methanol (10:1, 8:1, 6:1, 4:1, 2:1, and 1:1; v/v) to obtain 6 fractions (Fr 1-6). Fraction 1 was purified by LH20 column to afford 7 fractions. Fraction 1 was purified by a C18 ODS column chromatography to obtain sub-fractions (Fr 1A1-1A7). Fraction 1A2 was further purified by reversed-phase semi-preparative HPLC with MeOH/0.1 % aqueous TFA (40:60; v/v) to afford compounds **4** and **2** (R_t = 12 and 14 min, respectively). Fraction 3 was purified by semi-preparative HPLC with MeOH/0.1 % aqueous TFA (20:80 – 16:84; v/v) to afford

compound **5** ($R_t = 14.3$ min). Fraction 2 was separated by LH20 column to afford 8 subfractions (Fr2A1-2A8). Fraction 2A2 was purified by HPLC with MeOH/0.1 % aqueous TFA (30:70 – 43:57; v/v) to obtain compounds **3** and **1** ($R_t = 12.5$ and 14.1 min, respectively). Fraction 2A6 was purified by HPLC with MeOH/0.1 % aqueous TFA (11:89 – 37.8:62.2; v/v) to obtain compounds **6**, **7**, and **8** ($R_t = 10.3$, 15.5, and 23.6 min, respectively).

Cell Culture

Human neuroblastoma (SH-SY5Y) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained as previously described [6]. Compounds were dissolved in DMSO to achieve stock solutions (10 mM) and were further diluted to desired concentrations in serum free media for cell treatments. Cell viability was evaluated after 24 h of treatment of test compounds using a Cell Titer Glo 2.0 (CTG; Promega, Madison, WI, USA) assay. The neuroprotective effects of selected test compounds (at 10 and 20 μ M) were evaluated against neurotoxic agents including 6-hydroxydopamine (6-OHDA; 25 μ M), and 1-methyl-4-phenylpyridinium (MPP⁺; 2 mM) using a previously described method [6].

Acknowledgments

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Figure 1: Chemical structures of compounds **1-8** from *Mucuna pruriens* ethyl acetate (MPEA) seeds extract.

Figure 2: Evaluation of single isolated compounds in human neuroblastoma (SH-SY5Y). Cell viability of each compound was assessed after 24h (A), exposure to 6-OHDA (B) and exposure to MPP⁺ (C). All data expressed as mean $\pm$ standard error ($n = 4$), significance reported by analysis of variance (ANOVA) followed by Dunnett multiple comparison tests, as compared to toxic agent $p \leq 0.0001$ (****).

Table 1: NMR of Isolated compounds were compared to published literature to confirm structure and name of compounds.

Compound Number	Compound Name	Publication
1	Phaseic acid	[7]
2	Cis-dihydrophaseic acid	[7]
3	Trans-dihydrophaesic acid	[7]
4	Gancidin W	[8]
5	Maculosin	[9]
6	Levodopa	[10]
7	2,6-Dihydroxybenzoic acid	[11]
8	Protocatechuic acid	[11]

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CHAPTER 2

MANUSCRIPT 3

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Polyphenol Microbial Metabolites Exhibit Gut and Blood–Brain Barrier Permeability and Protect Murine Microglia against LPS-Induced Inflammation

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Abstract

Increasing evidence supports the beneficial effects of polyphenol-rich diets, including the traditional Mediterranean diet, for the management of cardiovascular disease, obesity and neurodegenerative diseases. However, a common concern when discussing the protective effects of polyphenol-rich diets against diseases is whether these compounds are present in systemic circulation in their intact/parent forms in order to exert their beneficial effects *in vivo*. Here, we explore two common classes of dietary polyphenols, namely isoflavones and lignans, and their gut microbial-derived metabolites for gut and blood–brain barrier predicted permeability, as well as protection against neuroinflammatory stimuli in murine BV-2 microglia. Polyphenol microbial metabolites (PMMs) generally showed greater permeability through artificial gut and blood–brain barriers compared to their parent compounds. The parent polyphenols and their corresponding PMMs were evaluated for protective effects against lipopolysaccharide-induced inflammation in BV-2 microglia. The lignan-derived PMMs, equol and enterolactone, exhibited protective effects against nitric oxide production, as well as against pro-inflammatory cytokines (IL-6 and TNF- α) in BV-2 microglia. Therefore, PMMs may contribute, in large part, to the beneficial effects attributed to polyphenol-rich diets, further supporting the important role of gut microbiota in human health and disease prevention.

Keywords: polyphenol; gut microbial metabolites; permeability; equol; enterodiol; enterolactone; inflammation

Introduction

In the United States, obesity is a growing epidemic. There have been many studies that link obesity to an increased risk of developing other diseases, such as cardiovascular disease, diabetes, and Alzheimer's disease [1]. A quantitative systematic review estimated that the United States spent \$113.9 billion dollars total, or 4.8% of all healthcare spending in 2008 on overweight and obesity care [2]. The standard American diet consisting of high levels of saturated fat, sodium and sugars, contributes to these staggering obesity numbers [3]. People abiding by alternative diets in countries such as Greece and Italy exhibit decreased risks of obesity as well as confounding diseases such as metabolic syndrome [4].

Increasing evidence supports the beneficial effects of a traditional Mediterranean diet in the management of cardiovascular disease, obesity and most recently, neurodegenerative diseases [5]. The Mediterranean diet is characterized by high intake of polyphenols and unsaturated fats, with most of the beneficial effects from this diet being attributed to the high polyphenol intake [6,7]. There has been an increased interest in polyphenols for the treatment of neurodegenerative diseases as they exhibit the ability to cross the blood–brain barrier (BBB) and have a wide range of bioactive properties including antioxidant, anti-inflammatory, anti-apoptotic and lipid-lowering properties [8,9].

Polyphenols are a large class of secondary metabolites produced via the shikimate-derived phenylpropanoid or polyketide pathways, and they are characterized by the presence of two or more benzene rings bearing hydroxyl group(s) and lack any nitrogenous functional group in their core structure [10]. Polyphenols are further divided into several subclasses including stilbenes, flavonoids, lignans, and phenolic acids [11]. Although polyphenols exhibit biological effects in a variety of assays, their poor bioavailability, extensive phase-2 metabolism,

and whether they achieve physiologically relevant concentrations as their intact/parent forms to exert their protective effects, have been questioned. Rather, a growing consensus is that dietary polyphenols are metabolized by microbiota in the colon to yield bioactive gut microbial metabolites [12–16]. Herein, two common classes of dietary polyphenols were investigated, isoflavones and lignans, as well as their known polyphenol microbial metabolites (PMMs).

The isoflavones, genistein (GEN) and daidzein (DAI), are two soy-derived polyphenols that have been extensively studied for their bioactivities. Structurally similar to estrogen, isoflavones are comprised of a 3-phenylchromen-4-one backbone, modified by glycosides, O-substituents, and prenylated derivatives [17]. They exhibit the ability to serve as antioxidants, alleviate oxidative stress, and reduce the risk of hormone-dependent cancers [18–20]. GEN has been identified as a potential nutraceutical for Alzheimer's disease as it exhibits the ability to inhibit mitochondria-dependent apoptosis, and alleviate β -amyloid neurotoxicity, providing neuroprotection [21–23]. DAI also shows pro-apoptotic and neurotoxic effects against glutamate treatment in mouse hippocampal and cerebral cell cultures [24]. When metabolized in the gut by intestinal microflora, these isoflavones are converted into equol (EQ) (Figure 1) [25]. EQ has exhibited bioactive properties in cardiovascular disease, bone health, and cancers [26]. A recent study identified EQ as protective against oxidative stress in microglia, through the downregulation of neuronal apoptosis, and increased neurite growth [14].

Lignans often have complex structures made up of C6 and C3 units [27]. In plant tissues, lignans are often found as dimers and can be in the free state or sugar bound [28]. Data suggests a lignan-rich diet has numerous benefits such as prevention of hormone-dependent tumors, decrease in plasma cholesterol and glucose profiles, and delaying type 2 diabetes [29–31]. The common and prototypical dietary lignan,

secoisolariciresinol (SECO), is known to be deglycosylated by bacteria and converted to the PMMs enterodiol (ED) and enterolactone (EL) (Figure 1) [32].

A common question regarding dietary polyphenols is whether they are present in systemic circulation in physiologically relevant concentrations to exert their biological effects. Polyphenol concentrations in the blood generally range from 0.1–1.0 μM [33]. However, certain phenolic metabolites such as pyrogallol sulfate and catechol sulfate reached plasma concentrations ranging from 5–20 μM , while their parent compounds were undetected [34]. Absorption through the GI tract is complex as the pH changes as compounds traverse through the stomach to the intestines, which changes the bioavailability of substances [35]. The high-throughput parallel artificial membrane permeability assay (PAMPA) is used to determine permeability properties related to the transcellular in vivo absorption process of large compound libraries [36]. PAMPA has become a robust, versatile method for predicting passive permeability of compounds through the gastrointestinal (GI) track, the BBB, and skin [37,38]. A major concern for the development of pharmaceuticals for CNS-related diseases is the ability of compounds to cross the BBB, therefore it is of high importance to evaluate this ability early in drug discovery [39]. The BBB is a lining of endothelial cells that protects the brain from the peripheral nervous system [40]. PAMPA uses a combination of phospholipids specific to the membrane being tested along with a microfiber filter to simulate the biological membrane [41].

Herein, we propose that certain PMMs derived from gut microflora metabolism of their parent polyphenols are gut and BBB permeable and may provide protection against neuroinflammatory stress. To explore this hypothesis, we first performed in silico screening for gut and BBB permeability and then PAMPA of the parent polyphenols and their respective PMMs. Neuroprotective activity for all of the compounds was then assessed by evaluating the levels of nitric oxide species (NOS)

and inflammatory cytokines against LPS-induced inflammation in BV-2 murine microglia.

Materials and Methods

Compounds and Chemicals

Dimethylsulfoxide (DMSO) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Daidzein (DAI), (±) enterodiol (ED) (Cat# 45198-5MG-F), (±) enterolactone (EL) (Cat# 45199-1MG-F), genistein (GEN) and (±) secoisolariciresinol (SECO) (Cat# 60372-5MG-F) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). S-Equol was purchased from Chembest (Shanghai, China). Antipyrine, corticosterone, ketoprofen, ranitidine, theophylline and verapamil were purchased as PAMPA controls from Pion, Inc (Billerica, MA, USA).

In Silico ADME Predictors

The *in silico* tool SwissADME was used as a measure to predict BBB and gut permeability. Predicted permeability was assessed for each individual compound and control, as previously explained [56].

Parallel Artificial Membrane Permeability Assay (PAMPA)

Parallel Artificial Membrane Permeability Assay was performed to analyze both blood–brain barrier (BBB) and gut passive permeability. All materials for PAMPA were purchased from Pion, Inc (Billerica, MA, USA). Both the gut and BBB assays were performed according to the manufacturer's instructions. Briefly, test compounds and standards were prepared at 10 mM concentrations in DMSO. For the gut assay, Prisma HT buffer was adjusted to pHs 5, 6.2, and 7.4 by adding 0.5 M NaOH. In the deepwell plate provided by Pion, 1 mL of each pH-adjusted buffer was added to separate wells. For each pH, 5 µL of the sample was added to the 1 mL of buffer then mixed thoroughly with a pipette, diluting the sample to a final concentration of 50 µM.

Next, 200 μ L of the diluted sample was added to the donor (bottom) plate from the Pion sandwich assay, 5 μ L of GIT lipid matrix was added to the membrane on the bottom of the acceptor plate, and 200 μ L of acceptor sink buffer was added in each well of the acceptor plate. The sandwich was assembled, and the plate incubated at room temperature for 4 h undisturbed. In BBB assays, compounds (10 mM) were diluted to final concentrations of 50 μ M in Prisma HT Buffer (pH = 7.4). The PAMPA sandwich was created with the donor (bottom), BBB artificial membrane, and brain sink buffer as the acceptor (top). The assay was performed for 1 h at room temperature with shaking (60 μ m) using the manufacturer-supplied GIT Box. After incubation, the UV profiles of the donor and acceptor plates were read on the SpectraMax plate reader connected to the PAMPA software. PAMPA software calculated the $-\log P_e$ values for each compound and standard using the UV profiles of the donor and acceptor plates. Standards were all within 0.25 units of the expected value provided by PAMPA and each sample was averaged with 4 replicates.

