2013

P-GLYCOPROTEIN TRANSPORT IN PESTICIDE PHARMACOKINETICS AND TOXICITY: INVESTIGATING A LINK TO PARKINSON'S DISEASE RISK

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TOXICITY: INVESTIGATING A LINK TO PARKINSON'S DISEASE RISK

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Dissertation

presented in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy in Toxicology

University of Montana
Missoula, Montana

August 2013

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P-glycoprotein Transport in Pesticide Pharmacokinetics and Toxicity:
Investigating a Link to Parkinson's Disease Risk

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  Variation in the gene that encodes P-glycoprotein (P-gp), *ABCB1*, has been associated with Parkinson's disease risk. The aim of my research was to evaluate P-gp transport of pesticides that have been associated with Parkinson's disease as a mechanism for the pharmacogenomic association. To facilitate this aim, a combination of models was utilized to determine P-gp transport of the pesticides diazinon, dieldrin, endosulfan, maneb, MPP+, paraquat, and rotenone.

  Paraquat pharmacokinetics and brain accumulation were measured in both FVB wild-type and $mdr1a^{(-/-)}/1b^{(-/-)}$ mice. P-gp deficient mice displayed no changes in paraquat brain accumulation, indicating that P-gp does not mediate paraquat disposition. I confirmed this observation *in vitro* using ATPase activity, cytotoxic sensitivity, transepithelial transport, and rhodamine-123 inhibition assays, in which I observed no interaction between P-gp and paraquat. There has been recent controversy regarding paraquat induction of Parkinson's disease in mice. I evaluated paraquat toxicity in both C57BL/6J and FVB wild-type mice. I observed no differences in any of the neurological endpoints in C57BL/6J and FVB mice.

  I next evaluated diazinon, dieldrin, endosulfan, maneb, MPP+, and rotenone as P-gp substrates or inhibitors. None of the pesticides inhibited P-gp. Only rotenone stimulated ATPase activity, suggesting that rotenone is a P-gp substrate. It is possible that exposure to rotenone may confer differential Parkinson's disease risk in individuals with genetic variation in *ABCB1*.

  Collectively, I screened the pesticides associated with Parkinson's disease as P-gp substrates and inhibitors. Indentifying rotenone as a P-gp substrate has provided the first piece of evidence towards identifying a potential mechanism for the association between genetic variation in *ABCB1* and Parkinson's disease. The observations gathered from the models of P-gp transport and pesticide toxicity evaluated herein will be pivotal in further evaluation of the association of genetic variation in *ABCB1* and Parkinson's disease risk. It is possible that this association may be a result of another exposure or endogenous molecule, however, the role of genetic variation in *ABCB1* and Parkinson's disease remains unclear. Further, the use of paraquat as a reproducible method to induce Parkinson's disease in mice is an unreliable model of Parkinson's disease and requires further investigation.

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ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Erica L. Woodahl for her inspiration, guidance, support, and creativity throughout my graduate career. Dr. Woodahl's contribution to my success was invaluable. I would like to express sincere appreciation to the members of my committee, Drs. Elizabeth Putnam, Fernando Cardozo-Pelaez, Dave Poulsen, Scott Wetzel, and Leslie Dickmann for contributing their time and knowledge to my research. I would also like to extend my gratitude to Dr. Andrij Holian for his support and mentorship throughout my time as a student in the Center for Environmental Health Sciences. I would like to thank members of the Department of Biomedical and Pharmaceutical Sciences, as well as the Center for Environmental Health Sciences who facilitated this research. I would also like to thank the members of the Woodahl lab for their help and friendship during my graduate research: Mindy Stone, Alana White, Chelsea Morales, Rachel Dalton, Kasse Skagen, Amanda Schlichenmayer, Meg Trahey, and Harmen Steele.

Most importantly, I would like to thank the many friends and family members who have given endless amounts of laughter, support, encouragement, and inspiration; without whom this research would have never been possible. Specifically I would like to extend a special acknowledgement to Drs. Marcy McNamara, Katie Hoffman, and Phillip Beske for their love and support during our time in the Department of Biomedical and Pharmaceutical Sciences as graduate students. In addition, many friends have contributed to my success in working towards completing my research including Josh Ballman, Amanda Bohlman, Alyssa Borchardt, Kirsten San Miguel, Becky Grassl-Smith, and Kellen Beck. Our outdoor, concert, barbeque, fishing, camping, and floating adventures together were vital for my mental health, and I anticipate many more to come. I especially want to extend a special acknowledgement to my parents Dan and Sue Lacher, and my brother David Lacher. Their love and support in every aspect of my life have given me the motivation to strive for the top and continue forward with my career and personal aspirations.

DEDICATION

To my family, Daniel, Mary Sue, and David. Their love and support fuel my success.

CHAPTER 1

INTRODUCTION

The distribution of nutrients, toxins, and drugs to the brain is strictly regulated. In order to maintain homeostasis in the brain, the central nervous system (CNS) has evolved effective mechanisms to simultaneously protect the brain from circulating agents and supplies the brain with vital nutrients. Even slight perturbation of the homeostatic mechanisms present at the brain can lead to disease. Although altered access to the brain alone does not account for the source of all brain disease, it is clear that deeper understanding of these protective mechanisms will largely influence brain disease prevention, diagnosis, and treatment (Daneman, 2012).

The brain is protected from circulating xenobiotics by an anatomic barrier, first described by German microbiologist Paul Ehrlich in 1885. Following injection of the hydrophilic dye trypan blue in rodents, Erlich observed that almost all tissues were stained except the brain. Alternatively, when the dye was injected directly into the brain, the dye stained the brain, however, the dye did not distribute to peripheral tissues. This observation clearly demonstrated the existence of a barrier between the blood and brain, now referred to as the blood-brain-barrier (BBB). While many drugs and toxicants are unable to cross the BBB and distribute to the brain, the BBB is not an absolute barrier to systemically circulating xenobiotics

The BBB contains multiple mechanisms that protect and regulate access to the brain. The BBB is comprised of capillaries in which endothelial cells form tight junctions with adjacent cells. The tight junctions provide strong contact between the cells, preventing paracellular transport and distribution of polar substances to the brain (Liu et al., 2012). Thus molecules that do gain access to the brain must be small and lipophilic in order to traverse through the endothelial cells via transcellular pathways. In addition,

transport proteins in the cell membranes of the endothelial cells mediate the efflux or influx of molecules across the BBB. Carrier-mediated transport mechanisms at the BBB facilitate the directional flux of xenobiotics across the endothelial cells. Thus, in addition to the physical barrier posed by the endothelial cells, the BBB contains many transport mechanisms that protect the brain from the toxic effects of circulating xenobiotics.

The Human Genome Project identified more than 400 membrane transporters primarily belong to either the solute carrier (SLC) or ATP-binding cassette (ABC) transporter superfamilies. SLC transporters can operate as bidirectional transporters, and the energy for the transport is supplied by a concentration or ion gradient. Thus these transporters are considered passive facilitated transporters. Conversely, ABC transporters operate as unidirectional transporters, and the energy required for transport is provided as ATP. Therefore, ABC transporters are considered active facilitated transporters. SLC and ABC transporters are expressed in many tissues including the BBB and mediate the uptake of important nutrients as well as the efflux of potentially dangerous xenobiotics.

SLC transporters transport a variety of xenobiotics including organic anions, organic cations, nucleotides, and amino acids. SLC transporters are commonly referred to as organic anion transporters (OAT) and organic cation transporters (OCT). SLC transporters have been shown to mediate nutrient and drug uptake at the BBB (Asaba et al., 2000; Gao et al., 2000; Kakyo et al., 1999). Upon consideration of the substrates of SLC transporters, it is clear that their main role at the BBB is to mediate the uptake of nutrients and xenobiotics.

Contrary to SLC transporters, ABC transporters efflux compounds out of the cell. In humans there are seven ABC gene subfamilies. The gene *ABCB1* (or *MDR1*) encodes

the most clinically relevant drug transporter, P-glycoprotein (P-gp). P-gp mediates the efflux xenobiotics in healthy tissues throughout the body including the intestine, liver, kidney, and at the BBB (Lin and Yamazaki, 2003a; Lin and Yamazaki, 2003b; Schinkel, 1997; Sharom, 2006; Sharom, 2011). P-gp plays an important role in the pharmacokinetics and disposition of drugs from many therapeutic classes [as reviewed in (Cascorbi, 2011; Giacomini, 1997; Giacomini et al., 2010; Sharom, 2011)]; however, there is less evidence for the role of P-gp in toxicant disposition.

Altered P-gp expression and activity have been associated with a number of exposure related diseases, including Parkinson's disease, childhood acute lymphoblastic leukemia, renal cancer, multiple myeloma, irritable bowl syndrome, Alzheimer's disease, progressive supranuclear palsy, and multiple system atrophy (Bartels et al., 2008b; Bartels et al., 2008a; Blokzijl et al., 2007; Drozdzik et al., 2003; Dutheil et al., 2010; Furuno et al., 2002; Jamroziak et al., 2004; Kuhnke et al., 2007; Le Couteur et al., 2001; Lee et al., 2004; Lee and Bendayan, 2004; Maggini et al., 2008; Semsei et al., 2008; Siegsmund et al., 2002; Tan et al., 2005; Tan et al., 2004; Urayama et al., 2007; van Assema et al., 2012; Vautier and Fernandez, 2009; Westerlund et al., 2009; Westerlund et al., 2008). One hypothesis of the association between P-gp and Parkinson's disease is that decreased P-gp transport of neurotoxicants at the BBB may lead to increased brain exposure and increased risk of Parkinson's disease (Bonnet and Houeto, 1999; Caudle et al., 2012a; Caudle et al., 2012b; Gatto et al., 2009; Langston et al., 1984; Semchuk et al., 1992; Steece-Collier et al., 2002). Pesticide manufacture, application, and exposure have been extensively associated with an increased risk to Parkinson's disease, however,

Parkinson's disease-associated neurotoxicants such as pesticides have not been evaluated as P-gp substrates.