Cell Culture Conditions

Murine microglia (BV-2) were generously provided by Dr. Grace Sun of the University of Missouri at Columbia. Cells were maintained in DMEM/F12 (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% (v/v) sterile filtered and heat inactivated Fetal Bovine Serum in addition to 1% (v/v) Penicillin/Streptomycin Antibiotic Solution (Life Technologies, Gaithersburg, MD, USA). Cells were incubated at approximately 65% relative humidity at 37 °C under atmospheric oxygen and 5% CO₂. Cells were passaged using Trypsin-EDTA (Life Technologies, Gaithersburg, MD USA), enumerated, and checked for viability using Trypan blue (Sigma Aldrich, St. Louis, MO, USA) dye exclusion. DMSO (Sigma

Aldrich, St. Louis, MO, USA) was used to dissolve compounds and did not exceed 0.1% when incubated with cells.

Cell Viability

The cytotoxicity of each compound was determined in BV-2. Briefly, cells were seeded in 96 white-walled, clear bottom plates at 100,000 cells/mL. Cells were allowed to adhere for 24 h. Compounds were prepared to 10 mM in DMSO, then diluted in serum-free media to yield concentrations of 20 and 10 μ M. Cells were treated with prepared concentrations of each compound for 24 h. Cellular viability was determined as a percentage of control (DMSO) by Cell Titer Glo 2.0 (CTG; Promega, Madison, WI, USA), read for luminescence on the SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

LPS Stimulation of Murine Microglia BV-2 Cells

As previously published, BV-2 cells were seeded at a density of 100,000 cells/mL in 24-well plates [57]. After reaching a confluency of 85%, cells were exposed to either DMSO, SECO (10 μ M), ED (10 μ M), EL (10 μ M), GEN (20, 10 μ M), DAI (20, 10 μ M), or EQ (20, 10 μ M), for 1 h prior to incubation of LPS (1 μ g/mL) for 23 h. Media were collected, aliquoted for nitric oxide and cytokine analysis.

Quantification of Nitric Oxide

Nitric Oxide (NO) was detected in BV-2 culture media following the stimulation of LPS for 24 h from the previously described experiment by way of the Griess Assay (Promega, Madison WI), according to the manufacturer's protocol.

Measurement of IL-6 and TNF- α

IL-6 and TNF- α were measured from BV-2 media using Enzyme Linked Immunosorbent Assay (ELISA) provided by Biolegend (San Diego, CA, USA) [57].

Statistical Analysis

All data are reported as mean $\pm$ standard errors of at least three independent biological samples. The analysis of all cellular data was conducted by ANOVA followed by Dunnett's test or Tukey's test for multiple comparisons of group means. The significance of the toxic agent compared to the control group is presented as $p \leq 0.001$ (###) and $p \leq 0.0001$ (####). The significance for tests compared to toxic treatment was defined as: $p \leq 0.03$ (*), $p \leq 0.002$ (**), $p \leq 0.0002$ (***) and $p \leq 0.0001$ (****). GraphPad Prism software 7.0 (GraphPad Software, Inc., San Diego, CA, USA) was used for all statistical analysis calculations and graphical representations.

Results

SwissADME Predicts Polyphenol Microbial Metabolites Are Highly Gut and BBB Permeable

Utilizing SwissADME in silico modeling, BBB and gut permeability were predicted (Table 1). All six of the compounds (parent polyphenols and their corresponding PMMs; Figure 1) were identified as having high gut absorbance. The positive PAMPA gut controls, verapamil, ranitidine, ketoprofen and antipyrine, all exhibited high gut permeability in the SwissADME predictor. ED, DAI and EQ were predicted to have BBB permeability. The PAMPA BBB controls, both BBB permeable and impermeable, were additionally screened in SwissADME, predicting passive permeability for verapamil and corticosterone, but no BBB passive permeability by theophylline. After this predictive measure, compounds were experimentally evaluated for passive permeability in the gut and BBB.

SECO, GEN, DAI, EL, and EQ Exhibit High Permeability through PAMPA Gut

Compounds were evaluated for their permeability through simulation membranes of the gut using PAMPA at pHs 5.0, 6.2, and 7.4 (Figure 2). Verapamil was used as the highly permeable control, ranitidine as the low permeability, and antipyrine was used as an intermediate control. Ketoprofen was used as a pH-dependent control. At pH 5, EL showed the highest permeability with a $-\log P_e$ value of 4.27 ± 0.05 , closely followed by EQ at 4.35 ± 0.11 . At pH 6.2, EQ showed the highest permeability with a $-\log P_e$ value of 4.20 ± 0.02 followed by EL at 4.37 ± 0.02 . At pH 7.4, the same trend followed as pH 6.2. SECO showed moderate permeability at all pHs tested, and DAI was most permeable at pHs 5 and 6.2. Parent compounds showed high permeability

in all three pH treatments, while the metabolite ED showed the lowest permeability of all test compounds in all three pH treatments tested (Figure 2).

GEN, EL, and EQ Show BBB Passive Permeability in PAMPA Assay

Isoflavones and lignans were evaluated for BBB penetration through PAMPA experiments (Figure 3). Verapamil, corticosterone and theophylline were controls for high, intermediate and low permeability, respectively, and yielded similar permeability measures as previously reported [35,39]. EL and EQ exhibited high permeability, with $-\log P_e$ values of 3.97 ± 0.14 and 3.52 ± 0.20 , respectively. Intermediate BBB permeability was seen for both GEN and DAI, with $-\log P_e$ values of 4.26 ± 0.16 and 4.53 ± 0.13 , respectively. SECO and ED exhibited low permeability through the BBB.

Isoflavones and Lignans Show No Cytotoxicity in Murine Microglia

Isoflavones were administered at 20 μ M, while lignans were dosed at 10 μ M. Parent polyphenols and PMMs were evaluated for cytotoxicity in murine microglia after a 24 h incubation period (Figure 4A,5A). There were no significant reductions in cellular viability at each of the test concentrations, thus indicating nontoxic levels.

Isoflavones Reduce NOS Production

Isoflavones were evaluated for their ability to reduce nitric oxide production after LPS induction, as determined by the Griess Reagent (Figure 4B). Cells treated with LPS produced 41.5 ± 5.6 μ M NOS, significantly more than the control (0.4 ± 0.02 μ M). GEN significantly reduced nitric oxide at both 20 and 10 μ M by 68% and 38%, respectively as compared to LPS alone. At 20 μ M, DAI and EQ also significantly reduced nitric oxide compared to LPS by 24 and 22%, respectively.

Isoflavones Reduce Pro-Inflammatory Cytokine Release

TNF- α was increased significantly to 687.7 ± 8.0 pg/mL after LPS stimulation, compared to the unstimulated control, 115.4 ± 2.8 pg/mL (Figure 4C). All three isoflavones significantly reduced TNF- α production compared to the LPS-induced treatment. GEN reduced TNF- α , by 29.1% and 16.6% at concentrations of 20 and 10 μ M, respectively. DAI and EQ exhibited similar, albeit less potent, protective abilities at both the high (20 μ M) and low (10 μ M) concentrations (Figure 4C). IL-6 production was significantly increased by LPS (103.7 ± 3.6 pg/mL) compared to the control (0.6 ± 0.05 pg/mL) (Figure 4D). All isoflavones were able to significantly reduce IL-6 production compared to the LPS-induced treatment. GEN was the most effective, reducing IL-6 production by 80.4 and 34.0% at 20 and 10 μ M, respectively (Figure 4D).

ANOVA followed by Tukey's post hoc test was used to determine significant differences amongst test compounds and anti-inflammatory potential. The 20 μ M dose of GEN resulted in significantly reduced concentrations of NOS, TNF- α , and IL-6 compared to all other test compounds and concentrations (Tables S1–S3 in supplementary files), clearly showing GEN as the most potent anti-inflammatory metabolite of the isoflavones and PMMs tested.

Lignans Limit NOS Production

Nitric oxide concentrations for vehicle-treated microglia were approximately $11.97 \mu\text{M} \pm 0.07$, whereas LPS stimulation increased this concentration to $51.01 \mu\text{M} \pm 1.23$. SECO, ED and EL decreased nitric oxide release by approximately 9.13 ($46.35 \mu\text{M} \pm 0.22$), 8.54 ($46.65 \mu\text{M} \pm 0.36$) and 30.07% ($35.67 \mu\text{M} \pm 0.74$), respectively (Figure 5B).

SECO, ED and EL Significantly Reduce IL-6 and TNF- α

As expected, IL-6 and TNF- α concentrations were elevated in LPS-treated microglia with concentrations of 665.7 pg/mL $\pm$ 5.8, and 518.4 pg/mL $\pm$ 8.8, respectively (Figure 5C, 5D). IL-6 concentration decreased by 1.05 % (672.8 pg/mL $\pm$ 13.9) in samples treated with SECO. ED and EL pre-treated conditions decreased the release of IL-6 by approximately 3.74% (690.6 pg/mL $\pm$ 5.30) and 26.41% (489.9 pg/mL $\pm$ 11.31), respectively. With respect to TNF- α , SECO, ED and EL reduced cytokine release by 3.97% (497.8 pg/mL $\pm$ 12.42), 0.5% (515.8 pg/mL $\pm$ 7.12) and 29.47% (365.6 pg/mL $\pm$ 4.91), respectively. Like GEN above, EL showed significantly greater reduction of inflammatory cytokines when compared to SECO and EL (Tables S4-S6).

Discussion

The overall health of humans and communities depends on many factors including genetics, various environmental factors, and diet. Increased adherence to the Mediterranean diet was recently correlated with a reduced risk of neurodegenerative diseases, including prodromal Parkinson's disease [5,42]. In this report, we sought to explore two common classes of polyphenols found in the Mediterranean diet, namely isoflavones and lignans, to investigate the bioavailability and bioactivity of these compounds and their metabolites.

Previously published literature suggests that for polyphenols to be bioactive, they must first be transformed in the colon by the gut microbiota into molecularly unique metabolites [16]. Compounds which are permeable through the gut mucosa enter the bloodstream and circulate in the body. The *in vivo* permeability may change if these substances are compatible with active transporters, as well as differences based on the individual's GI environment and diet [43]. A number of factors contribute to the absorption ability of a compound, namely, physicochemical (e.g., pKa, solubility, polarity), physiological (e.g., GI pH, GI blood flow), and dosage form (e.g., tablet, capsule) [44].

To investigate the role of lignans, isoflavones, and PMMs in systemic circulation, we explored gut permeability through *in silico* (SwissADME) and *in vitro* (PAMPA) models. Gut PAMPA results largely coincided with those of *in silico* predictions and showed that the test compounds, with the exception of ED, passively permeate the gut. The presence of O-methyls on SECO and lactone moiety on EL may provide these molecules with additional lipophilicity needed to cross the membrane (Figure 1), whereas ED contains free hydroxyl groups, which may reduce passive permeability. These data are supported by previously published results such as the

evaluation of GI absorption of alkaloids from *Coptis* [45]. PAMPA is a useful tool for predicting a compound's permeability through the human gut, however, it only considers the rate of passive transport [36].

The BBB serves as a protective barrier of the brain, comprised of endothelial cells that create tight junctions, allowing for extremely selective passive permeability [40]. This typically results in higher penetrability by lipophilic compounds through the BBB [46]. Compounds that pass through the BBB may influence the brain state and physiology. We sought to identify if the aforementioned isoflavones, lignans, and PMMs can passively cross the BBB and could be relevant in modulating the pathogenic features of neurodegenerative diseases.

Computational predictive software, SwissADME, identified EL, DAI and EQ as BBB-penetrable molecules. Interestingly, neither GEN nor SECO were considered permeable. Furthermore, our PAMPA in vitro study supported these results, reporting high permeability of EL and EQ. SECO and ED exhibited no apparent permeability. The parent compounds of isoflavones, GEN and DAI, were identified as intermediate permeable compounds. Similar investigative strategies have identified high permeability of compounds derived from natural sources through PAMPA–BBB [47]. While there are many other factors that contribute to BBB permeability, such as plasma concentration, plasma binding, and metabolic modifications by barrier enzymes, PAMPA is a good predictor of passive movement across the BBB [35,46]. The relationship between gut and BBB permeability is further complicated by presystemic metabolism. For instance, previous studies have shown that following gut metabolism, EL predominantly exists as a glucuronide conjugate, certainly affecting its BBB permeability and overall bioavailability [48].