The goals of this research were to evaluate P-gp transport of the pesticides associated with Parkinson's disease and investigate the link between genetic variation in *ABCB1* and Parkinson's disease. If P-gp transport at the BBB limits pesticide brain exposure, then genetic variation in *ABCB1* could alter the pesticide brain concentrations and potential Parkinson's disease risk. Identifying whether pesticides are P-gp substrates could provide a mechanism to explain the observed epidemiological association between genetic variation in *ABCB1* and Parkinson's disease.

In the following sections, background information regarding the role of P-gp in xenobiotic disposition will be presented, as well as a thorough description of the protective mechanisms present at the BBB. Next, information regarding Parkinson's disease phenotypes and risks will be discussed with an emphasis on the risk posed by pesticide exposure. Finally, current research regarding the role of *ABCB1* genetic variation in Parkinson's disease risk will be addressed. This introductory chapter will conclude with gaps in the understanding of P-gp transport of Parkinson's disease associated-pesticides, and the specific aims of this research.

1.1   ATP BINDING CASSETTE SUPERFAMILY: FROM DRUG TO TOXIN TRANSPORT

Multiple influx and efflux transporters are expressed at the BBB to supply nutrients to the brain and protect it from circulating toxicants. These transport mechanisms include passive facilitated (energy independent) in which molecules move

down a concentration gradient or active (energy dependent) in which transporters utilize

energy to facilitate the movement of a molecule against its concentration gradient. The

majority of carrier-mediated transporters involved in xenobiotic efflux utilize ATP and

possess an ATP-binding domain.

The ATP-binding cassette (ABC) transporter superfamily is the largest family of

membrane transporters. ABC transporters are responsible for actively transporting a wide

variety of compounds across cell membranes including but not limited to phospholipids,

ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile salts, drugs,

and toxins. In humans *ABC* genes are organized into seven subfamilies (*ABCA, ABCB,*

ABCC, ABCD, ABCE, ABCF, and *ABCG*) (Klein et al., 1999). Of the seven families, the

ABCB, ABCC, and *ABCG* subfamilies are the most clinically relevant ABC transporters.

The protein products of these genes are expressed in tissues important for the processes

of absorption (intestine) and elimination (liver and kidney), and are also responsible for

maintaining the regulatory function of blood-tissue barriers such as the BBB, blood-testes

barrier, and placental-barrier. A summary of the three most clinically relevant ABC

families can be found in (Table 1.1., page 25).

ABC transporters possess nucleotide-binding domains (NBD) that bind and

hydrolyze ATP to move substances against their concentration gradient. ABC

transporters also contain multiple transmembrane domains that are involved in substrate

recognition. ABC transporters are highly expressed at the BBB (Figure 1.1., page 27)

where they prevent passage of xenobiotics across the capillary membrane into the brain

(Begley, 2004; Dauchy et al., 2008; de Boer et al., 2003; Fricker and Miller, 2004;

Loscher and Potschka, 2005; Miller, 2010). The first identified and best studied ABC

transporter, P-gp, is encoded by the gene *ABCB1*.

1.1.1 DISCOVERY AND CHARACTERIZATION OF P-GLYCOPROTEIN

The presence of P-gp was first discovered in drug-resistant Chinese hamster ovary

cells (Juliano and Ling, 1976). Studies demonstrated that selecting cells in the presence

of the chemotherapy agent colchicine resulted in clonal cell lines with pleiotropic cross-

resistance to chemically unrelated drugs (Ling, 1975; Ling and Thompson, 1974). This

observation led to the conclusion that some currently unidentified mechanism was

responsible for the reduced accumulation of drugs within cancer cells, thus rendering

them resistant to chemotherapy drugs. Following isolation of the plasma membrane of

these resistant clonal cells, a protein band was recognized at 170 kDa with intensity

proportional to the level of drug resistance (Riordan and Ling, 1979). In 1986, the full-

length multidrug resistant gene 1 (*MDR1)* was cloned and was found to be homologous to

bacterial ATP-dependent transporters (Gros et al., 1986; Ueda et al., 1986). Since then,

MDR1 has become commonly referred to as *ABCB1*, the name assigned to it following

the ABC superfamily nomenclature.

1.1.2 P-GLYCOPROTEIN: BEYOND MUTLIDRUG RESISTANCE

Since its discovery in cancer cells, P-gp is expressed in healthy tissues throughout

the body including the intestine, liver, kidney, and at the BBB where it mediates the

efflux of a variety of structurally diverse xenobiotics (Lin and Yamazaki, 2003a; Lin and

Yamazaki, 2003b; Schinkel, 1997; Sharom, 2006; Sharom, 2011). Localization of P-gp

expression suggests that P-gp has evolved to protect the body from xenobiotic exposure by effluxing these compounds into the intestinal lumen, bile, urine, and by preventing accumulation in the brain and other sensitive tissues. Thus, P-gp plays a major role in the pharmacokinetic processes of absorption, distribution, metabolism, and excretion [as reviewed in (Lin and Yamazaki, 2003b)].

Translated as a single 1280 amino acid polypeptide, P-gp is made up of two semi homologous halves, each of which contains six transmembrane domains and an ATP-binding domain (Gottesman and Pastan, 1993). Mutagenesis and antibody mapping studies suggest that the two halves of P-gp interact together to form a single functional unit (Loo and Clarke, 1994; Muller et al., 1996). In addition, studies have demonstrated that P-gp extrudes its substrates directly from the cell membrane prior to entering the cytoplasm (Higgins and Gottesman, 1992; Homolya et al., 1993; Rosenberg et al., 1997; Shapiro and Ling, 1997). Although crystal structures of a bacterial ABC transporter had been published, it was not until 2009 that a crystal structure of mammalian P-gp (murine-87% sequence identity to human) was published (Aller et al., 2009).

One of the most fascinating characteristics of P-gp is that it is able to recognize and transport numerous drugs with a large variety of chemical structures and therapeutic uses. Though there are exceptions, the majority of drugs transported by P-gp are neutral and basic. One unifying feature is that the majority of P-gp substrates are lipophilic, supporting that partitioning into the lipid cell membrane is the first step towards efflux via P-gp. Although much is known regarding the role the P-gp plays in drug distribution, little is known regarding toxicant substrates of P-gp.

1.2   THE BLOOD-BRAIN-BARRIER IN HEALTH AND DISEASE

Neuronal homeostasis is essential for the maintenance of normal brain function. The structure responsible for maintaining homeostasis and protecting the brain from neurotoxic compounds is the BBB. Since the discovery of the BBB over 100 years ago most neuroscientists are familiar with the idea that the BBB has evolved as a mechanism to allow vital nutrients into the brain and keep neurotoxic substances out. Therefore, it is important to address the unique properties of the endothelial cells comprising the BBB as well as the contribution of drug transporters in brain penetration of xenobiotics.

1.2.1   THE BIOLOGY OF THE BLOOD-BRAIN-BARRIER

The three-cell model of the BBB is depicted in Figure 1.1., (page 27) where the endothelial cells of the BBB are closely connected to two other cell types, pericytes and astrocytes. The endothelial cells are polarized, form tight and therefore and express differential protein profiles on either the luminal (apical – blood facing) and abluminal (basolateral - brain facing) membranes. Pericytes are phagocytic cells, and in isolated brain capillaries there is estimated to be one pericyte for every three endothelial cells (Cancilla et al., 1972). The astrocytes contain foot processes that comprise the large majority of the capillary basement membrane. Analysis of the intimate relationship of astrocyte foot processes and endothelial cells clearly showed astrocyte foot processes forming rosette-like structures at the abluminal surface of the endothelium. In this same evaluation, each astrocyte was found to send processes to a single endothelial cell, although one endothelial cell may receive input from multiple astrocytes (Kacem et al., 1998). Astrocytes and pericytes are responsible for physically reinforcing the endothelial

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cell-to-cell junctions, as well as producing factors that induce the endothelial cells' ability to generate tight junction proteins (Arthur et al., 1987; Janzer and Raff, 1987). Thus, the function of the endothelial cells of the brain capillaries is further supported by the relationship with pericytes and astrocyte foot processes.

The tight junctions that form between adjacent endothelial cells at the BBB are the most complex tight junctions of the entire vascular system (Claude, 1978). High electrical resistance (1500-2000 Ohm cm^2) and low permeability are features of stable tight junction morphology *in vivo*. Several tight junction-associated proteins have been identified in endothelial cells including occludin, claudin-1 and -2, ZO-1 and -2, Zo-3/p130, 7H6, and cingulin (Kniesel and Wolburg, 2000). Collectively the BBB forms and maintains a strict barrier to the brain that prevents polar or large molecules from gaining access and having potential neurotoxic effects.

1.2.2   DISTRIBUTION ACROSS THE BLOOD-BRAIN-BARRIER

Many factors contribute to the distribution of a xenobiotic to the brain. The pharmacokinetic processes of absorption, distribution, metabolism, and elimination all determine the amount of xenobiotic available for uptake and transport at the BBB. For highly permeable xenobiotics, blood flow is a limiting factor, whereas other xenobiotics are limited by their permeability. In addition, plasma protein binding determines the unbound fraction of xenobiotics, thus influencing the amount of available xenobiotic available that is free to penetrate the BBB. Xenobiotic metabolism can occur at the BBB (Decleves et al., 2011), as some drug-metabolizing enzymes are present in the brain capillaries, although at significantly lower levels than in the liver. The endothelial cells of

the BBB are critical for the uptake and efflux of both endogenous and exogenous compounds by preventing passive hydrophilic diffusion and by expressing an array of transport mechanisms (Figure 1.1., page 27) (de Boer et al., 2003).

1.2.3   P-GLYCOPROTEIN: PROTECTION AT THE BLOOD-BRAIN-BARRIER

P-gp has been shown to play an important role in protecting the brain from circulating drugs and neurotoxicants. In humans, *ABCB1* is the sole gene that encodes for P-gp, while in mice there are two genes, *mdr1a* and *mdr1b*. Important evidence for the role of P-gp at the BBB was initially obtained through studies with *mdr1a* knock-out mice; *mdr1a* is the primary P-gp isoform detected in the brain vasculature in mice. Following exposure to ivermectin, researchers reported that they "serendipitously" discovered that the *mdr1a* deficient animals were much more sensitive to the neurotoxic effects (100-fold) and had increased brain uptake (88-fold) of ivermectin than the wild-type (Schinkel et al., 1994). Since this discovery, many reports have described the role of P-gp in brain distribution of therapeutics and many attempts are being made to modulate P-gp activity at the BBB to increase delivery of drugs to the CNS (Miller et al., 2008). Little is known, however, about the role that P-gp plays in brain exposure of toxicants and subsequent risk of neurodegenerative diseases.

It is well established that exposure to environmental toxicants can result in neurodegenerative diseases. Exposure to environmental agents combined with genetic pre-disposition and aging, increases the risk for developing CNS diseases such as Parkinson's disease, Alzheimer's disease, and Creutzfeldt-Jakob Disease (Drozdzik et al., 2003; Hartz et al., 2010; Hoffmeyer et al., 2000; Kortekaas et al., 2005; Vogelgesang et

al., 2006; Wolf et al., 2012). Decreased P-gp function at the apical membrane of the BBB could potentially increase the risk of neurological disease as a result of increased exposure to exogenous and/or endogenous toxins. However mechanistic knowledge in each of these diseases is greatly lacking to determine the main risk factors for neurodegenerative diseases (Bartels et al., 2009).

1.3   CHARACTERISTICS AND RISK FACTORS OF PARKINSON'S DISEASE

Parkinson's disease is an incurable degenerative disorder of the central nervous system that results in crippling motor dysfunction. The pathology of Parkinson's disease results from progressive loss of the dopaminergic neurons in the substantia nigra pars compacta (SNpc), a loss of dopamine input to the striatum, the presence of cytoplasmic alpha-synuclein protein aggregates known as Lewy Bodies, depigmentation of the locus ceruleus, and sympathetic denervation of the heart (Bennett et al., 1999; Goldstein, 2007; Olanow and Tatton, 1999). In addition, it has been shown that Parkinson's disease patients exhibit systemic inhibition of mitochondrial complex I, which results in depletion of ATP and generation of toxic reactive oxygen species (Schapira et al., 1989). The cause of the dopaminergic cell death is unknown, but it is thought that both genetic and environmental factors may contribute. Some common risk factors for developing Parkinson's disease are aging, genetic predisposition, and exposure to toxicants such as heavy metals (manganese and iron), industrial solvents, and pesticides.

The National Institute for Neurological Disorders and Stroke (NINDS) estimated that 50,000 new cases of Parkinson's disease are diagnosed in the US each year, and the total number of cases in the US is at least 500,000. However the total number of

Parkinson's disease cases is difficult to assess, as the disease is not diagnosable until it is far advanced. The prevalence of Parkinson's disease is expected to double by 2030 (Dorsey et al., 2007). In addition, the NINDS estimates that the economic cost of Parkinson's disease in the US currently exceeds $6 billion per year. Little is known about Parkinson's disease prevention and early diagnosis. Current treatment options offer temporary reduction of symptoms; however, these often result in devastating side effects such as increased involuntary movements (dyskinesia), hallucinations, impaired memory, and compulsive behavior. Thus, further research regarding risk factors of exposure related Parkinson's disease is of obvious interest.

1.3.1 EXPOSURE AND PARKINSON'S DISEASE RISK: MPTP

The contribution of environmental exposure to the development of Parkinson's disease was strongly supported after the discovery of a group of intravenous drug users who injected a synthetic analog of demerol that was unknowingly contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al., 1983). This exposure resulted in an acute and permanent parkinsonian state that was temporarily responsive to the dopamine replacement therapy, levodopa. Upon gaining access to the brain, MPTP is converted to 1-methyl-4-phenyl-4-phenylpyridinium ion (MPP+, cyperquat). MPP+ displays neurotoxicity in the SNpc and results in many parkinsonian pathological characteristics (Langston et al., 1984). In addition, it has been shown that MPP+ interacts with mitochondrial complex I and generates harmful reactive oxygen species (Richardson et al., 2005). These observations prompted researchers to identify

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compounds in the environment that could be similar to MPTP, and upon exposure could lead to the development of Parkinson's disease.

1.3.2   EXPOSURE AND PARKINSON'S DISEASE RISK: PESTICIDES

Pesticides are compounds designed to deter or kill insects and plants. Five billion pounds of pesticides were used worldwide from 2006-2007 (Grube et al., 2011). Pesticide exposures have not only been associated with an increased risk for developing Parkinson's disease in epidemiological studies, but are also capable of inducing neurotoxicity such as increased dopaminergic neurodegeneration in the brain, oxidative stress, and loss of dopaminergic neurons in the substantia nigra in murine models of Parkinson's disease. The most commonly associated pesticides associated with Parkinson's disease are paraquat, rotenone, and maneb. Although pesticides are a very structurally and functionally complex class of chemicals (Figure 1. 2., page 28), epidemiological and laboratory evidence implicate only a subset of these pesticides in Parkinson's disease pathology [as reviewed in (Hatcher et al., 2008)]. These include the pesticides diazinon, dieldrin, endosulfan, maneb, MPP+, paraquat, and rotenone.

1.3.2.1   PARAQUAT

The herbicide paraquat is the most thoroughly evaluated pesticide as a parkinsonian neurotoxicant. Paraquat was first produced in 1961 and quickly gained attention as a toxicant due to cases of toxicity in human exposure. It was also noted that paraquat is chemically homologous to the known parkinsonian neurotoxicant MPP+ (Figure 1.3., page 29). These observations led to the prediction that paraquat was toxic to

dopaminergic neurons in a similar manner as MPP+ (Dawson and Dawson, 2003; Snyder and D'Amato, 1985). Paraquat exposure has been associated with an increased Parkinson's disease risk in epidemiological studies (Hertzman et al., 1990; Liou et al., 1997; Semchuk et al., 1992). Despite these epidemiological observations, it was initially suggested that paraquat was not a likely candidate for inducing Parkinson's disease due to its polar charge, limited absorption, and poor BBB penetration (Koller, 1986). However, more recent studies have reported that paraquat is able to cross the BBB possibly through a neutral amino acid transporter, and may accumulate in the brain of mice (Prasad et al., 2009; Prasad et al., 2007; Shimizu et al., 2001).

In addition to the epidemiological evidence that paraquat exposure results in increased Parkinson's disease risk, it has been demonstrated *in vitro* that paraquat is a likely Parkinson's disease inducing neurotoxicant. Paraquat (PQ^{2+}) accepts an electron from a reductant during glycolysis and forms paraquat monocation ($PQ^{+\cdot}$). This quickly reacts with O_2 to form a superoxide radical ($O_2^{\cdot-}$), which then regenerates PQ^{2+} (Choi et al., 2006). The formation of the superoxide radical initiates the generation of other reactive oxygen species, and this is likely the main cause of paraquat toxicity. Mitochondrial complex I and III have both been suggested to be directly involved in the generation of paraquat reactive oxygen species (Castello et al., 2007; Cocheme and Murphy, 2008).

The ability of paraquat to induce Parkinson's disease in animals has recently become a topic of controversy. Some groups have been successful in demonstrating that chronic administration of paraquat leads to dopaminergic dysfunction, while others have argued that paraquat is not a dopaminergic neurotoxicant. For example, chronic paraquat

administration has been shown to result in a 20-30% selective dopamine neuronal loss in the SNpc and increased expression and aggregation of alpha-synuclein (Manning-Bog et al., 2002; McCormack et al., 2002). To further this observation, it was shown that over-expression of either wild-type or mutant alpha-synuclein is protective against paraquat toxicity (Manning-Bog et al., 2003). Finally, others have reported that paraquat exposure in mice leads to loss of dopaminergic neurons in the SNpc, degeneration of striatal terminals, and decreased locomotor activity (Brooks et al., 1999; Ossowska et al., 2006). In combination these reports support the ability of paraquat exposure to result in a loss of dopaminergic neurons.