We identified that these PMMs, specifically EL and EQ, passively cross both the gut and BBB barriers. To further identify the potential bioactivities of these molecules,

we examined their protective ability against inflammation in microglia. Microglia are the resident macrophages of the brain that work to eliminate debris from the brain [48]. However, continuous activation of microglia can lead to the over-production of inflammatory cytokines, as identified in postmortem brains of Alzheimer's and Parkinson's disease patients [49]. One mechanism of microglia activation is the increase in LPS levels in peripheral blood, due to poor diet [50,51]. The continued elevated levels of these pro-inflammatory cytokines, specifically IL-6 and TNF- α , can stimulate the recruitment and activation of other microglia, further inducing the production of reactive oxygen species and nitric oxide species [52]. NOS at low doses is critical for maintaining healthy microglia and neuron function, but at high doses can induce necrosis or apoptosis [53]. Phenolic compounds have previously been linked to reduced microglia-induced neuroinflammation [54]. At 20 μ M, all tested isoflavones were able to significantly reduce nitric oxide production, and TNF- α and IL-6 concentrations compared to LPS alone. These results are consistent with previous reports of isoflavones [14]. However, GEN clearly showed the greatest anti-inflammatory properties when compared to SECO and EQ. Furthermore, we subjected SECO and its microbial-derived metabolites to neuroprotective assays. Among the lignans tested, only the gut-derived metabolite EL was able to significantly reduce IL-6 and TNF- α production. Additionally, EL showed significantly greater reduction in nitric oxide concentrations than its parent metabolite and ED. Recent studies with certain polyphenol metabolites, namely, gallic acid derivatives, demonstrated that these metabolites pass across BBB endothelium and provide neuroprotective effects through modulation of the NF- κ B pathway [55]. It is clear from these previous studies and the work presented here that the neuroprotective potential

of BBB-permeable metabolites is an important area for further investigation to understand the relationship between dietary intake of polyphenols and brain health.

In summary, the fate of microbial metabolites is largely governed by their ability to permeate through biological barriers. After investigating two classes of polyphenols often found in the Mediterranean diet, namely, isoflavones and lignans, our data suggest that their gut microbial metabolites, but not parent compounds, may enter the blood, cross the BBB and provide protection against neuroinflammation. The isoflavone parent compound, DAI, exhibits high gut permeability, but intermediate BBB permeability. DAI's microbial metabolite, EQ, exhibits high permeability in both the gut and BBB, as well as significantly reducing nitric oxide and the production of pro-inflammatory cytokines in murine microglia. GEN shows permeability through both the gut and BBB and very strong anti-inflammatory effects. However, gut microbial metabolism would likely limit the circulating levels of GEN in the blood. The lignan SECO has extremely limited membrane permeability, but its gut microbial metabolite EL passively penetrates both the gut and BBB. Additionally, EL was most effective out of the lignans tested in inhibiting NOS and pro-inflammatory cytokines in vitro.

These data, supported by previously published literature, suggest the further investigation of gut-microbial-derived metabolites, specifically EL and EQ, for the treatment of neurodegenerative diseases both in vitro and in vivo. Furthermore, using a system like PAMPA can aid in refining in vitro testing to ultimately inform the design of more efficacious animal studies.

Supplementary Materials: The following are available online at <http://www.mdpi.com>, Tables S1–S6, which detail additional statistical analysis.

Author Contributions: N.P.S and M.J.B conceived and designed the experiments; S.L.J., R.D.K., N.A.D., H.M., performed the experiments; S.L.J, R.D.K., N.P.S., and M.J.B., analyzed the data; N.P.S and M.J.B contributed reagents and materials. S.L.J., R.D.K., N.A.D., H.M., N.P.S and M.J.B wrote the paper and approved the final manuscript.

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Figure 1. Overview of the polyphenol isoflavones and lignan parent compounds and their respective polyphenol microbial metabolites produced by gut microflora. (Genistein = GEN, daidzein = DAI, equol = EQ, secoisolariciresinol = SECO, enterodiol = ED, enterolactone = EL).

Table 1. In silico SwissADME predictive permeability of parent polyphenols and their microbial metabolites in the gut and blood–brain barrier (BBB).

Compound	Molecular Weight (g/mol)	Gut Absorption	BBB Permeability
SECO	362.42	High	No
ED	302.36	High	No
EL	298.33	High	Yes
GEN	270.24	High	No
DAI	254.24	High	Yes
EQ	242.27	High	Yes
Antipyrine	188.23	High	Yes
Corticosterone	346.46	High	Yes
Ketoprofen	254.28	High	Yes
Ranitidine	314.40	High	No
Theophylline	180.16	High	No
Verapamil	454.60	High	Yes

Figure 2. Parallel artificial membrane permeability assay (PAMPA) gut passive permeability at three relevant pH levels: 5.0 (**A**), 6.2 (**B**), and 7.4 (**C**). Controls verapamil, antipyrine and ranitidine exhibit high, medium and low penetrability, respectively. Ketoprofen shows variable permeability with pH change.

Figure 3. Assessment of blood–brain barrier passive permeability of polyphenols determined by PAMPA. Positive (verapamil), intermediate (corticosterone) and low (theophylline) permeability controls.

Figure 4. Effects of isoflavones against LPS-induced oxidative stress in BV-2 murine microglia. Isoflavones exhibited no cytotoxic effects in murine microglia (**A**). Isoflavones (20 μ M and 10 μ M) reduce nitric oxide production, as determined by the Griess Reagent (**B**). Isoflavones inhibited the production of the pro-inflammatory cytokines, TNF- α (**C**) and IL-6 (**D**), in murine microglia. All data expressed as mean $\pm$ standard error ($n \geq 3$), significance was reported by analysis of variance (ANOVA) followed with Dunnett multiple comparison testing. Significance as compared with control $p \leq 0.0001$ (####); as compared with LPS, $p \leq 0.03$ (*), $p \leq 0.002$ (**), $p \leq 0.0002$ (***) and $p \leq 0.0001$ (****).

Figure 5. Effects of lignans in murine microglia cells. Lignans were evaluated for cytotoxicity in BV-2 (**A**). Effect of lignan treatment (10 μM) on nitric oxide production, as determined by the Griess Reagent (**B**). Lignans reduce the production of pro-inflammatory cytokines in murine microglia, TNF-α (**C**) and IL-6 (**D**). All data expressed as mean ± standard error ($n \geq 3$), significance was reported by analysis of variance (ANOVA) followed with Dunnett multiple comparison testing. Significance as compared with control $p \leq 0.0001$ (####); as compared with LPS, $p \leq 0.002$ (**), $p \leq 0.0002$ (***) and $p \leq 0.0001$ (****).

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CHAPTER 2

MANUSCRIPT 4

Phytoestrogen, Equol, provides protection against toxin-induced models of Parkinson's disease

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Abstract

Parkinson's disease is a complex neurodegenerative disease that attacks dopaminergic neurons of the substantia nigra, resulting in a variety of locomotor deficits. Disease severity, progression, and drug response appear to be influenced by gender, suggesting a protective role of estrogen. Phytoestrogens, genistein, daidzein, and gut-derived microbial metabolite equol, exhibit antioxidant activity, alleviate oxidative stress, and most recently show neuroprotective ability. Herein, we investigate these three phytoestrogens' for their protective abilities against toxin-induced models of Parkinson's disease. In a co-culture model, isoflavones significantly improved cellular viability as compared to LPS-induced murine microglia cell supernatant. Equol was most successful in protecting against neuronal cell death induced by 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP⁺), improving cell viability by 31% and 18% at 20 μ M, respectively. Parent compounds, genistein, and daidzein, failed to consistently improve cellular viability. Therefore, equol was assessed in *Caenorhabditis elegans* and *Drosophila melanogaster*. In *C. elegans*, equol significantly improved lifespan as compared to toxic agent MPP⁺. In *D. melanogaster*, equol treatment for 7 days exhibited minor improvements in climbing ability as compared to 6-OHDA alone. Moreover, our data suggest a potentially protective role of equol, but not parent compounds, against toxic models of Parkinson's disease.

Keywords: Parkinson's disease, phytoestrogens, isoflavones

Abbreviations: PD: Parkinson's disease, ER β : Estrogen receptor β , EST: 17 β -Estradiol, GEN: Genistein, DAI: Daidzein, EQ: S-Equol, LPS: Lipopolysaccharide, 6-OHDA: 6-Hydroxydopamine, MPP⁺: 1-Methyl-4-phenylpyridinium

Introduction

Parkinson's disease (PD) is a multifaceted neurodegenerative disease that can present as a wide variety of symptoms, but a clinical diagnosis is determined by the presence of motor difficulties such as tremors, bradykinesias, rigidity and postural instability, and exclusion of any other diseases¹. PD is anticipated to affect 930,000 by 2020². Pathological characteristics of PD are the loss of dopamine specifically in the substantia nigra, as well as the accumulation of α -synuclein³. Pharmaceutical interventions primarily focus on replenishing the lost dopamine, typical treatment includes levodopa coupled with a dopamine decarboxylase inhibitor⁴. Recent focus has shifted to stop the progression of the disease by exploring alternative targets such as genetic mutations or neuroinflammation^{5,6}. Interestingly, PD progression, incident rates, and pharmaceutical response seem to be influenced by gender⁷. For instance, male prevalence rates are 1.5 times higher than in females⁸. Additionally, risk substantially increases in females after menopause, suggesting a potential protective role of estrogen against PD⁹.

The protective role of estrogen in PD has been explored in multiple recent studies. In mice, estrogen treatment modified the activation of astroglia and microglia, and nigrostriatal disruption against toxin-induced PD¹⁰. Similarly, a recent clinical study compared striatal dopamine transporter (DAT) availability in females, reporting lower DAT in the brain, suggesting estrogen may have a beneficial effect on dopaminergic neurons¹¹. Taken together, these studies support a protective role for estrogen against PD. A systematic meta-analysis found no positive relationship between hormone replacement therapy and a decreased risk in PD¹². This discrepancy may be due to the massive role estrogen plays throughout the body.

Therefore, we hypothesize that naturally occurring estrogens may have similar neuroprotective abilities without performing all estrogenic activities. Phytoestrogens classified as isoflavones exhibit excessive antioxidant activity, alleviate oxidative stress, and selectively bind to ER- β ^{13–17}. The two most common isoflavones, genistein (GEN) and daidzein (DAI) are found primarily in soy and have been extensively studied. Previous reports show GEN inhibits mitochondria-dependent apoptosis, and alleviates β -amyloid neurotoxicity, providing neuroprotection^{18–20}. Similarly, DAI demonstrates pro-apoptotic and neurotoxic effects against glutamate treatment²¹. The bioavailability of these compounds after consumption has been disputed, evidence suggests that gut microbial-derived metabolites are much more abundant²². Gut microbial metabolite, equol (EQ), has been shown to protect against cardiovascular disease, bone health, and specific cancers^{23,24}. In our recent publication, we showed that of these isoflavones, EQ is most likely to cross the blood-brain barrier and protects against inflammation in murine microglia models²⁵.

As phytoestrogens and known agonists of estrogen receptor beta (ER- β), we hypothesized that these isoflavones, EQ, in particular, will provide protection in Parkinson's disease models. Herein, we evaluate positive control 17 β -estradiol, genistein, daidzein and equol in toxin-induced models of Parkinson's disease in cells, *Caenorhabditis elegans* and *Drosophila melanogaster*.

Materials and Methods

Chemicals

Dimethylsulfoxide (DMSO), lipopolysaccharide (LPS), 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP⁺) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DMEM/F-12, phenol red-free DMEM medium, and trypsin-versene were purchased from Life Technologies (Grand Island, NY, USA).

Compounds

17 β -Estradiol (EST), daidzein (DAI), and genistein (GEN) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). S-Equol (EQ) was purchased from Chembest (Shanghai, China).

Cell Culture Maintenance

Murine microglia (BV-2) were generously gifted by Dr. Grace Y. Sun (University of Missouri at Columbia, MO, USA). Human neuroblastoma (SH-SY5Y) were obtained from American Type Culture Collection (ATCC, VA, USA). Cells were maintained in DMEM/F-12 high glucose (4.5 g/L), supplemented with 10% inactivated FBS and 1% P/S (100 U/mL penicillin, 100 mg/mL streptomycin) (Life Technologies, Gaithersburg, MD, USA) at 37°C in 5% CO₂. EST and isoflavones were prepared to 10 mM in DMSO, and further diluted in serum-free DMEM/F-12 media. Control wells contained 0.1% DMSO.

Cytotoxicity Screen

Cytotoxicity of compounds at 20 μ M in SH-SY5Y were assessed with Cell Titer Glo 2.0 (CTG) (Promega, Madison, WI, USA) after 24 h treatment, cell viability was determined as percentage of control²⁶.

Non-Contact Co-Culture

The non-contact co-culture experiment was performed using treated media from BV-2 cells on human neuroblastoma, as previously described²⁷. BV-2 were pretreated with isoflavones for 1 h, then induced with lipopolysaccharide (1mg/mL) for 23 h. Media was removed from BV-2 and placed on SH-SY5Y, seeded in white-walled 96-well plates at 100,000 cells/mL. SH-SY5Y were then incubated for 24 h, cell viability was then assessed using CTG.

Cellular Viability against Toxic Agents

Human neuroblastoma were seeded in white-walled 96-well plates at 100,000 cells/mL for 24 h. Cells were incubated with corresponding treatments for 2 h in serum-free media, then induced with toxic agents for 24 h: 6-OHDA (100 μ M) or MPP⁺ (2 mM). Cellular viability was determined by CTG, illustrated as percentage of control.

Caenorhabditis elegans

C. elegans were maintained and assayed for lifespan against MPP⁺ as previously described²⁸. Briefly, wild type (N2) were maintained on normal nematode growth media at 20°C. Nematodes were age synchronized, and at L1 40 μ L worms were plated in a 96-well microplate (approximately 20 worms/well). Worms were plated with *Escherichia coli* OP50 (5 mg/mL), MPP⁺ (750 μ M), and Equol (17.5°C or 30 μ M). Control groups received S-complete media. Post treatment, live worms were counted every 12 h until no live worms remained.

Drosophila Melanogaster Maintenance and Treatment

Canton S (w[*]) flies were a kind gift from Dr. Belinda Barbagallo (Salve Regina University, Newport, RI). Flies were maintained and age synchronized on Nutri-Fly BF (Genesee Scientific, San Diego, CA). On day 1 post eclosion, flies were separated by gender to approximately 20 flies per vial. Treatments were added to the media on day 3 post eclosion, control received 5% sucrose and equol received 100

μM diluted in 5% sucrose. Treatment vials were exchanged every other day for 7 days.

Climbing Assay

After 7 days of treatment, flies were assayed for climbing ability^{28,29}. Briefly, flies were placed in empty vials marked at 5 cm and allowed to acclimate for 30 minutes. Flies were gently tapped to the bottom of the vial and a picture was taken after 10 seconds using a Nikon D2200 camera (Nikon, Tokyo, JP). Percent climbing was calculated by ((# flies above line / total # flies)*100).

Statistical Analysis

All data are reported as mean ± standard errors of at least three independent biological samples. Analysis of all cellular and *Drosophila* data were conducted by ANOVA followed by Dunnett's test for multiple comparisons of group means. Significance of the toxic agent compared to the control group is presented as $p \leq 0.01$ (##) and $p \leq 0.0001$ (####). Significance for tests compared to toxic treatment was defined as: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Lifespan analysis for *C. elegans* identified using the Kaplan–Meier, tested for statistical significance using the log-rank test (Mantel-Cox). GraphPad Prism software 8.0 (GraphPad Software, Inc., San Diego, CA) was used to create all graphs and for all statistical analysis calculations.

Results

Cytotoxicity Screen

Compounds were tested for cytotoxicity at 20 μ M in neuroblastoma (Figure 1). No significant difference was seen between control and isoflavone treatment, indicating safe concentrations.

Effects of Isoflavones in Non-Contact Co-Culture

Treatment with LPS media reduced SH-SY5Y viability to $56.9 \pm 1.2\%$, as compared to control, $100 \pm 2.5\%$ (Figure 2). Isoflavones at all concentrations exhibited significantly increased cellular viability as compared to LPS treatment alone. GEN significantly increased cellular viability by 11 and 6%, at 20 μ M and 10 μ M, respectively. Treatment with DAI (20 and 10 μ M) increased cellular viability by 11 and 16%. EQ significantly increased cellular viability by 17 and 14% at 20 and 10 μ M, respectively.

Human Neuroblastoma PD Model

Human neuroblastoma (SH-SY5Y) were analyzed against PD toxic insults 6-OHDA and MPP⁺ (Fig. 3). 6-OHDA significantly decreased cellular viability to $35.3\% \pm 6.8$, as compared to control (100 ± 6.5). DAI (20 μ M) improved viability by 26%, compared to 6-OHDA. EQ at both concentrations increased cellular viability by 31 and 27%, compared to toxic treatment.

In the MPP⁺ model, control exhibited $100 \pm 3.0\%$ viability, whereas MPP⁺ treatment significantly reduced cellular viability to $57.8 \pm 2.3\%$. DAI significantly increased cellular viability by 20 and 15% at 20 μ M and 10 μ M, respectively. 20 μ M and 10 μ M of EQ significantly improved cellular viability by 18% and 19%, as compared to MPP⁺.

C. elegans Lifespan

We further evaluate EQ against toxic agent, MPP⁺, in the *C. elegans* model. MPP⁺ treatment alone significantly reduced lifespan to 72 h (Fig. 4A). EQ at 17.5 and 35 μ M significantly improved the median lifespan to 108 h (Fig. 4B &C).

Drosophila melanogaster Negative Geotaxis

D. melanogaster were evaluated for EQ (100 μ M) protection against 6-OHDA toxicity in the negative geotaxis assay (Fig. 5). 6-OHDA treatment for one week failed to significantly reduce the climbing ability of male flies. EQ treatment exhibited increased climbing ability as compared to 6-OHDA, however not significantly.

Discussion

There has been an increased interest in flavonoids for the prevention of neurodegenerative diseases, as they exhibit the ability to cross the BBB and have a wide range of bioactive properties including antioxidant, anti-inflammatory, antiapoptotic and lipid-lowering properties^{30,31}. PD is a neurodegenerative disease characterized by the loss of dopaminergic neurons in the substantia nigra, leading to tremors, bradykinesias, and further dementias. Dopaminergic neuronal death in PD has also been heavily linked to increases in neuroinflammation³². Endogenous estrogen exhibits the ability to reduce neuroinflammation and protects against neurodegeneration in PD³³. Herein, we explored the effects of naturally occurring phytoestrogens, namely, isoflavones, in the protection against toxin-induced PD models in neuroblastoma and drosophila.

Oxidative stress and inflammation induced by activated microglia have become key pathological features of PD, resulting in increased levels of cytokines and dopaminergic neuronal loss localized to the substantia nigra^{34,35}. Multiple recent publications have identified a protective role of gut-derived microbial metabolite, equol, in astrocytes and microglia induced with LPS^{36,37}. Further, we explored the relationship between activated microglia and neuronal death *in vitro*²⁷. We show that EQ treatment in murine microglia followed by LPS-induced inflammation produces lower neuronal death in our co-culture experiment. This indicates that EQ may reduce oxidative and inflammatory stress in microglia, protecting neurons against toxic agents.

Toxin-induced models of PD have proven to be useful in discoveries regarding the treatment and progression of the disease. Human neuroblastoma cell line SH-SY5Y is heavily used in PD research as it has dopaminergic properties allowing for

PD-like phenotypes when induced with toxic agents such as 6-OHDA, or MPP⁺³⁸. 6-OHDA leads to the death of dopaminergic neurons, proposed to involve the generation of free radicals³⁹. MPP⁺ mimics PD symptoms by altering mitochondrial function, leading to increased oxidative stress and abnormal protein aggregation^{39,40}. EQ exhibited significant protection against both 6-OHDA and MPP⁺ induced toxicity in the neuroblastoma model. Although EQ has not previously been published in PD models of SH-SY5Y, it is in agreement with previous reports of a combination of GEN, DAI and EQ neuroprotective abilities⁴¹. Further *in-vivo* investigation supports this protective role of EQ against MPP⁺, but not 6-OHDA. Future investigations of EQ should explore both toxic and transgenic models of PD to determine mechanisms of protection.

Herein, we report that gut microbial metabolite, Equol, exhibits neuroprotective activities in Parkinson's disease models *in vitro* and *in vivo*. Highlighted by the reduction in toxin-related cell and *C. elegans* death. Our results warrant further investigation of Equol as a future intervention for PD.

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Conflict of Interest

The authors have declared no conflict of interest.

Figure 1. Cytotoxicity evaluation of isoflavones at 20 μ M after 24h on human neuroblastoma (SHSY5Y). Data are expressed as mean $\pm$ standard error, presented as percentage compared to control ($n \geq 3$).

Figure 2. Effects of isoflavone treated, LPS-induced murine microglia cell supernatant on human neuroblastoma cellular viability. Data are expressed as mean $\pm$ standard error ($n \geq 3$), reported as percentage compared to control. Statistical significance was evaluated using ANOVA followed with Dunnett's multiple comparison test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ as compared to control cells. Toxin insult was compared to control, ## $p < 0.01$.

Figure 3. Isoflavones protect against toxic inducers of PD in human neuroblastoma. Cells were treated, then exposed to 25 μM 6-OHDA (A) and 2 mM MPP⁺(B) for 24h, where cellular viability was assessed. Data are expressed as mean ± standard error, exhibited as percentage compared to control (n≥6). Statistical significance was evaluated using ANOVA followed with Dunnett's multiple comparison test, *p < 0.05, ** p < 0.01, ***p < 0.001, and ****p < 0.0001. Toxin insult was compared to control, ####p < 0.0001.

Figure 4. Effects of Equol (17.5 and 35 μ M) on lifespan of *C. elegans* exposed to MPP⁺ (750 μ M). Toxin reduces lifespan in control group (A). EQ at 17.5 (B) and 35 μ M (C) exhibit protective effects versus MPP⁺. Survival curves were statistically analyzed by log rank test (Mantel Cox), as compared to MPP⁺ treatment (n >100).

Figure 5. Effects of Equol on climbing ability of *Drosophila melanogaster*. Influence of equol (100 μM) treatment on wildtype flies alone and induced with 6-OHDA (1 mM) on climbing ability after 7 days of treatment.

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CHAPTER 3

MANUSCRIPT 5

Direct thrombin inhibitor, Dabigatran, protects against neuroinflammation in LRRK2 transgenic *Drosophila melanogaster* model of Parkinson's disease

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Abstract

Parkinson's disease (PD) is a complex neurodegenerative disease characterized by the presence of tremors, loss of dopaminergic neurons and accumulation of α -synuclein. While there is no single direct cause of PD, genetic mutations, exposure to pesticides, diet and traumatic brain injury have been identified as risk factors. Increasing evidence supports that activated microglia, and the resulting neuroinflammation plays a critical role in disease progression. Our lab has previously identified the neurotoxic role of thrombin, linked to neuroinflammation. For the first time, we explore direct thrombin inhibitor, dabigatran, in a transgenic Parkinson's disease *Drosophila melanogaster* model. After 7 days of dabigatran treatment, male flies exhibited significant improvement in climbing ability, as compared to the vehicle. To elucidate the mechanism of locomotor protection, we investigate the production of nitric oxide (NOS) and reactive oxygen species (ROS). Dabigatran significantly reduced both NOS and ROS in male transgenic fly head homogenates. Western blot analysis confirmed that iNOS correlates with NOS production in male flies. SOD1 and NOX4 expression were reduced with dabigatran treatment, potentially contributing to ROS reduction. Our results indicate that dabigatran treatment improves motor function by reducing oxidative stress. We propose that inhibiting thrombin may provide a new therapeutic opportunity for potentially reducing PD associated pathologies. These initial findings should be investigated in alternative transgenic models of PD and evaluated for effects on behavior as well as biochemical markers of pathology to further elucidate mechanisms of action.

Keywords: Parkinson's disease, Dabigatran, Neuroinflammation

Introduction

Neurodegenerative diseases currently cost the United States billions of dollars annually^{1,2}. The two most common neurodegenerative diseases are Alzheimer's (AD) and Parkinson's disease (PD)^{3,4}. Currently, there are no interventions that can stop disease progression. Although the symptoms and disease progression of AD and PD are vastly different, both diseases are associated with protein aggregation, genetic predisposition, mitochondrial dysfunction, and neuroinflammation⁵.

Neuroinflammation has been recognized as a major factor in the pathogenesis AD and PD^{1,2,6–10}. The neuroinflammatory response in AD and PD is similar, characterized by phenotypic alterations in several cell types, including astrocytes, microglia, and endothelial cells⁵. Under normal conditions, microglia work to detect pathogens or tissue damage and elicit a response to clear cellular debris and degenerating cells¹⁰. However, when microglia are continuously activated by external stimuli, such as aggregated protein or foreign pathogens, this response can exacerbate disease conditions and lead to further neuronal damage¹¹. This toxic microglial inflammatory response includes the release of a variety of inflammatory mediators, such as chemokines, cytokines, cyclooxygenase-2 (COX-2), and reactive mediators, namely, reactive oxygen species (ROS) and nitric oxide species (NOS)^{10,12,13}. The chronic production of these inflammatory mediators increases oxidative stress and contributes to neuronal death. Although quite complex, neuroinflammation plays a major role in neurodegenerative diseases and has been identified as a worthy target for pharmaceutical interventions in neurodegenerative diseases.