However, there is a growing body of contradictory evidence that suggests that paraquat is not able to induce Parkinson's disease phenotypes in mice (Breckenridge et al., 2013; McCormack et al., 2002; McIntosh et al., 2010; Miller, 2007; Widdowson et al., 1996; Woolley et al., 1989). For example, in the striatum of C57BL/6J mice, the concentrations of dopamine, dopamine metabolites, and the rate of dopamine turnover have been reported to be either unaffected or only marginally affected following paraquat exposure (Breckenridge et al., 2013; McCormack et al., 2002; Woolley et al., 1989). Locomotor activity assays have also shown conflicting results following paraquat administration, again ranging from unaffected to marginally affected (Shepherd et al., 2006; Yang et al., 2007). In addition, a recent report showed that paraquat-induced dopamine loss in the SNpc is highly dependent on genetic background. Researchers found that C57BL/6J, but not Swiss-Webster, mice are susceptible to dopaminergic toxicity following chronic paraquat administration (Jiao et al., 2012). Further adding to the controversy, the Office of Research Integrity of the United States Department of

Health and Human Services announced on June 28, 2012 that a paper regarding chronic paraquat toxicity in C57BL/6J mice had been retracted due to "intentional fabrication of stereological cell count data" (Thiruchelvam et al., 2005). This event has further clouded the field of paraquat and Parkinson's disease toxicity, and warrants further investigation of paraquat as a dopaminergic neurotoxicant.

1.3.2.2   ROTENONE

The second most commonly associated pesticide with Parkinson's disease is rotenone. Rotenone is very lipophilic and easily crosses plasma membranes without the need for an uptake transporter. Initial evaluation of rotenone exposure found minimal SNpc damage (Ferrante et al., 1997; Thiffault et al., 2000). Other studies showed that rotenone administration resulted in parkinsonian symptoms including selective dopaminergic degeneration and cytoplasmic inclusions similar to Lewy Bodies (Betarbet et al., 2000). Much like MPP+, rotenone interacts with mitochondrial complex I and generates harmful reactive oxygen species throughout the entire brain, however, cell toxicity was only observed in the SNpc (Richardson et al., 2005). In addition, rotenone exposure has been shown to lead to increased oxidative stress, inflammation, ubiquitin accumulation, and proteasomal inhibition (Kim and Joh, 2006; Liu et al., 2003; Sherer et al., 2002; Wang et al., 2006), all of which are pathological features of Parkinson's disease.

1.3.2.3   MANEB

Exposure to the manganese containing pesticide, maneb, has also been implicated as a Parkinson's disease risk factor. It is possible that the association between maneb exposure and Parkinson's disease is due to the manganese metal core and leads to symptoms similar to those seen in manganism (Meco et al., 1994). Manganism is a neurological disease with many similar disease phenotypes as seen in Parkinson's disease. One study has suggested that maneb produces reactive oxygen species via mitochondrial complex III (Zhang et al., 2003). Additionally, maneb has shown to exacerbate toxicity of other agents in mouse models, such as paraquat (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b), although these publications come from the researcher disbarred for data fabrication, thus the interpretation of these data are difficult.

1.3.2.4   ORGANOCHLORINES AND ORGANOPHOSPHATES

Organochlorine pesticides are a class of chlorinated pesticides that have been banned in the United States for decades, however, they are persistent in the environment and are resistant to degradation. The association of organochlorines with Parkinson's disease risk is driven by several studies in dopaminergic cell lines. These studies have shown the ability of the organochlorines endosulfan or dieldrin to generate reactive oxygen species due to interaction with mitochondrial complex III, induction of ubiquitin-proteasome dysfunction, promotion of alpha-synuclein aggregation, depletion of intracellular dopamine, and activation of caspases (Jia and Misra, 2007b; Kanthasamy et al., 2005; Kitazawa et al., 2003; Sun et al., 2005). Exposure to endosulfan or dieldrin in mice has been shown to reproduce many of the phenotypes seen in Parkinson's disease

such as increased alpha-synuclein expression and dopaminergic dysfunction (Hatcher et al., 2007; Jia and Misra, 2007a). Finally, studies have demonstrated increased levels of dieldrin in the post-mortem brain tissue of Parkinson's disease patients (Fleming et al., 1994).

Organophosphate pesticides are known neurotoxicants that inhibit acetylcholinesterase. Organophosphates have been evaluated as Parkinson's disease risk factors and although there are case reports of people who develop severe parkinsonism following organophosphate exposure (Bhatt et al., 1999). While the symptoms of organophosphate exposure are reversible, they are not responsive to levodopa, which are not features of Parkinson's disease. It is possible that the effects of organophosphate exposure involves disturbance of the balance between dopaminergic and acetylcholinergic systems rather than specific death of the dopaminergic neurons in the SNpc (Hatcher et al., 2008). Diazinon is one organophosphate in particular that has been shown to contribute to Parkinson's disease risk in a gene-environment interaction study (Slotkin and Seidler, 2011).

1.4   A ROLE FOR *ABCB1* PHARMACOGENOMICS IN PARKINSON'S DISEASE

The *ABCB1* gene is large and complex spanning more than 200 kb on human chromosome 7. The *ABCB1* gene is made up of 28 exons that give rise to a 3.8 kb coding region. It has become evident that P-gp expression and activity is at least in part influenced by genetic polymorphisms in the *ABCB1* gene, referred to as single nucleotide polymorphisms (SNPs) (Hitzl et al., 2001; Hoffmeyer et al., 2000; Kim et al., 2001). The four most studied functional *ABCB1* SNPs are 1199G>A, 1236C>T, 2677G>T/A, and

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3435C>T. The 1236C>T and 3435C>T SNPs are synonymous and do not alter amino acid sequence. The mechanism by which these synonymous gene variants alter P-gp expression is unknown, although a possible effect could be altered mRNA stability or delayed protein translation (Kimchi-Sarfaty et al., 2007; Wang et al., 2005; Wang and Sadee, 2006).

The non-synonymous SNPs 1199G>A and 2677G>T/A code for a serine to asparagine change at amino acid 400 and the triallelic change from an alanine to a serine or an alanine to a threonine change at amino acid 893, respectively (Woodahl et al., 2009b; Woodahl and Ho, 2004; Woodahl et al., 2004; Woodahl et al., 2005). One issue in interpreting data regarding *ABCB1* SNPs and P-gp expression and activity is that most studies have focused on evaluating individual SNPs and not haplotypes. Haplotypes are a combination of alleles in a gene that are in linkage disequilibrium and are inherited together. For example, most studies have investigated the role of 3435C>T on P-gp expression and function without accounting for the strong linkage disequilibrium with 1236C>T, and 2677G>T/A (Woodahl and Ho, 2004). The strong linkage disequilibrium between *ABCB1* SNPs is well known (Chowbay et al., 2003; Furuno et al., 2002; Horinouchi et al., 2002; Illmer et al., 2002; Johne et al., 2002; Kroetz et al., 2003; Siddiqui et al., 2003; Tanabe et al., 2001; Zheng et al., 2002), yet many evaluations have failed to account for the linkage in their study design.

In addition to altered P-gp transport, *ABCB1* genetic variation has also been associated with a number of neurological diseases in addition to Parkinson's disease including Alzheimer's disease, epilepsy, stroke, and HIV-associated dementia. It is possible that *ABCB1* genetic variation causes alterations in P-gp expression and/or

function at the BBB, and thus alters neurotoxicant transport and distribution in the brain. Therefore, alterations in P-gp expression and transport due to *ABCB1* genetic variation could be an important predictor of disease.

1.4.1 *ABCB1* PHARMACOGENOMICS IN PARKINSON'S DISEASE RISK

Epidemiologic studies have found an association between *ABCB1* pharmacogenomics and susceptibility to Parkinson's disease (Bartels et al., 2008a; Drozdzik et al., 2003; Dutheil et al., 2010; Furuno et al., 2002; Le Couteur et al., 2001; Lee et al., 2004; Lee and Bendayan, 2004; Tan et al., 2005; Tan et al., 2004; Vautier and Fernandez, 2009; Westerlund et al., 2009; Westerlund et al., 2008). A table summarizing the epidemiological associations of *ABCB1* SNPs and Parkinson's disease risk can be found in Table 1.2. (page 26) Studies in Asian patients showed that the 2677T/3435T or 1236T/2677T/3435T haplotypes were significantly associated with a reduced risk of Parkinson's disease (Lee *et al.*, 2004; Tan *et al.*, 2005). In European populations, the 1236C>T and 3435C>T SNPs have been shown to have an increased frequency in Parkinson's disease patients, while another study evaluating the 2677G>T/A and 3435C>T SNPs found no association between allele frequencies and Parkinson's disease (Furuno *et al.*, 2002; Tan *et al.*, 2004) Finally, a recent study in Europeans observed an association between *ABCB1* variation and Parkinson's disease risk, but only after including occupational exposure to organochlorine pesticides in the analysis; the researchers observed a higher risk of Parkinson's disease in patients with 2677TT or TA genotypes who were also exposed to organochlorines (Dutheil et al., 2010). Although the authors did not report what specific organochlorine was responsible for this association, a

personal contact with the corresponding author suggested that dieldrin is the

organochlorine that is commonly used in the area of the population evaluated. One

possible mechanism to explain this association may be that certain *ABCB1* genetic

variants decrease P-gp activity at the blood-brain-barrier, leading to increased brain

accumulation of neurotoxicants and an increased incidence of Parkinson's disease.

1.4.2 BRAIN DISTRIBUTION OF VERAPAMIL IN PARKINSON'S DISEASE PATIENTS

In addition to the pharmacogenetic evidence of an association between P-gp and

Parkinson's disease, other work has attempted to understand the role of P-gp in

Parkinson's disease neuropathology using the radiolabeled P-gp substrate verapamil.