Cerebrovascular activation has been identified as a toxic contributor to both neuroinflammation and neurodegenerative diseases. Vascular activation is the result

of endothelial cell damage and leads to the upregulation of a number of noxious factors. One such factor is the protease thrombin, which is produced by injured endothelial cells both *in vivo* and *in vitro*, initiating a neurotoxic cascade^{14–16}. Elevated levels of thrombin can be lethal to neurons through the interaction with protease activated receptor 1 (PAR-1), leading to the release of ROS and increases in an array of inflammatory proteins^{17,18}. Cerebrovascular activation is likely present in both AD and PD, and increased levels of both thrombin and PAR-1 have been identified in both diseases¹⁹.

Our lab has been interested in evaluating the direct thrombin inhibitor, Dabigatran etexilate (Pradaxa®, Boehringer Ingelheim), for the treatment of vascular dysfunction in AD^{14,18}. Multiple studies have shown dabigatran treatment mitigates the neurotoxic effects of thrombin in transgenic AD models^{14,20,21}. A single publication previously explored dabigatran etexilate in a rotenone-induced rat model of PD, exhibiting neuroprotective effects through nuclear receptor-related 1 protein²². Dabigatran treatment is yet to be explored in transgenic models of PD.

Therefore, we utilize a *Drosophila melanogaster* model of PD expressing mutant leucine-rich repeat kinase 2 (LRRK2). *Drosophila melanogaster* have been heavily used in neuroscience research as their neuronal structures and circuits and neurotransmitters, such as dopamine, are similar to those found in the human brain^{23–25}. When dopamine is dysfunctional in *Drosophila*, locomotion is impaired, similar to symptoms seen in human PD²⁶. Leucine-rich repeat kinase 2 (LRRK2) has been identified in both idiopathic and inherited PD, accounting for as high as 40% of familial cases²⁷. Postmortem analysis revealed LRRK2 is heavily expressed in the Lewy bodies of the brain stem and cortex, as well as in the brain vasculature, axons, and neuronal cell bodies^{28,29}. LRRK2 mutant *Drosophila* exhibit reduced lifespan,

compromised motility, and mitochondria morphological changes as compared to wildtype³⁰.

Herein, we hypothesize that dabigatran treatment in LRRK2 transgenic *Drosophila melanogaster* will protect against motor function deficits by reducing oxidative stress.

Materials and Methods

Drosophila Melanogaster Maintenance and Treatment

LRRK2 mutant *Drosophila melanogaster* (#34750, w[*]; Lrrk[ex1]/TM6B, Tb[1]) were purchased from Bloomington Stock Center (Indiana University Bloomington, Bloomington, IN). Canton S (w[*]) flies were a kind gift from Dr. Belinda Barbagallo (Salve Regina University, Newport, RI). Flies were maintained and age synchronized on Nutri-Fly BF (Genesee Scientific, San Diego, CA). Flies were separated by gender to approximately 20 flies per vial. On day 3 post eclosion, corresponding treatments were added to the media, control received 5% sucrose and dabigatran received 25 μ M diluted in 5% sucrose. Treatment vials were exchanged every other day for 7 days.

Toxicity Analysis

Toxicity of dabigatran at 25 μ M was identified by recording deaths per vial every other day. At least 100 individual flies per treatment were monitored and evaluated over the course of one week.

Negative Geotaxis Assay

After 7 days of treatment, flies were assayed for climbing ability^{31,32}. Briefly, flies were placed in empty vials marked at 5 cm and allowed to acclimate for 30 minutes. Flies were gently tapped to the bottom of the vial and a picture was taken after 10 seconds using a Nikon D2200 camera (Nikon, Tokyo, JP). Percent climbing was calculated by ((# flies above line / total # flies)*100) and standardized as a percentage to control (gender-specific).

Drosophila Head Homogenization

Drosophila were flash-frozen on dry ice and heads were collected through brass sieves, as previously reported³³. Heads were counted and 10 µL/head of phosphate buffered saline was added for NOS and ROS. For western blots, fly heads were homogenized in phosphate buffered saline with 1X protease inhibitors. Samples were frozen immediately on dry ice and stored at -80°C. To homogenize the heads for assays, they were sonicated in a water bath for 5 min, centrifuged (10,000 RCF, 5 min, 4°C), then placed at -80°C until frozen. This process was repeated 3 times and the supernatant was collected.

Nitric Oxide Quantification

Nitric oxide species was quantified in fly head homogenates using the Griess Reagent System (Promega, Madison, WI, USA)³⁴.

Reactive Oxygen Species Relative Quantification

For reactive oxygen species relative quantification, homogenates were incubated with 20 µM 2',7'-Dichlorofluorescein Diacetate (DCFDA) at 37°C for 1h in a black-walled, clear-bottom 96-well plate. Fluorescence was then read on Spectramax (M2) plate reader at 485 nm excitation and 535 nm emission³⁵.

Western Blotting

Fly head homogenate samples were prepared for western blot by adding 40% 4x Sample buffer (4x Laemmli Sample Buffer (BioRad, Hercules, CA, USA) with 10% 2-mercaptoethanol), heated at 100°C for 10 minutes. 15 µg of total protein was loaded on a 4-20% Gel (Invitrogen, Carlsbad, CA) and ran for 1h and 5min at 105 V

in 1x SDS-running buffer. Proteins were transferred to nitrocellulose membranes using the iBlot2 Gel Transfer Device (ThermoFisher Scientific, Waltham, MA), and membranes were blocked for 1h using 5% bovine serum albumin (BSA) in 0.05% TBS-T. Primary antibodies were incubated at room temperature for 3 hours, and then over 3 nights at 4°C. Primary antibodies used for this study include iNOS (Abcam, MA, 1:500), NOX4 (Sigma-Aldrich, MO, 1:500), SOD1 (Abcam, MA, 1:1,000), Tyrosine Hydroxylase (TH) (Novus Biologicals, Co, 1:500), and B-actin (Santa Cruz, CA, 1:10,000). Membranes were washed three times for 10 minutes with 0.05% TBS-T and incubated with infrared secondary antibodies at room temperature for 1 h. Membranes were washed and imaged on a LiCor Odyssey (LI-COR Biosciences, Lincoln, NE). Western blots were quantified in ImageJ. Values for each protein were normalized to β -actin loading control on the same blot. Data are represented as fold change, as compared to gender-specific wild type control.

Statistical Analysis

All statistical analysis was performed in GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). For toxicity analysis, log-rank (Mantel-Cox) tests were performed to identify significant differences between vehicle and dabigatran treatment. All other data were analyzed by unpaired one-tailed t-tests. Significance was compared and reported as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)

Results

Toxicity Analysis

Throughout the experiment, fly deaths were recorded to evaluate toxicity related to dabigatran treatment (Figure 1). Dabigatran did not induce significant death in either gender or genotype. Therefore, treatment at 25 μ M was regarded as safe in this experiment.

Negative Geotaxis

Motor impairments in *Drosophila* were evaluated using the negative geotaxis assay (Figure 2). In male flies, LRRK2 mutants exhibited a significant reduction in climbing ability as compared to wildtype control, reported as 22% $\pm$ 12.1 and 110.8% $\pm$ 16.8, respectively (Figure 2A). Additionally, in LRRK2 mutant flies, dabigatran significantly improved climbing ability to 52.2% $\pm$ 7.3, as compared to transgenic control. Significant motor impairments were identified in LRRK2 female control flies, as compared to wildtype (LRRK2, 56.2% $\pm$ 11.1; wildtype, 100% $\pm$ 12.2) (Figure 2B). Dabigatran did not exhibit any significant effects as compared to control in female flies.

Nitric Oxide Species Production

NOS production was evaluated in fly head homogenates using the Griess reagent (Figure 3). Interestingly, LRRK2 mutant flies did not exhibit increased NOS as compared to wildtype control. In male LRRK2 flies, dabigatran exhibited a significant reduction in NOS as compared to control (control 38.8 $\pm$ 5; dabigatran 26.8 $\pm$ 3.3) (Figure 3A). NOS was unaltered by dabigatran treatment in female LRRK2 flies (Figure 3B).

Reactive Oxygen Species

ROS production in fly head homogenates was also investigated (Figure 4). LRRK2 mutant flies did show a change in ROS production as compared to wildtype control. In male LRRK2 mutant flies, dabigatran significantly reduced ROS as compared to control (control: 455.6 ± 26.8 ; dabigatran 351.8 ± 18.4). Female LRRK2 flies exhibited no difference in ROS production with dabigatran treatment (Figure 4B).

Western Blot Analysis

Tyrosine hydroxylase (TH; Figure 5) expression was significantly reduced in male transgenic flies (0.54 ± 0.09) as compared to wildtype control (1.08 ± 0.1). Female flies also exhibited significant loss of TH (wildtype 1.0 ± 0.17 ; transgenic 0.69 ± 0.01). Dabigatran treatment did not significantly alter TH expression in either gender.

Inducible nitric oxide synthase (iNOS) (Figure 6) is directly related to nitric oxide production. Herein, we evaluated the expression of iNOS in fly head homogenates. Transgenic male flies exhibit increased expression of iNOS, as compared to wildtype control, reporting 1.6 ± 0.32 and 0.89 ± 0.18 , respectively. Dabigatran treatment in transgenic male flies reduced iNOS expression, though not significantly. Female flies exhibited no significant differences between groups.

Superoxide dismutase 1 (SOD1) is heavily involved in ROS production, herein, we evaluated protein expression by western blot (Figure 7). SOD1 was significantly elevated in male transgenic homogenates, as compared to wild type (transgenic 1.4 ± 0.16 ; wildtype 0.68 ± 0.07). Dabigatran treatment reduced SOD1 expression to 0.84 ± 0.05 . Female fly homogenates followed a similar pattern. Wild type control flies exhibited 0.83 ± 0.1 , whereas transgenic control showed 1.2 ± 0.09 . In females, dabigatran treatment reduced SOD1 expression to 0.82 ± 0.09 .

NADPH oxidase 4 (NOX4) expression was evaluated in *Drosophila* head homogenates (Figure 8). Transgenic male flies exhibit significantly increased expression of NOX4 compared to wildtype control, reporting 1.5 ± 0.06 and 1.0 ± 0.03 , respectively. Dabigatran treatment in transgenic male flies reduced NOX4 expression to 0.61 ± 0.14 . Females exhibited no significant changes in NOX4 expression.

Discussion

Currently, there are no treatments that stop or even slow the progression of neurodegenerative diseases. Therefore, it is imperative to explore novel approaches aside from classical targets. Neuroinflammation has been identified as a major contributor in AD and PD that may begin years before an official diagnosis⁵. Activated brain vasculature can lead to an increase in disease severity^{36–38}. In PD, a variety of genetic mutations have recently been identified in disease pathology. One of the most common mutations, LRRK2, is heavily expressed in the vasculature of the brain³⁹. It was hypothesized that by inhibiting the vascular protein thrombin, neuroinflammation would be reduced in a transgenic PD model.

In PD, motor impairments are the main disease identifier, typically shown by tremors or motor function instabilities in humans. *Drosophila melanogaster* models of PD exhibit similar motor impairments that are believed to be linked to loss of dopaminergic neuronal function³⁰. Therefore, we first assayed motor function in wild type and transgenic flies in the negative geotaxis assay. As anticipated, in both genders, a significant reduction in climbing ability was seen in the transgenic as compared to the wildtype control. Dabigatran treatment in male transgenic flies alone exhibited improvement in climbing.

Parkinson's disease loss of motor control is heavily regulated by the loss of functional neurotransmitter dopamine. *Drosophila melanogaster* exhibit many similarities to humans, namely, there are distinct dopamine clusters that correspond to locomotor control, and dopamine synthesis is conserved⁴⁰. Regulation of dopamine is limited by protein tyrosine hydroxylase (TH), which converts tyrosine to dopamine⁴¹. Therefore, we performed western blotting to quantify tyrosine hydroxylase expression in fly head homogenates to identify the correlation between TH levels and the

observed motor function. In transgenic male flies, there was a significant reduction in TH as compared to wildtype control. However, dabigatran treatment did not improve the levels of TH. This suggests that alternative processes may be involved.

The production of nitric oxide and reactive oxygen species are linked to both neuroinflammation and damaged cerebrovasculature in neurodegenerative diseases^{5,42,43}. Dabigatran treatment has been shown to decrease oxidative stress markers in diseased models^{14,20,21}. LRRK2 has also been associated with increases in these mediators of oxidative stress⁴⁴. In *Drosophila melanogaster* NOS and ROS production can be altered with dietary modifications^{35,45,46}. Here, surprisingly, NOS was reduced in transgenic flies as compared to wildtype. However, dabigatran treatment significantly reduced NOS production as compared to control in transgenic male flies. No differences were found in the females. Additionally, ROS was significantly reduced in male LRRK2 flies following dabigatran treatment. To further investigate these results, we analyzed the expression of proteins in the regulatory pathways of each NOS and ROS production.

NOS is essential to normal function, but the continuous production of NOS leads to excessive toxicity in the central nervous system⁴⁷. iNOS is one of three isoforms that contributes to the production of NOS. Activated microglia induce cytokine activity, leading to iNOS production which has been deemed neurotoxic^{48,49}. We analyzed iNOS to get a better understanding of toxic NOS production. In transgenic male *Drosophila*, iNOS expression correlates to NOS levels. Females did not follow a similar trend.

Similar to NOS, ROS is toxic when overproduced. ROS is regulated by a variety of mediators. We chose two markers, namely, SOD1 and NOX4. Superoxide dismutases are essential in the breakdown of superoxide into oxygen and hydrogen peroxide⁵⁰. Typically, an increase in the expression of SOD1 indicates an elevation in

ROS. In both male and female *Drosophila*, SOD1 was elevated in transgenic as compared to wildtype. Additionally, dabigatran treatment reduced SOD1 expression. Interestingly, these differences did not exactly mimic identified ROS levels. In *Drosophila melanogaster*, it has been contested whether SOD directly correlates to ROS⁵⁰. NADPH oxidases are another family of enzymes that regulate ROS production⁵¹. NOX4 is heavily expressed in endothelial cells and contributes directly to ROS production⁵². Male *Drosophila* exhibited increased NOX4 in transgenic flies. Additionally, dabigatran treatment reduced NOX4. Similar to SOD1, NOX4 expression does not directly correlate to ROS production.

Interestingly, our data reports differences in treatment response by gender. In human PD, the incidence, progression, and symptom severity present differently in males and females^{53,54}. These differences have been largely attributed to hormonal differences^{55,56}. Sex differences in *Drosophila* have frequently been overlooked. However, reports on lifespan indicate the potential of extreme differences between gender in flies⁵⁷.

Conclusions

Here, we explore direct thrombin inhibitor, dabigatran, in a transgenic *Drosophila melanogaster* model for the first time. Our results indicate that dabigatran treatment improves motor function by reducing oxidative stress. Future research should evaluate dabigatran treatment in additional models of Parkinson's disease, focusing on the progression and pathology. These initial findings should be investigated in alternative transgenic models of PD and evaluated for effects on behavior as well as biochemical markers of pathology to further elucidate mechanisms of action.

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Conflicts of Interest

Authors declare no conflicts of interest.

Figure 1. Analysis of treatment induced toxicity in wildtype and transgenic *Drosophila melanogaster*. Flies were exposed to sucrose or dabigatran (25 μ M) over 1 week of treatment in males (A) and females (B) ($n \geq 100$).

Figure 2. Effects of dabigatran on negative geotaxis in *Drosophila melanogaster*. Male (A) and female (B) flies were evaluated on negative geotaxis after seven days of treatment ($n \geq 8$). Data expressed as percent of wildtype control, significance determined by t-test, $p \leq 0.05$ (*); $p \leq 0.001$ (**).

Figure 3. Evaluation of nitric oxide species production in *Drosophila melanogaster* head homogenate. Male (A) and female (B) homogenates treated for 1 week with control or dabigatran (25 μM) (n≥8). Statistical significance determined by t-test, $p \leq 0.05$ (*).

Figure 4. Effects of dabigatran (25 μ M) on reactive oxygen species production in wildtype and transgenic *Drosophila melanogaster*. Head homogenates in males (A) and females (B) after 1 week treatment ($n \geq 8$). Significance determined by t-test, $p \leq 0.01$ (**).

Figure 5. Quantification of tyrosine hydroxylase (TH) by western blot in *Drosophila melanogaster* head homogenates. Expression was evaluated in male (A) and female (B) homogenates after 1 week of control or dabigatran treatment ($n \geq 3$). Significance determined by t-test, $p \leq 0.05$ (*); $p \leq 0.01$ (**).

Figure 6. Western blot analysis of inducible nitric oxide synthase (iNOS) in *Drosophila melanogaster* head homogenate. Control or dabigatran (25 M) treatment for 1 week in males (A) and females (B) ($n \geq 3$). Statistical significance was determined by t-test, $p \leq 0.01$ (**).

Figure 7. Superoxide dismutase 1 (SOD1) expression in *Drosophila melanogaster*. Evaluation of SOD1 after 1 week treatment with control or dabigatran in male (A) and female (B) *Drosophila* ($n \geq 3$). Statistical significance was determined by t-test, $p \leq 0.05$ (*); $p \leq 0.01$ (**).

Figure 8. Evaluation of NADPH (NOX4) by western blot in *Drosophila melanogaster*.

Male (A) and female (B) flies were subject to treatment for 1 week ($n \geq 3$). Statistical significance was determined by t-test, $p \leq 0.01$ (**); $p \leq 0.0001$ (***).

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CHAPTER 4

MANUSCRIPT 6

Direct thrombin inhibitor, dabigatran etexilate, reduces oxidative stress *in vivo* in a transgenic mouse model of Alzheimer's disease

Running Title: Thrombin inhibition in a tau-based AD model

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Abstract

Background. Thrombin is neurotoxic and is elevated in the brain and cerebrovasculature in Alzheimer's disease (AD). We have identified thrombin as a key mediator of vascular inflammation in AD with data demonstrating that thrombin inhibition is protective in an amyloid based animal model. Here, we investigate the role of thrombin as a pathological mediator in a tau-based animal model of AD. We hypothesize that thrombin inhibition will reduce oxidative stress, inflammation, and pathology related to tau *in vivo*.

Methods. 15-month-old Tg4510 mice were treated with the direct thrombin inhibitor dabigatran etexilate or control for 7 days. Brains were collected, and western blot and LC-MS/MS SWATH analysis were performed to evaluate proteins related to coagulation, oxidative stress, intracellular signaling, and tau-pathology.

Results. Dabigatran etexilate treatment reduces thrombin and thrombin-related proteins. MAPK related proteins exhibited significant differences following dabigatran etexilate treatment. Treatment also reduced oxidative stress-related proteins and increased expression of antioxidant proteins. Total tau and related phosphorylated tau species were significantly increased in transgenic mice compared to wild type. Dabigatran treatment slightly reduced expression of total tau and significantly altered the tau phosphorylation pattern. Proteins identified as downregulated in human AD were also significantly altered with dabigatran treatment compared to control.

Conclusions. Taken together, these data indicate short-term treatment with a direct thrombin inhibitor modulates protein expression in the brains of aged Tg4510 mice. This finding supports our hypothesis that targeting thrombin, a key mediator of neuroinflammation and neurotoxicity may be effective in reducing AD-related

pathology. Further studies are needed to better understand the mechanisms of action and possible neuroprotective benefits of thrombin inhibition.

Keywords: Alzheimer's disease, Inflammation, Oxidative Stress, Thrombin, Tau

Introduction

Cardiovascular disease and cardiovascular risk factors (CVRFs) are strongly associated with an increased risk of developing dementia, particularly Alzheimer's disease (AD). Hypertension, hypercholesterolemia, hyperhomocysteinemia, diabetes, atherosclerosis, and hypoxia are all linked with an increased risk of developing AD [1-5]. While the connection between CVRFs and AD is well-documented, the mechanism by which the disorders are connected is not well understood. It is likely there are several, pathological mediators involved in the progression of both disorders.

One such mediator is the serine protease thrombin, the main driver of the coagulation cascade [6]. Thrombin is indicated as a potential pathological mediator in both cardiovascular disease and AD. Thrombin has been implicated in atherosclerosis and diabetes-related pathology [7-11]. Thrombin is also elevated in the Alzheimer's brain. Past studies found increased thrombin and reduced levels of the thrombin inhibitor protease nexin-1 in the brain of Alzheimer's patients compared to healthy controls; this elevation was largely localized in vessels, amyloid deposits, and neurofibrillary tangles [12-14]. Thrombin is also elevated in AD patient-derived microvessels, and brain endothelial isolated from AD patients synthesize their own thrombin [15, 16]. Thrombin is a mediator of vascular-derived oxidative stress and inflammation. Aside from its pro-coagulant activity, thrombin has well-documented pro-inflammatory and pro-oxidative effects on several cell types in the body, largely through its activation of protease-activated receptors (PARs) [17, 18]. In the periphery, increased thrombin in disease is related to the formation of atherosclerotic plaques, endothelial cell activation, and an increase in both inflammatory cytokines and reactive oxygen species (ROS) [7, 19-21].

Thrombin has similar effects in the brain as it does in the periphery. Thrombin treatment induces alteration of endothelial cell phenotype at the blood-brain barrier (BBB), leading to changes in adhesion and tight junction formation [22, 23]. Thrombin also stimulates endothelial cells to release inflammatory factors and ROS; similar increases are found in the AD cerebrovasculature [15, 24-29]. Thrombin treatment of microglia induces a pro-inflammatory phenotype characterized by increases in ROS, NO, and cytokine production [30-33]. Thrombin is also associated with increased pro-inflammatory, reactive astrocytes in the brain [34-37]. Additionally, thrombin exerts direct neurotoxicity by several mechanisms, including alterations in the neuronal cell cycle, induction of pro-apoptotic proteins, and NADPH-oxidase mediated oxidative stress [38, 39].

Thrombin is also associated with AD-related hallmarks in the brain, including tau, amyloid aggregation, and apolipoprotein E [40-44]. Rats treated with thrombin *in vivo* exhibit cognitive deficits along with cell death and glial scarring [35]. Thrombin may be involved in the altered processing and secretion of amyloid precursor protein (APP), and thrombin cleavage of apolipoprotein E4 results in a neurotoxic fragment [42-44]. Thrombin accumulation has been identified in neurofibrillary tangles, and thrombin induces rapid tau aggregation [40, 41].

Thrombin is related to altered processing and aggregation of tau, but the role of thrombin as a pathological mediator has not yet been explored in a tau-based model of AD. Currently, a number of tauopathy animal models are being used to study AD. One such model is the Tg4510, which overexpresses human tau with a P301L mutation at 13:1 versus murine tau [45]. These mice exhibit profound tau pathology and neuronal loss in the hippocampus and cortex, as well as cognitive deficits and metabolic changes. The pathology starts early at about 2 to 4 months of

age and progresses with age [45, 46]. The pathological features, including tau hyperphosphorylation synapse loss, are more pronounced in females than males [47]. Additionally, the tg4510 mouse model displays blood vessel abnormalities accompanied by alterations in oxidative and inflammatory markers [48].

We suggest that thrombin is a mediator of cerebrovascular-derived inflammation and neurotoxicity in AD, and therefore targeting thrombin may be a worthy therapeutic strategy to combat Alzheimer's disease-related pathology. Our lab has focused on the direct thrombin inhibitor Dabigatran etexilate (Pradaxa®, Boehringer Ingelheim), a commonly prescribed anticoagulant that is administered orally. In a previous paper, we identified that dabigatran etexilate treatment for 34 weeks in 3xTg AD mouse model reduced levels of oxidative stress and markers of cerebrovascular inflammation in the brain [49].

The objective of this study is to investigate the role of thrombin as a pathological mediator and the potential therapeutic benefits of inhibiting thrombin in an animal model of tauopathy and AD. We explore the effects of short-term treatment with direct thrombin inhibitor, dabigatran etexilate, in aged Tg4510 mice. We hypothesize that inhibiting thrombin will reduce oxidative stress and inflammation-related indicators corresponding to an overall reduction in tau-related dysfunction in the brain.

Materials and Methods

Animals and Treatment

Transgenic Tg4510 AD mice overexpressing human mutant tau (P301L) and background matched controls were a kind gift from MindImmune Therapeutics, Inc. originally obtained from Charles River (Wilmington, MA, USA). Mice were maintained on normal chow (ENVIGO 2020X, Huntingdon, UK) with water available ad libitum. At 15 months of age, mice were treated via oral gavage with vehicle (2.5% DMSO, 2.5% koliphor EL, 90% diH₂O) or dabigatran etexilate (100 mg/kg in uniform suspension, Cayman Pharm, Ann Arbor, MI) for 7 days. After 7 days, mice were deeply anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) administered I.P. and euthanized.