Global ^{11}C-verapamil distribution was compared in Parkinson's disease patients and

healthy volunteers in imaging studies using positron emission tomography. Because ^{11}C-

verapamil is actively transported out of the brain by P-gp, the brain distribution volume

of ^{11}C-verapamil is inversely related to P-gp activity at the BBB (Bart et al., 2003;

Lubberink et al., 2007). These studies demonstrated a 20% increased brain accumulation

of verapamil in Parkinson's disease patients compared to control subjects, indicating that

P-gp activity at the BBB was decreased (Bartels et al., 2008a; Kortekaas et al., 2005).

This finding strongly suggests P-gp activity at the BBB is reduced in patients with

Parkinson's disease, but whether it is due to *ABCB1* pharmacogenomics or mechanisms

of down-regulation is unclear.

1.4.3 P-GLYCOPROTEIN TRANSPORT OF PESTICIDES

Some studies have evaluated pesticides as substrates of P-gp (Bain and LeBlanc, 1996; Bircsak et al., 2012; Lecoeur et al., 2006; Pivcevic and Zaja, 2006). Specifically, the organochlorine endosulfan has been shown to be both a P-gp substrate and inhibitor (Bircsak et al., 2012; Shabbir et al., 2005). The organophosphate diazinon is transported by P-gp in Caco-2 cells and has been shown to induce P-gp expression in rats (Lecoeur et al., 2006). In another rat model, paraquat-induced lung toxicity was reduced by induction of P-gp with dexamethasone, while verapamil increased paraquat accumulation and toxicity in the lung (Dinis-Oliveira et al., 2006a; Dinis-Oliveira et al., 2006c). If pesticides are P-gp substrates, decreased P-gp activity at the BBB due to genetic variation in *ABCB1* would be expected to lead to increased brain accumulation of pesticides and an increased risk of Parkinson's disease.

1.5 SPECIFIC AIMS

The objectives of this research were to test the hypothesis that decreased P-gp transport at the BBB, due to genetic variation in *ABCB1,* results in an increase in brain accumulation of neurotoxicants and may be a risk factor for Parkinson's disease. Using a combination of *in vitro* and *in vivo* models, this research aimed to identify a potential mechanism for the role of P-gp in Parkinson's disease by evaluating P-gp mediated transport of pesticides. In Specific Aim 1, a pharmacokinetic analysis of plasma and brain samples following single oral doses of paraquat in FVB wild-type and FVB *mdr1a$^{(-/-)}$/1b$^{(-/-)}$* mice was performed. Additionally, in Specific Aim 1, paraquat was evaluated as a P-gp substrate using *in vitro* models of transport. In Specific Aim 2, rotenone, maneb,

endosulfan, diazinon, dieldrin, and ivermectin were evaluated as potential P-gp substrates or inhibitors. Finally, in Specific Aim 3, C57Bl/6J and FVB wild-type mice were chronically exposed to paraquat, following which dopamine loss, oxidative stress, and motor dysfunction were evaluated.

The following chapters detail the experimental design and research results completed to accomplish the goals of the research. In combination, the models chosen allowed for a complete and comprehensive analysis of pesticides as potential P-gp substrates. Understanding mechanisms of pesticide penetration at the BBB and accumulation in the CNS as well as which pesticides are substrates of P-gp will allow us to move closer to a therapy or preventative mechanism for Parkinson's disease.

Table 1.1. Clinically Relevant ABC Transporters

Gene	Protein	Tissue	Substrate	Inhibitor	Inducer
ABCB1	P-gp, MDR1	intestine, liver, kidney, BBB, placenta, adrenal, testes	colchicine, cyclosporine, digoxin, doxorubicin, fexofenadine, HIV protease inhibitors, ivermectin, paclitaxel, verapamil, vincristine,	cyclosporine, erythromycin, HIV protease inhibitors, GF120918 (elacridar), ketoconazole, LY335979, PSC 833 (valspodar), verapamil, quinidine	dexamethasone, grapefruit juice, rifampin, St. John's wort
ABCB4	MDR3	liver	digoxin, paclitaxel, vinblastine		
ABCC1	MRP1	intestine, liver, kidney, BBB	adefovir, indinavir		
ABCC2	MRP2	intestine, liver, kidney, BBB	indinavir, cisplatin	probenecid	
ABCC3	MRP3	intestine, liver, kidney, BBB	etoposide, methotrexate, tenoposide		
ABCG2	BCRP	intestine, liver, breast cancer, placenta	danorubicin, doxorubicin, topotecan, rosuvastatin, sulfasalazine	GF120918 (elacridar)	

Table adapted from the U.S. FDA Drug Development and Drug Interactions website and the University of Washington Drug Interaction Database

Table 1.2. Genetic Variation in *ABCB1* and Risk of Parkinson's Disease

Population Evaluated	*ABCB1* SNPs			Parkinson's Disease Risk
	1236C>T	2677G>T/A	3435C>T	
European (Furuno et al., 2002)	✓		✓	↑
European (Tan et al., 2004)		✓	✓	⊘
Asian (Lee et al., 2004)		✓	✓	↓
Asian (Tan et al., 2005)	✓	✓	✓	↓
European (Dutheil et al., 2010)	✓	✓	✓	⊘
*When included pesticide exposure		✓		↑
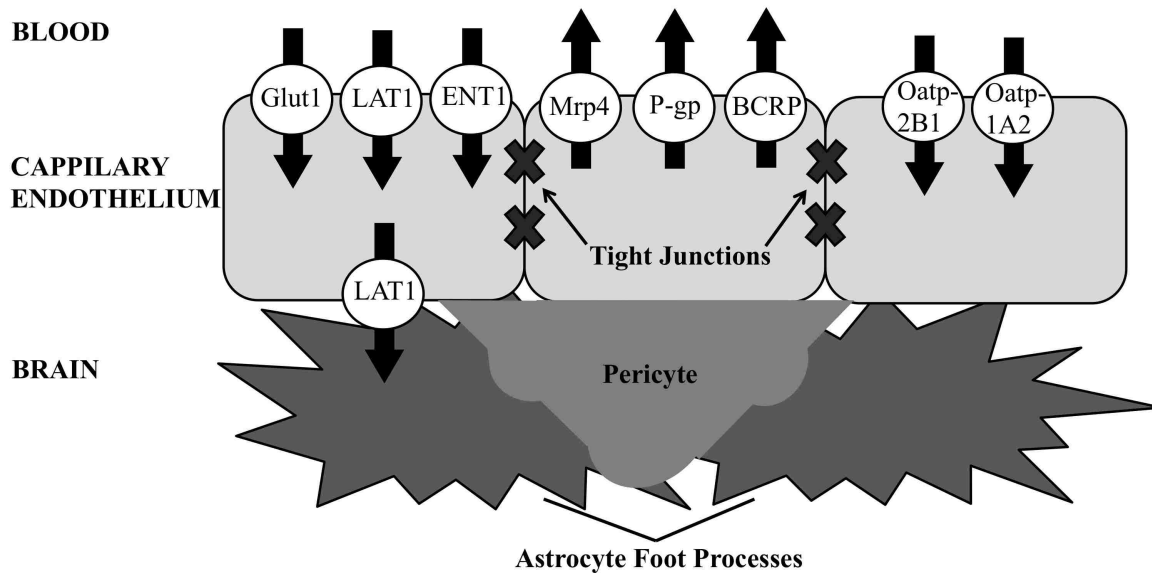
Figure 1.1. Model Highlighting the Composition of the BBB. Endothelial cells form tight junctions with adjacent cells, preventing diffusion of polar compounds through paracellular pathways. Astrocytes and pericytes are present to physically reinforce the endothelial cell-to-cell junctions, as well as produce factors that induce the endothelial cells' ability to generate tight junction proteins. Finally, an array of influx and efflux transporters is present and mediate transcellular transport of endogenous and xenobiotic compounds. Glucose transporter 1 (Glut1), Large amino acid transporter 1 (LAT1), Equilibrate nucleoside transporter 1 (ENT1), Multidrug resistance protein 4 (MRP4), P-glycoprotein (P-gp), Breast cancer resistance protein (BCRP), Organic anion transporting polypeptide 2B1 (Oatp2B1), Organic anion transporting polypeptide 1A2 (Oatp1A2). *Image adapted from Figure 5-10 of* (Doull, 2008).

Pesticides

Fungicides

Dithiocarbamates
 -Maneb, Ziram

Hexachlorobenzene

Pentachlorophenol

Phthalamides

Herbicides

Bipyridyls
 -Paraquat, Diquat, Cyperquat

Phosphomethyl amino acids

Chloroacetanilides

Chlorophenoxy Compounds

Insecticides

Organophosphates

Parathion

Chlorpyrifos

Diazinon

Organochlorines

Dieldrin

Endosulfan

DDT/DDE

Botanicals

Rotenone

Nicotine

Avermectins

Ivermectin

Pyrethroids

Permethrin

Cypermethrin

Figure 1.2. Classification of Pesticides. Pesticides are classified based on their target species. Of the compounds associated with Parkinson's disease, all are classified as either fungicides, herbicides, or insecticides.
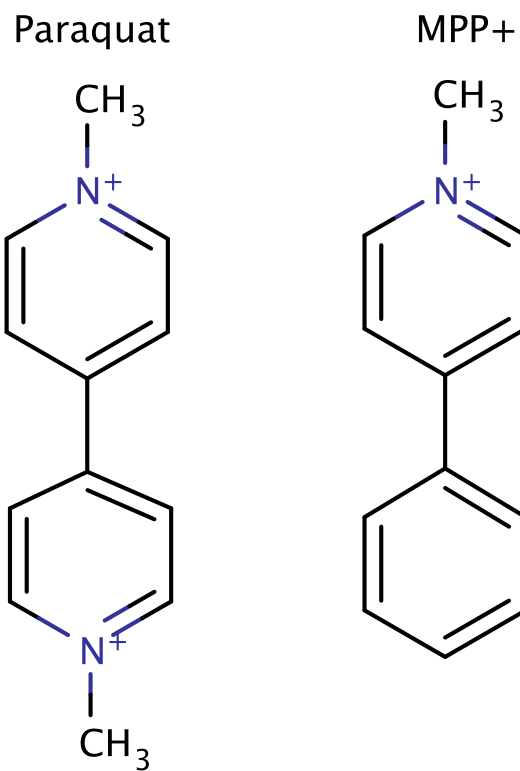
Figure 1.3. Chemical Structures of MPP+ and Paraquat. MPTP is converted to MPP+
(cyperquat) in the brain. One of the pyridine rings of MPP+ is charged, whereas both
pyridine rings in paraquat are charged.

CHAPTER 2

ABSENCE OF P-GLYCOPROTIEN TRANSPORT IN THE

PHARMACOKINETICS AND TOXICITY OF THE HERBICIDE PARAQUAT

ABSTRACT

P-glycoprotein (P-gp) is an efflux transporter encoded by the *ABCB1* (or *MDR1*) gene. *ABCB1* genetic variation has been associated with Parkinson's disease, which may be due to altered neurotoxicant disposition. Our goal was to investigate the role of P-gp transport in the disposition of the herbicide paraquat, a Parkinson's-associated neurotoxicant. We utilized *in vitro* transporter methods of ATPase, cytotoxicity, transepithelial permeability, and rhodamine-123 inhibition. We also measured paraquat pharmacokinetics and brain distribution in FVB wild-type and P-gp-deficient (*mdr1a*$^{(-/-)}$/*mdr1b*$^{(-/-)}$) mice following 10, 25, 50, and 100 mg/kg oral doses. *In vitro* data showed that: 1) paraquat failed to stimulate ATPase activity; 2) paraquat-induced cytotoxicity was unchanged in P-gp-expressing cells in the absence or presence of P-gp inhibitors verapamil and GF120918 (EC$_{50}$ values of 37.0 (33.2 – 41.4), 46.2 (42.5 – 50.2), and 34.1 (31.2 – 37.2) μM, respectively); 3) transepithelial transport of paraquat in P-gp-expressing cells was not altered by GF120918 (efflux ratios of 1.55 ± 0.39 and 1.58 ± 0.36, respectively); and 4) paraquat did not inhibit rhodamine-123 transport. We observed differences in pharmacokinetic parameters between FVB wild-type and *mdr1a*$^{(-/-)}$/*mdr1b*$^{(-/-)}$ mice. A clearance of 0.78 (95% CI: 0.58 – 0.98) and 0.47 L/hr (0.42 – 0.52), was observed in the FVB wild-type and *mdr1a*$^{(-/-)}$/*mdr1b*$^{(-/-)}$ mice, respectively, and volume of distributions of 3.36 (2.39 – 4.33) and 1.77 L (1.50 – 2.04), respectively. Importantly, paraquat brain accumulation was the same across all doses. Together these studies indicate that paraquat is neither a substrate nor an inhibitor of P-gp. Therefore, the association between *ABCB1* genetic variation and risk of Parkinson's disease is not due to alterations in efflux of paraquat.

2.1. INTRODUCTION

P-glycoprotein (P-gp) is an efflux drug transporter encoded by the multidrug resistance gene *ABCB1* (also known as *MDR1*). P-gp is a member of the ATP-binding cassette (ABC) superfamily of transporters, and mediates the efflux of a variety of structurally diverse xenobiotics in healthy tissues throughout the body including the intestine, liver, kidney, and at the blood-brain-barrier (Lin and Yamazaki, 2003a; Lin and Yamazaki, 2003b; Schinkel, 1997; Sharom, 2006; Sharom, 2011). P-gp plays an important role in the pharmacokinetics and disposition of drugs from many therapeutic classes [as reviewed in (Cascorbi, 2011; Giacomini, 1997; Giacomini et al., 2010; Sharom, 2011)]; however, there is less evidence for the role of P-gp in toxicant disposition. P-gp has also been associated with a number of diseases, including Parkinson's disease (Bartels et al., 2008a; Drozdzik et al., 2003; Dutheil et al., 2010; Furuno et al., 2002; Le Couteur et al., 2001; Lee et al., 2004; Lee and Bendayan, 2004; Tan et al., 2005; Tan et al., 2004; Vautier and Fernandez, 2009; Westerlund et al., 2009; Westerlund et al., 2008). While exposure to neurotoxic xenobiotics is a well-known risk factor in Parkinson's disease, it is unknown whether P-gp mediates the transport of these compounds (Bonnet and Houeto, 1999; Caudle et al., 2012a; Caudle et al., 2012b; Gatto et al., 2009; Langston et al., 1984; Semchuk et al., 1992; Steece-Collier et al., 2002).

Paraquat dichloride, or methyl viologen, is an herbicide that has been highly implicated as a risk factor for Parkinson's disease (Costello et al., 2009; Dhillon et al., 2008; Dinis-Oliveira et al., 2006b; Liou et al., 1997; McCormack et al., 2002; Tomenson and Campbell, 2011). Although production and commercial sale of paraquat has been banned in several countries due to toxicity, paraquat remains one of the most commonly

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used herbicides worldwide, including in the United States. In rodents, paraquat induces toxicity associated with the progression of Parkinson's disease, such as increased dopaminergic neurodegeneration in the brain, oxidative stress, and a loss of dopaminergic neurons in the substantia nigra (Betarbet et al., 2000; Dinis-Oliveira et al., 2006b; Gatto et al., 2009; Kang et al., 2009; Koller et al., 1990; Li et al., 2005a; Li et al., 2005b; Liou et al., 1997; Semchuk et al., 1992; Somayajulu-Nitu et al., 2009; Tomenson and Campbell, 2011; Yang et al., 2007).

Some studies have suggested a link between P-gp activity and paraquat exposure. Specifically, two studies found that induction of P-gp in rat lungs was protective against paraquat-induced lung toxicity (Dinis-Oliveira *et al.*, 2006a; Dinis-Oliveira *et al.*, 2006c). If paraquat was a P-gp substrate, decreased P-gp activity at the blood-brain-barrier could lead to increased brain accumulation of paraquat and an increased risk of Parkinson's disease. One mechanism for decreased P-gp function is due to genetic variation in the *ABCB1* gene, which is highly polymorphic with more than 300 single nucleotide polymorphisms (SNPs) in the coding region alone. Of these SNPs, four in particular (1199G>A, 1236C>T, 2677G>T/A, and 3435C>T) have been shown to alter P-gp expression or activity [as reviewed in (Cascorbi, 2011; Chinn and Kroetz, 2007; Wang and Sadee, 2006; Woodahl and Ho, 2004)].

The goal of this study was to evaluate P-gp-mediated transport of paraquat in a combination of *in vitro* and *in vivo* models to evaluate a potential mechanism for the role of P-gp in Parkinson's disease. We characterized P-gp transport of paraquat *in vitro* using cell- and membrane-based models. We also used an animal model to determine paraquat pharmacokinetics and brain accumulation in FVB wild-type and P-gp deficient mice

($mdr1a^{(-/-)}/1b^{(-/-)}$) to evaluate the role of P-gp in paraquat disposition *in vivo*. This is the first comprehensive study to evaluate P-gp-mediated disposition of paraquat.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

Paraquat dichloride, doxorubicin, verapamil, rhodamine-123 (R123), cyclosporine, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). GF120918 [N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] was kindly provided by GlaxoSmithKline (Research Triangle Park, NC). ^{14}C-paraquat was purchased from American Radiolabeled Chemicals (St. Louis, MO).

2.2.2. ATPase Activity

Stimulation of ATPase activity was measured using SB MDR1/P-gp Sf9 ATPase membranes (SOLVO Biotechnology, Budaors, Hungary) according to the manufacturer's instructions. ATPase activity in the presence of xenobiotics was estimated by measuring the release of inorganic phosphate (P_i) in a colorimetric reaction. P-gp membranes were incubated with either paraquat or the known P-gp substrate verapamil over a concentration range of 0.05 - 100 μM. ATPase activity was determined as the difference in P_i liberation measured in the absence or presence of 1.2 mM sodium orthovanadate, a non-specific ATPase inhibitor. The reactions were incubated at 37°C for 20 min and stopped by the addition of 10% SDS. The detection reagent containing ammonium molybdate was added and incubated for 25 min at 37°C, and absorbance was read at 690

nm using a SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA). The amount of P_i liberation was calculated from a phosphate standard curve. ATPase activity was reported as nmol P_i liberated per min incubation time per mg total protein (nmol P_i/min/mg protein). Compounds were evaluated in duplicate, and the assay was repeated twice. Michaelis-Menten parameters (V_{max} and K_m) were estimated using a nonlinear regression least squares model fit on Prism 5.0 software (GraphPad, San Diego, CA).