Western blot

Whole brains were removed post-sacrifice and the cerebellum was detached. Tissues were homogenized by sonication (Branson SX150 Sonifier®, Branson Ultrasonics, Danbury, CT) in 9x brain volume of 1X PBS with protease inhibitors. To prepare samples for western blot, samples were mixed with equal parts 2x Sample Buffer (4x Laemmli sample buffer with 10% 2-mercaptoethanol, 50% diH₂O) and heated at 100°C for 10 minutes. Samples containing 25 µg total protein were loaded on a 4-20% Gel (NOVEX) that was run at 105 V for 1 hour and 5 minutes in 1x SDS-running buffer. Proteins were then transferred to nitrocellulose membranes using iBlot2 Gel Transfer Device (ThermoFisher Scientific, Waltham, MA). Membranes were blocked using antibody-specific concentration of BSA or Milk in 0.05% TBS-T for one hour. Primary antibodies for this study include thrombin (Abcam, MA; 1:500), fibrin (Santa Cruz, CA; 1:250), NOX4 (Sigma, MA; 1:1,000), iNOS (Abcam, MA; 1:250), SOD1 (Abcam, MA; 1:1,000), SOD2 (Abcam, MA; 1:2,000), GFAP (Abcam, MA;

1:10,000), Iba1 (GeneTex, CA; 1:1,000). Tau and p-tau species were detected using phosphor-tau family antibody sampler kit (Cell Signaling, MA). Primary antibodies were added overnight at 4°C. Membranes were washed three times for 5 minutes with 0.05% TBS-T and incubated with infrared secondary antibodies for 1 hour at room temperature. Membranes were washed as before and then imaged on a LiCor Odyssey (LI-COR Biosciences, Lincoln, NE). Quantification of western blots was done in ImageJ. Values for each protein were normalized to actin or GAPDH loading control on the same blot. Data represented as fold change, as compared to TgV.

Pressure Cycling Technology Based Protein Digestion

Whole brain homogenates were further digested using pressure cycling technology (PCT) for LC-MS/MS SWATH acquisition following the method by Jamwal et al. 2017 with slight modifications [50]. Each sample, containing 500 µg of protein, was spiked with 2 ng of bovine serum albumin (BSA). Samples were then incubated with dithiothreitol (100 mM) at 90°C for 15 minutes (100 rpm). Iodoacetamide (200 mM) was added and samples were incubated at room temp in the dark for 30 minutes. Protein was then precipitated by using the ice-cold chloroform, methanol and water method (1:2:1) followed by centrifugation at 12,000 RPM for 5 minutes at 10°C. The protein pellet was rinsed with methanol and resuspended in 3% w/v sodium deoxycholate (DOC) in 50 mM ammonium bicarbonate. Samples were placed in MicroTubes (Pressure BioSciences Inc, South Easton, MA) with trypsin at a 1:20 ratio of trypsin:protein. Digestion was performed at 55°C for 75 cycles (50 sec at 35kpsi, 10 sec at ambient pressure) in a Barocycler NEP2320-45k (Pressure BioSciences Inc). A second digestion was performed by adding fresh trypsin at the same ratio and running the barocycler for an additional 60 cycles. Samples were then transferred to microcentrifuge tubes where digestion was stopped and DOC was

precipitated by the addition of formic acid in acetonitrile at a final percentage of 0.5%. Samples were centrifuged and supernatant was collected for analysis.

Mass Spectrometry Data Acquisition with SWATH-MS

Mass spectrometry was performed as previously described with minor modifications [50]. Samples were analyzed on a SCIEX TripleTOF® 5600 mass spectrometer using a DuoSpray™ ion source (SCIEX, Framingham, MA) coupled to an Acquity HClass UHPLC system (Waters Corp., Milford, MA). Separation was achieved on an Acquity UPLC Peptide BEH C18 column (2.1 x 150 mm, 300 Å, 1.7 µm) with an Acquity VanGuard pre-column (2.1 x 5 mm, 300 Å, 1.7 µm). The column temperature was set to 50°C and the autosampler was set to 10°C. A linear gradient was used with a flow rate of 100 µL/min for 90 min. Mobile phase A consisted of 99.9% acetonitrile and 0.1% formic acid. Mobile phase B consisted of 99.9% water and 0.1% formic acid. The gradient was as follows: 98% A from 0 to 5 min, 98% to 75% from 5 to 55 min, 75% to 50% A from 55 to 60 min, 50% to 20% from 60 to 70 min. Mobile phase A was held at 20% from 70 to 75 min and returned to 98% A at 80 min. The column was held at 98% A for 10 min to equilibrate prior to the next sample. A mixture of trypsin-digested β-galactosidase peptides were used between every 8 samples to calibrate masses and monitor the TOF detector.

Positive ionization mode was used for data dependent acquisition. The mass spectrometer parameters are as follows: gas 1, gas 2, curtain gas, temperature and ion spray voltage floating were 55 psi, 60 psi, 25 psi, 450°C, 5500 V, Respectively. Declustering potential was 10, collision energy 10 and collision energy spread 15. For data acquisition, a maximum of 50 candidate ions were monitored for each survey scan. All ions had a charge state from 2 to 4. A range of m/z 300-1250 was used for exclusion criteria and all ions that had an intensity greater than 25 cps were chosen

for MS/MS analysis. The temperature was set at 450°C and the total cycle time was 3.5 sec with a mass tolerance of 50 mDa during the first 0.75 sec survey scan.

For SWATH analysis, all parameters were the same as above except for the following: Seventy SWATH windows per cycle were collected over m/z 400-1100 with each window size being m/z 10 and TOF masses were collected from m/z 300 to 1500.

Mass Spectrometry Data Analysis

LC-MS/MS SWATH data was used to generate spectral libraries through ProteinPilot (SCIEX, Framingham, MA). Proteins of interest were identified and investigated. FASTA files were downloaded from UniProt and imported into Skyline (MacCoss Lab, University of Washington). In Skyline, at least 3 transitions were selected per peptide, and at least 3 peptides per protein were chosen. Once data was analyzed, the MPPreport (MacCoss Lab, University of Washington) was generated and exported to excel. In excel, transitions were averaged, and the sum of each peptide was calculated to yield the total area under the curve representative of each protein. These were then standardized to internal standard, BSA.

Statistical analysis

Data from each experiment are expressed as mean +/- standard error (SEM), unless otherwise indicated. All tests were performed in GraphPad prism. Analysis was done by unpaired, one-tail t-test. Statistical significance was determined at $p < 0.05$. Significance for all tests was defined as follows: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****).

Results

One-week dabigatran etexilate treatment reduces expression of coagulation related proteins

The expression of major proteins involved in coagulation, namely, thrombin, prothrombin and fibrin were evaluated by western blot. Thrombin levels remained unaltered in wild type as compared to Tg-Vehicle. Although not significant, dabigatran treatment decreased thrombin by 33% as compared to Tg-Vehicle (Figure 1A). Prothrombin was significantly lower in wild type mice by 44% compared to Tg-Vehicle (Figure 1B). Dabigatran treatment significantly reduced prothrombin by 46.7% as compared to Tg-Vehicle. Fibrin levels were slightly reduced in both wildtype (54.9%) and dabigatran etexilate (44.4%) as compared to Tg-Vehicle (Figure 1C).

Dabigatran etexilate reduces oxidative-stress related proteins

Inducible nitric oxide synthase (iNOS) and NADPH oxidase 4 (NOX4) are enzymes involved in the activation of oxidative stress by catalyzing the production of NOS and ROS. The expression of iNOS was significantly reduced in wild type and Tg-Dabigatran, as compared to Tg-Vehicle by 23.5% and 17.4%, respectively (Figure 2A). Tg-Dabigatran also significantly reduced NOX4 expression by 24.7% as compared to Tg-Vehicle (Figure 2B).

Dabigatran etexilate increases expression of superoxide dismutases

Expression of antioxidant related proteins, superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2) was also evaluated by western blot (Figure 3). No differences were reported between wild type and Tg-Vehicle. Tg-Dabigatran significantly increased the expression of both SOD1 and SOD2 by 34.2% and 20.7%, respectively.

Effect of Dabigatran etexilate on neuroinflammatory proteins

Glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (IBA1) are markers of activated astrocytes and microglia, respectively. These neuroinflammatory markers were significantly elevated in Tg-Vehicle as compared to wild type mice (Figure 4). Tg-Dabigatran exhibited slightly lower GFAP (0.78 ± 0.12) and Iba1 (0.95 ± 0.12) expression than Tg-Vehicle, but reductions were not significant.

Dabigatran etexilate treatment alters tau phosphorylation but not overall expression

Total tau and phosphorylated forms S396, S404 and S416 were evaluated by western blot (Figure 5). Tg-Vehicle expressed 75.9% more total tau than wild type mice (Figure 5A). Tg-Dabigatran exhibited 22.1% less total tau as compared to Tg-Vehicle. Wild type mice exhibited significantly lower levels of all three phosphorylated tau forms. Tg-Dabigatran showed decreased levels of S396 and S416, by 32.8 and 29.2%, respectively. Interestingly, S404 was elevated by 14.9% in Tg-Dabigatran, as compared to Tg-Vehicle.

LC-MS/MS SWATH acquisition identifies significant proteomic differences between wild type and Tg-Vehicle mice

Major proteins involved in coagulation, AD, inflammation and tau-related pathology were identified and evaluated using LC-MS/MS SWATH acquisition (Table 1). Significant differences in protein expression were first evaluated between wildtype and transgenic mice, visualized with a volcano plot (Figure 6A). Coagulation related proteins exhibited significant differences in FIBB, ICAM5, KPCD, MMP9, and RACK1. Differences were seen in inflammation related proteins DLG4, GFAP, NOS3 and TLR4. Alzheimer's disease related proteins that were significantly altered in Tg4510 mice were A4, APOE, BACE1, TAU (Human), TAU (Mouse), and TAU (Total). A recent publication identified proteomic signatures in the human brain affected by tau

pathology (Mendoza, 2019). From this publication we identified a list of tau-related pathology proteins and explored these differences. Wildtype to Tg4510 brains exhibited significant differences in G6PD1, HPCA, KCC2A, NAC2, SV2B, TBB3 and VIME.

LC-MS/MS SWATH Acquisition identifies significant proteomic expression between vehicle and dabigatran etexilate treated transgenic mice

Alzheimer's disease, coagulation, inflammation and Tau-related identified proteins were further evaluated to identify differences between vehicle and dabigatran etexilate treated transgenic mice (Figure 6B). The pathway most influenced by dabigatran etexilate treatment was the coagulation cascade. Significant differences were seen in ANT3, ICAM1, ITAL, ITB2, KPCB, KPCD, MK08, MK09, MP2K1, MP2K2, ROCK2 and VCAM (Figure 7A). Inflammatory proteins were also significantly altered, namely, COX2, DLG4, and DYN1 (Figure 7B). BACE1 was the single altered protein from the group of AD-related proteins. Significant differences were seen in Tau-related proteins, ANS1B, G3BP2, HPCA, NCKP1, SV2B, SYGP1 and TBB3 (Figure 7C).

Discussion

Increasing evidence has shown elevated levels of thrombin and thrombin-related proteins of the coagulation cascade in the brains of Alzheimer's disease patients [12-14, 40-44, 51]. Further, thrombin accumulation is co-localized with tau aggregation [40, 41]. Herein, we propose that inhibiting thrombin in a tau-pathology mouse model may identify an alternative approach to combat Alzheimer's disease-related pathology.

Our data shows the efficacy of treatment with a direct thrombin inhibitor, dabigatran etexilate, in a tau mouse model. As expected, the drug treatment reduces thrombin and proteins increased by thrombin activity (prothrombin, fibrin), confirming normal drug action. In addition to coagulation, dabigatran etexilate treatment also produced alterations in the expression of proteins involved in oxidative stress, inflammation, and tau-related pathology. These results suggest that inhibition and reduction of thrombin may affect aspects of AD pathology through the alteration of different thrombin-mediated signaling mechanisms in the brain.

Thrombin's pro-inflammatory effects throughout the body, including in the brain, are largely mediated through the signaling of its receptors, protease-activated receptors (PARs) 1, 3, and 4. PARs represent a unique family of G-protein coupled receptors that are activated by a self-ligand [17]. PARs have been found in a number of cell types in the periphery and the brain, including endothelial cells, neurons, astrocytes, and microglia [52]. When cleaved and activated by thrombin, PAR1 initiates a variety of intracellular signaling. Among others, PAR1 activates the mitogen-activated protein kinase (MAPK) pathway which can lead to cell growth, proliferation or migration [53]. MAPK related proteins were analyzed by LC-MS/MS SWATH analysis, identifying significant differences between Tg-Vehicle and Tg-

Dabigatran etexilate in ANT3, ITB2, KPCB, MK08, MP2K1 and MP2K2. Interestingly, Dabigatran etexilate treatment did not alter proteins to wild type levels.

Thrombin activation of PARs is responsible for pro-inflammatory and oxidative effects in both the periphery and brain. Here, we identified significantly elevated levels of iNOS, GFAP, and IBA1 in transgenic mice as compared to wild type. Dabigatran etexilate treatment reduced iNOS, and NOX4, as compared to vehicle. Dabigatran etexilate significantly increased COX2, DYN1, SOD1, and SOD2. These increases may be due to the timing in the antioxidant cycle [54]. Together, these results indicate a shift towards reduced oxidative stress following dabigatran etexilate treatment.