2.2.3. Cell Culture

The porcine renal epithelial cell line LLC-PK1 was utilized in the *in vitro* models of transport. LLC-PK1 vector cells (LLC-vector) and recombinant *ABCB1/MDR1* cells (LLC-MDR1-WT), generously provided by Michael M. Gottesman in the Laboratory of Cell Biology at the National Cancer Institute (Bethesda, MD), were cultured in complete Media 199 (Mediatech, Manassas, VA) supplemented with 3% (v/v) fetal bovine serum (FBS) (Mediatech), 1% (v/v) L-glutamine (Mediatech), 1% (v/v) penicillin/streptomycin (Mediatech), and 1% (v/v) geneticin (Life Technologies, Carlsbad, CA) and grown at 37°C in the presence of 5% CO_2.

2.2.4. Cytotoxic Sensitivity

Sensitivity to cytotoxic agents was evaluated in LLC-vector and LLC-MDR1-WT cells plated overnight at a density of 1000 cells/well in 96-well plates (Fischer Scientific, Hampton, New Hampshire). Cells were treated with either paraquat (concentration range of 15 - 250 μM), doxorubicin (concentration range of 0.05 nM - 250 μM), or colchicine (concentration range of 0.01-50,000 μM) for 72 hrs at 37°C. Cell viability was evaluated

using the CellTiter-Glo® Cell Viability Assay (Promega, Fitchburg, WI) according to the manufacturer's instructions. Inhibition was performed at 0.5 µM GF120918 and 10 µM verapamil. Luminescence was measured using a SynergyMX microplate reader (Biotek, Winooski, VT). Compounds were evaluated in triplicate. Viability was estimated as the effective concentration necessary for 50% cell death (EC_{50}) based on a nonlinear regression log (agonist) vs. response variable slope least squares model fit using Prism 5.0.

2.2.5. Transepithelial Permeability

Transepithelial permeability assays were completed according to methods developed previously (Woodahl et al., 2009a; Woodahl et al., 2004; Woodahl et al., 2005). Briefly, LLC-vector and LLC-MDR1-WT recombinant cells were plated at a density of 2 x 10^6 cells/24 mm well on permeable supports (Transwell; 3.0 μm membrane pore size; Corning, (Tewksbury, MA)) and grown for 4 days prior to the initiation of the experiment. Transepithelial electrical resistance (TEER) values were measured with a Millicell-ERS (Millipore, Billerica, MA). All transport assays were carried out at 37°C and corrections were made for the difference in volume in the apical and basolateral compartments. Transport of the known P-gp substrate, R123 (5 μM), or ^{14}C-paraquat (450 nM) was performed in serum-free Media 199. Inhibition of transport was performed at 1 μM GF120918. Aliquots of 50 μl were taken from the apical and basal compartments at 0.5, 1, 2, 3, and 4 hrs. Experiments were run in triplicate. R123 was quantified by measuring fluorescence with an excitation of 488 nm and an emission of 525 nm using a Gemini XS Fluorescent microplate reader (Molecular Devices, Sunnyville CA). ^{14}C-

paraquat was quantified by liquid scintillation counting (Beckman LS 6500, Brea, CA). Apparent permeability (P_{app}) was calculated as $P_{app} = (1/(A \times C_0)) \times (dQ/dt)$ where A is the surface area of the permeable support, C_0 is the initial concentration in the donor compartment, and dQ/dt is the rate of transfer of compound into the acceptor compartment; P_{app} was estimated in both the apical-to-basolateral ($P_{app\ A \to B}$) and basolateral-to-apical ($P_{app\ B \to A}$) directions. The permeability efflux ratio of ($P_{app\ B \to A}$)/ ($P_{app\ A \to B}$) was estimated to evaluate P-gp-mediated directional efflux. Permeability ratios >2 are expected for P-gp substrates, and the ratio should reduce to approximately 1.0 in the presence of the P-gp inhibitor GF120918.

2.2.6. Intracellular Accumulation

Inhibition of R123 intracellular uptake in LLC-vector and LLC-MDR1-WT cells was performed based on a previously developed assay (Woodahl et al., 2004), with the following modifications. Cells were plated overnight at a density of 1×10^6 cells/well in 6-well plates (Invitrogen). Cells were incubated in triplicate in 5 μM R123 with and without inhibitors in serum-free Media 199 at 37°C and then allowed to efflux in the presence of inhibitor in complete Media 199. Concentrations ranged from 0.1 - 1000 μM for paraquat, 0.1 - 500 μM for verapamil, 0.1 - 100 μM for cyclosporine, and 1.56 nM - 1 μM for GF120918. After the efflux period, cells were washed in ice-cold phosphate buffered saline (PBS), trypsinized, and resuspended in PBS containing 5 mM EDTA, 25 mM HEPES, and 1% FBS (pH 7.0). Immediately prior to flow cytometry, DAPI was added to the cells as a measure of cell viability. Cells were analyzed with a FACSAriaII flow cytometer (BD Biosciences, San Jose, CA) using FACSDiva software. Ten thousand

cells from each sample were analyzed for forward scatter, side scatter, DAPI, and R123 accumulation. Inhibition was estimated as the inhibitor concentration necessary for 50% inhibition (IC_{50}) based on a nonlinear regression log (inhibitor) vs. normalized response variable slope least squares model fit on Prism 5.0.

2.2.7. Animals

Male FVB wild-type and $mdr1a^{(-/-)}/1b^{(-/-)}$ (FVB background) mice age 1-8 months (Taconic Farms, Germantown, NY) were used in this study. Mice were maintained on a 12 hr light-dark cycle, housed in microisolators, and were given food and deionized water *ad libitum*. All procedures were approved by the University of Montana Institutional Animal Care and Use Committee.

2.2.8. Paraquat Dosing and Sample Collection

Paraquat was prepared fresh in sterile water for each treatment. Paraquat was administered via oral gavage at doses of 10, 25, 50, or 100 mg/kg (n = 5-10 per dose group). Blood samples were collected via the saphenous vein at 1, 2, 4, and 8 hrs following paraquat administration, and plasma was separated from whole blood via centrifugation. Paraquat administration occurred at approximately the same time of day for each treatment. Following the 8 hr blood collection, mice were euthanized via cervical dislocation. Whole brains were extracted and washed twice in PBS. Plasma and brain samples were frozen at -80°C until analysis.

2.2.9. Quantitation of Paraquat in Plasma and Brain Samples

After thawing, sterile water was added to the brain samples at a 2:1 (w:v) ratio. The brain:water mixture was first homogenized for 1 min and then sonicated for 5 sec intervals for a total of 1 min; samples were kept on ice throughout the entire homogenization and sonication period. Ethyl viologen (100 μg/L) was used as an internal standard in plasma and brain samples prior to protein extraction. Standard curves were simultaneously prepared from brain and plasma samples from untreated mice at a range of 10- 2500 μg/L paraquat and 100 μg/L ethyl viologen. Plasma and brain samples were extracted with 1:2 (v:v) acetonitrile (-20°C), vortexed, and centrifuged at 13,000 rpm for 10 min. The supernatant was collected and the extraction was repeated two more times for a total of three extractions. Supernatants were combined and filtered using Ultrafree-MC centrifugal filter units (Millipore). Samples were stored at -80°C prior to quantitation.

Levels of paraquat in mouse plasma and brain samples were determined by liquid chromatography tandem mass spectroscopy (LC-MS/MS) using an Agilent 1200 Series HPLC and 6300 series MSD/XCT (Waldbronn, Germany), utilizing a method adapted from previously published work (Ariffin and Anderson, 2006). Separations were accomplished using a Phenomenex Kinetex® C18 column (Torrance, CA), 2.1x100 mm, 2.6 μm particles, and a flow rate of 0.1 mL/min. A gradient from 25% to 90% of methanol (mobile phase B) over 4 min was employed with 20 mM ammonium formate and 15 mM heptafluorobutyric acid in water (mobile phase A; pH 3.2). MS detection was made from 5-10 min for plasma samples and 3.5-10 min for brain samples with all other flow diverted to waste. Quantitation of paraquat in plasma or brain samples was

determined using multiple reaction monitoring to characterize fragmentation of singly-charged analytes with transitions m/z 185→171 for paraquat and m/z 213→157 for ethyl viologen. Basic MS settings included nebulizer pressure 40 psi, dry gas flow 8 L/min, dry gas temperature 350°C, isolation window 4.0 amu, and fragmentation amplitude 0.41 V. Plasma and brain concentrations were reported as μg/L and ng/g brain tissue, respectively.

2.2.10. Pharmacokinetic Analysis

Initial pharmacokinetic parameter estimates were made using standard non-compartmental methods (Phoenix™ WinNonlin® 6.3, Pharsight, St. Louis, MI) (Gabrielsson and Weiner, 2012). When possible the linear trapezoidal method with the "partial area" option of 0 to 8 hrs was used in each strain of mouse to estimate the elimination rate constant based on available observations and the area under the plasma concentration-time curve (AUC). In instances where calculation of the elimination rate constant was not possible, the AUC was calculated up to the last observation. Initial estimates of apparent oral clearance (CL/F) and apparent volume of distribution (V/F) were calculated using standard equations. The concentration-time profile of paraquat was explored graphically.