Total tau and related phosphorylated (S396, S404, S416) tau species were significantly increased in transgenic mice compared to wild type, as was expected. Dabigatran treatment slightly reduced expression of total tau, significantly decreased S396, S416; but led to an increase in S404. S396 and S404 phosphorylation are found early in the disease course of AD [55, 56] and are related to destabilization of microtubules [57, 58]. S396 is also linked with abnormal truncation of the tau protein, indicating altered functionality [55]. Phosphorylation at S416 by CamKII is largely found within the neuronal soma, rather than localized to microtubules, and has been found to be associated with the promotion of AD-related cell death [59, 60]. Together, our findings indicate that dabigatran etexilate treatment may reduce AD-related tau dysfunction through altered phosphorylation, particularly decreased phosphorylation at S396 and S416.

To further explore tau pathology, we performed LC-MS/MS SWATH analysis on a variety of previously identified tau related proteins. ANS1B, NCKP1, SV2B and SYGP were significantly altered with dabigatran etexilate treatment, compared to Tg-

Vehicle. These proteins were previously found to be downregulated in tau pathology in AD brains across multiple proteomic studies [61]. These data indicate additional changes in tau-related dysfunction following dabigatran etexilate treatment.

It's important to note that there may be alternative explanations for the effects seen with dabigatran etexilate treatment, particularly for the decreased expression of oxidative and inflammatory mediators. Fibrin, a coagulation protein found downstream of thrombin, has also been identified as a potential pathological mediator in Alzheimer's disease [62]. Higher levels of fibrin have been identified in the AD brain compared to healthy controls [51]. Fibrin accumulation in AD, similar to thrombin, has been linked with increases in inflammation and oxidative stress, as well as alterations in both amyloid and tau pathology [62]. Our findings showed a decrease in fibrin expression as a result of dabigatran etexilate treatment, which is expected given thrombin catalyzes the conversion of fibrinogen into fibrin. It is possible that some of the other changes identified with dabigatran etexilate treatment, including decreases in oxidative stress-related and inflammatory proteins, are the result of decreased fibrin accumulation rather than reduced thrombin signaling activity.

Alternatively, there are anti-coagulant proteins, such as activated protein C (APC), that act in opposition to thrombin. These proteins may alternatively catalyze PARs and subsequently alter the signaling mechanism activated, producing anti-inflammatory and cytoprotective effects [63]. Further studies have shown anti-inflammatory effects of APC-like ligands mediated through PAR signaling [64]. Just as reduced thrombin activity may result in reduced fibrin accumulation and therefore reduced inflammation, reduced levels of thrombin may allow for APC and other anti-coagulant proteins to bind to PARs and activate anti-inflammatory signaling processes.

Thrombin has increasingly become a protein of interest in Alzheimer's, with other investigators also investigating thrombin as a potential pathological mediator in AD, and therefore a potential therapeutic target. Studies have found that indirect thrombin inhibitors, such as warfarin, exert anti-inflammatory and neuroprotective effects in models of AD [65]. However, direct thrombin inhibition with dabigatran etexilate may be a safer treatment option, with a reduced risk of intracerebral hemorrhage [66]. In a longitudinal, community-based study use of dabigatran etexilate was associated with a lower risk of new-onset dementia compared to warfarin [67]. More recently, treatment with dabigatran etexilate TgCRND8 transgenic AD mice improved spatial memory deficits, reduced neuroinflammation and amyloid plaque formation [68]. These studies demonstrate beneficial effects of thrombin inhibition in AD models, further supporting our hypothesis that targeting thrombin, a key mediator of vascular inflammation and neurotoxicity, may be effective in reducing AD-related pathology.

While these findings are interesting, it is worthy to note that this study is limited in scope. The cohort analyzed was made up of a specific gender at a late age for a short treatment time. Future studies should analyze longer dabigatran etexilate treatment at various life stages to better identify the best time and length for pharmaceutical intervention for the alteration of AD-related pathology.

Here, we investigate the role of thrombin as a pathological mediator in Alzheimer's disease, and the efficacy of a direct thrombin inhibitor, dabigatran etexilate, in a transgenic tau-based AD mouse model. After seven days of treatment, dabigatran etexilate treatment altered proteins related to coagulation, inflammation, oxidative stress and tau pathology. Further, data suggests that thrombin inhibition

may mediate AD pathology through multiple thrombin-mediated signaling mechanisms.

Acknowledgements and Conflict of Interest

The authors would like to thank MindImmune Therapeutics, Inc. for generously contributing animals for this project. Authors declare no conflicts of interest.

Table 1. List of proteins and corresponding abbreviations. Proteins analyzed in Skyline, data obtained by LC-MS/MS with SWATH acquisition.

Pathway	Abbreviation	Full Protein Name
Alzheimer' s Disease	A4	Amyloid-beta A4 protein (ABPP) (APP)
	APOE	Apolipoprotein E (Apo-E)
	BACE1	Beta-secretase 1
	BACE2	Beta-secretase 2
	TAU (Human)	Microtubule-associated protein tau
	TAU (Mouse)	Microtubule-associated protein tau
	TTBK2	Tau-tubulin kinase 2
Coagulation Cascade	ANT3	Antithrombin-III
	FA5	Coagulation factor V (Activated protein C cofactor)
	FA8	Coagulation factor VIII (Procoagulant component)
	FA10	Coagulation factor X
	FIBA	Fibrinogen alpha chain
	FIBB	Fibrinogen beta chain
	ICAM1	Intercellular adhesion molecule 1
	ICAM5	Intercellular adhesion molecule 5
	ITAL	Integrin alpha-L
	ITAM	Integrin alpha-M
	ITAV	Integrin alpha-V
	ITB2	Integrin beta-2
	ITB3	Integrin beta-3
	KPCA	Protein kinase C alpha type
	KPCB	Protein kinase C beta type
	KPCD	Protein kinase C delta type
	MK01	Mitogen-activated protein kinase 1
	MK03	Mitogen-activated protein kinase 3
	MK08	Mitogen-activated protein kinase 8
	MK09	Mitogen-activated protein kinase 9
	MMP2	Matrix metalloproteinase-2; 72 kDa type IV collagenase
	MMP9	Matrix metalloproteinase-9
	MP2K1	Dual specificity mitogen-activated protein kinase kinase 1
	MP2K2	Dual specificity mitogen-activated protein kinase kinase 2
	RACK1	Receptor of activated protein C kinase 1
	ROCK1	Rho-associated protein kinase 1
	ROCK2	Rho-associated protein kinase 2
	VCAM1	Vascular cell adhesion protein 1
Inflammation Related	CDK5	Cyclin-dependent-like kinase 5
	COX2	Cytochrome c oxidase subunit 2
	DLG4	Disks large homolog 4
	DYN1	Dynamin-1
	GFAP	Glial fibrillary acidic protein (GFAP)
	MILK2	MICAL-like protein 2
	NFKB1	Nuclear factor NF-kappa-B p105 subunit
	NOS3	Nitric oxide synthase, endothelial
	PARK7	Protein/nucleic acid deglycase DJ-1
	PTPRC	Receptor-type tyrosine-protein phosphatase C
	TLR4	Toll-like receptor 4
Tau Related	ACTN2	Alpha-actinin-2
	ANS1B	Ankyrin repeat and sterile alpha motif domain-containing protein 1B (Amyloid-beta protein intracellular domain-associated protein 1)
	G3BP2	Ras GTPase-activating protein-binding protein 2
	G6PD1	Glucose-6-phosphate 1-dehydrogenase X
	GNAZ	Guanine nucleotide-binding protein G(z) subunit alpha
	HPCA	Neuron-specific calcium-binding protein hippocalcin
	KCC2A	Calcium/calmodulin-dependent protein kinase type II subunit alpha
	NAC2	Sodium/calcium exchanger 2
	NCKP1	Nck-associated protein 1
	PACN1	Protein kinase C and casein kinase substrate in neurons protein 1 (Syndapin-1)
	Q8CFX3	Protocadherin 1
	SV2B	Synaptic vesicle glycoprotein 2B
	SYGP1	Ras/Rap GTPase-activating protein SynGAP
	TBB3	Tubulin beta-3 chain
	TLN1	Talin-1
	VIME	Vimentin

Figure 1. One-week dabigatran etexilate treatment reduces expression of coagulation-associated proteins in Tg4510 mouse brain. Western blot evaluation of expression for proteins involved in coagulation, namely, thrombin (A), prothrombin (B) and fibrin (C). All data expressed as mean $\pm$ standard error (n=4-6), significance reported by t-test, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. One-week dabigatran etexilate treatment reduces expression of oxidative-stress related proteins in Tg4510 mouse brains. Evaluation of expression of oxidative stress proteins by western blot, iNOS (A) and NOX4 (B). All data expressed as mean $\pm$ standard error (n=4-6), significance reported by t-test, * $p \leq 0.05$, ** $p < 0.01$.

Figure 3. One-week dabigatran etexilate treatment increases expression of antioxidant proteins in Tg4510 mouse brain. Western blot analysis of antioxidant proteins SOD1 (A) and SOD2 (B). All data expressed as mean $\pm$ standard error (n=4-6), significance reported by t-test, * $p \leq 0.05$, **** $p \leq 0.0001$.

Figure 4. Dabigatran etexilate treatment does not alter expression of neuroinflammation-associated proteins in Tg4510 mouse brain. Investigation of proteins involved in neuroinflammation by western blot GFAP (A) and IBA1 (B). All data expressed as mean $\pm$ standard error (n=4-6), significance reported by t-test, *** $p \leq 0.001$; **** $p \leq 0.0001$.

Figure 5. Evaluation of total Tau, and phosphorylated protein expression by western blot. Total Tau (A), phosphorylated Tau at sites S396 (B), S404 (C) and S416 (D). All data expressed as mean $\pm$ standard error ($n \geq 4$), significance reported by t-test, * $p \leq 0.05$, ** $p \leq 0.01$; **** $p \leq 0.0001$ (****).

Figure 7. LC-MS/MS SWATH data analyzed for coagulation (A), inflammation (B) and tau-pathology related (C). Data expressed as fold change (n=5).

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CONCLUDING REMARKS

An exponential increase in the prevalence of neurodegenerative diseases has led to a worldwide health crisis. The two most common neurodegenerative diseases, Alzheimer's and Parkinson's disease constitute the largest majority of cases. Although research has been ongoing, no successful interventions have been discovered that stop the progression of the disease. Focus has shifted from targeting disease characteristic protein aggregations, namely β -amyloid and α -synuclein, to identifying targets earlier in disease progression, such as neuroinflammation or cognitive decline. Herein, we utilize two independent strategies: the use of natural products and repurposed pharmaceuticals to target neuroinflammation in models of Alzheimer's and Parkinson's disease.

Natural products have been utilized for centuries in traditional medicines to combat a wide variety of illnesses. The therapeutic potential of thousands of plants has yet to be fully elucidated, posing a major source of untapped resources. Our research analyzed *Mucuna pruriens* and a common class of plant compounds, polyphenols, for their protective effects against neuroinflammation and potential as nutraceutical interventions. A levodopa-reduced *Mucuna pruriens* seed extract exhibited protective effects against toxin-induced inflammation and neuronal death. After isolation, no single compound exhibited these protective effects, supporting a potential synergistic effect in the extract. Further, polyphenol microbial metabolites, but not parent compounds showed protective effects *in vitro* against toxins. Equol, a gut-derived metabolite, was also effective *in vivo* against Parkinson's disease-specific toxic agents. These natural products exhibit protective effects in Parkinson's disease models, suggesting potential therapeutic in neurodegenerative diseases.

A newer approach to identify new therapeutics is to repurpose previously approved pharmaceuticals for new indications. Repurposing pharmaceuticals is a drug discovery approach that may fast track pharmaceuticals to a new targeted patient population. We analyzed direct thrombin inhibitor, dabigatran etexilate (Pradaxa®), against neuroinflammation in AD and PD models. In a transgenic *Drosophila melanogaster* model of Parkinson's disease, dabigatran improved motor control, related to the reduction in neuroinflammation as exhibited by decreased levels of reactive oxygen and nitric oxide species. Short-term treatment with dabigatran in a transgenic tau-based animal model of Alzheimer's disease alters protein expression. These studies identify that repurposed pharmaceuticals may be effective in reducing neuroinflammation in neurodegenerative diseases.

While our results indicate these interventions may reduce neuroinflammation in models of neurodegeneration, extensive research is still necessary. Further elucidation of the mechanism of action in additional animal models is necessary before clinical trials can be conducted. Realistically, these natural products may be extremely far from reaching the clinic. However, dabigatran may be much closer to the targeted population. Moreover, these results support our initial hypothesis that natural products and repurposing pharmaceuticals are strategies that can target neuroinflammation in neurodegenerative diseases.