Final pharmacokinetic parameters were estimated using a population pharmacokinetic analysis of paraquat and nonlinear mixed effect modeling (NONMEM, version 7.2, ICON Dev. Soln., Ellicott City, MD) with PDx-Pop® (version 5, ICON Dev. Soln., Ellicott City, MD) interfaced with Xpose® (version 4.0, release 6, update 1, Uppsala, Sweden) (Yuh et al., 1994). Using the subroutines of NONMEM: ADVAN 2 (one compartment linear model with first order absorption) and TRANS 2 (CL/F and

V/F), population pharmacokinetic estimates of CL/F, V/F, and absorption rate constant

(k_a) were estimated by combining all dose groups in each mouse strain. Robust estimates

for interindividual variability (IIV), which is the source of variability determined between

mice, were predicted. Residual unexplained variability (RUV), the coefficient of variance

that accounts for the random and unexplained variability, was also determined. Paraquat

brain-to-plasma partitioning ratios were calculated using the paraquat concentrations in

brain (ng/g) and plasma (μg/L) at the 8 hr time point.

2.2.11. Model Evaluation

The population pharmacokinetic models were compared using the Akaike and

Schwarz information criterion to discriminate between non-hierarchical models in the

selection of the structural model. During model development, diagnostic plots of

observed vs. population predicted or individual predicted values were used to visually

assess model fit (Sherwin et al., 2012). Plots of residuals and weighted residuals vs. time

or predicted values were also examined. The population pharmacokinetic models were

evaluated using a non-parametric re-sampling bootstrap method to assess model accuracy

and stability. PDx-Pop® (Dublin, Ireland) was used to generate bootstrap runs generated

by random sampling using the original dataset. Standard errors for the estimated

population parameters and random effects error models were also assessed. Relative

standard error (RSE) was estimated as the standard error divided by the mean and

expressed as a percentage. Random error is considered to always be present and is

unpredictable (Sheiner and Beal, 1981). Empirical Bayesian estimates for the predicted

concentrations were obtained using the POSTHOC option in NONMEM. The

performance of the final model was further evaluated by generating a visual predictive

check, which compares statistics derived from the distribution of observations and the distribution of predictions (Bergstrand et al., 2011).

2.2.12. Statistical Analysis

Student's one- or two-tailed t test was used to determine differences between two sets of data for transepithelial permeability. An extra sum of squares F-test was utilized to compare individual parameters for the cytotoxic sensitiviy assay. The null hypothesis was that the EC_{50} estimates are the same within a cell type between xenobiotic treatment group alone and in the presence of a P-gp inhibitor, and the null hypothesis was only rejected if the inhibitor decreased cytotoxic resistance. A two-tailed Grubbs' test for outliers was utilized prior to analysis of paraquat pharmacokinetics in plasma and brain samples. Levels of paraquat in brain samples were analyzed using an analysis of variances (ANOVA). For all analyses, p-values of < 0.05 were considered statistically significant.

2.3. RESULTS

ATPase activity stimulated by paraquat was measured in P-gp membranes using the P-gp substrate verapamil as a positive control (Figure 2.1., page 54). V_{max} and K_m parameters for verapamil were estimated as 48.4 ± 2.6 nmol P_i/min/mg protein and 2.54 ± 0.68 µM, respectively. Paraquat did not stimulate ATPase activity above the level of the sodium orthovanadate-sensitive control (11.4 ± 4.3 nmol P_i/min/mg protein), indicating that paraquat is not a P-gp substrate.

Xenobiotic-induced cytotoxicity was measured in LLC-vector and LLC-MDR1-WT cells. Dose-response curves were generated following exposure to paraquat two

cytotoxic P-gp substrates, doxorubicin and colchicine, and EC_{50} values were estimated (Figure 2.2. and Table 2.1., pages 55 and 51 respectively). We also utilized P-gp inhibitors GF120918 and verapamil to confirm changes in cellular sensitivities were due to P-gp. As expected, LLC-MDR1-WT cells exhibit significantly increased resistance to doxorubicin (61-fold) and colchicine (48-fold) when compared with the LLC-vector cells (Figures 2.2., a-b and c-d, respectively, page 55). In addition, P-gp inhibitors, GF120918 and verapamil, reversed cellular resistance to doxorubicin and colchicine in LLC-MDR1-WT cells. Conversely, we observed a slight increase in resistance to paraquat toxicity in LLC-MDR1-WT cells compared to LLC-vector (3-fold) (Table 2.1., page 51). Importantly, the P-gp inhibitors GF120918 and verapamil, however, had no effect on paraquat-induced cytotoxicity (Figure 2.2. e-f, page 55) indicating that the modest increase in resistance to paraquat in LLC-MDR1-WT cells was not P-gp-mediated. This provided further evidence that paraquat is not a P-gp substrate.

P-gp-mediated directional transport in LLC-vector or LLC-MDR1-WT cells was evaluated by estimating transepithelial permeability efflux ratios using the P-gp substrate R123 as a positive control (Table 2.2., page 52). TEER values were measured as 676 ± 157 and $782 \pm 174 \ \Omega*cm^2$ in LLC-vector and LLC-MDR1-WT cells, respectively, prior to the start of experiments confirming integrity of the monolayers. Experiments were performed up to 4 hours to ensure that permeability rates were estimated in the linear range. As expected, we observed directional P-gp-mediated transport of R123 in LLC-MDR1-WT when compared LLC-vector cells, and the efflux was inhibited by GF120918. There was no directional transport of ^{14}C-paraquat in LLC-vector or LLC-

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MDR1-WT cells and no effect of GF120918, confirming that paraquat is not a P-gp substrate.

Paraquat was also evaluated as an inhibitor of P-gp. The ability of paraquat to inhibit the intracellular accumulation of R123 in LLC-vector and LLC-MDR1-WT cells was measured and compared to known P-gp inhibitors verapamil, cyclosporine, and GF120918 (Figure 2.3., page 56). Verapamil, cyclosporine, and GF120928 inhibited R123 efflux with IC_{50} values of 1.98 ± 0.12 μM, 1.39 ± 0.07 μM, and 22.9 ± 1.9 nM, respectively. Paraquat did not inhibit R123 accumulation in P-gp-expressing cells across a broad concentration range.

The systematic screening or paraquat *in vitro* indicates that paraquat is not a substrate of P-gp, but to confirm this finding we next investigated the role of P-gp in the disposition of paraquat *in vivo*. Paraquat pharmacokinetics and brain accumulation in FVB wild-type and *mdr1a*$^{(-/-)}$/*1b*$^{(-/-)}$ mice were evaluated. The absence of *mdr1a* and *mdr1b* in the knock-out mice was confirmed by genotyping both FVB wild-type and *mdr1a*$^{(-/-)}$/*1b*$^{(-/-)}$ mice using methods obtained from Taconic Farms (Figure 2.4., page 57). Both wild-type and knock-out strains of mice have similar plasma concentration-time curves following oral administration of 10, 25, 50, or 100 mg/kg paraquat (Figures 2.5a and 2.5b, page 58). After validating the model by non-compartmental analysis and generating initial parameter estimates (data not shown), we used the estimates in the generation of a one-compartment pharmacokinetic model to estimate final pharmacokinetic parameters (Table 2.3., page 53). Visual inspection of the plasma concentration time curves suggested that the majority of absorption had occurred prior to obtaining the first sample making it difficult to estimate the absorption rate constant,

particularly in the *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ mice. Therefore, the estimate for absorption in the

population pharmacokinetic model used the predicted estimate obtained from the non-

compartmental analysis and is expressed as a fixed estimate in the *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ mice.

There were modest differences in CL/F and V/F between FVB wild-type and *mdr1a*$^{(-/-}$

$^{)}$*/1b*$^{(-/-)}$ mice; however, the effect was small with less than a 2-fold increase in each

parameter in the *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ mice. Importantly, there were no differences between

FVB wild-type and *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ mice in brain-to-plasma partitioning ratios of paraquat

calculated from combined dose groups (2.39 ± 1.57 and 1.92 ± 1.62, respectively) or total

brain paraquat accumulation across dose groups (Figure 2.6., page 59). The lack of a

difference in brain distribution between FVB wild-type and *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ mice

confirms that paraquat is not a P-gp substrate

2.4 DISCUSSION

We have systematically evaluated the P-gp-mediated transport of paraquat using a

combination of *in vitro* and *in vivo* models. We determined that paraquat is neither a

substrate nor an inhibitor of P-gp. Although there is evidence to show that P-gp is

associated with the development of Parkinson's disease, these data demonstrate that the

causal relationship is not due to the P-gp-mediated disposition of the herbicide paraquat.

Several methods confirmed that P-gp does not transport paraquat. Paraquat was

screened as a substrate for P-gp in three experimental models: ATPase activity,

xenobiotic-induced cytotoxicity, and transepithelial permeability, all of which have been

well characterized to study P-gp (Brouwer et al., 2013; Feng et al., 2008; Giacomini et

al., 2010; Polli et al., 2001). First, paraquat did not stimulate the hydrolysis of ATP in P-

gp expressing membranes. Although the ATPase assay is a great screening tool to

investigate potential substrates of P-gp, it has been reported to generate false negatives (Bircsak et al., 2012), and this assay may not be appropriate for slowly transported substrates. Thus, we moved forward in assessing paraquat as a P-gp substrate in a cell based model. LLC-PK1 have low endogenous expression levels of transporters as well as differentiate in to polarized monolayers, and express tight junction proteins, similar to the cells of the BBB (Giacomini et al., 2010), making the LLC-PK1 cell line the gold standard cell line for transport evaluations. We utilized recombinant LLC-MDR1-WT and LLC-Vector cells as well as a combination of cytotoxic P-gp substrates and known P-gp inhibitors to assure our cell models contained multiple controls and were as robust as possible. We found that P-gp expression did not alter sensitivity to paraquat-induced cytotoxicity in cells, and there was no directional transepithelial transport of paraquat mediated by P-gp. Paraquat also did not inhibit R123 efflux in P-gp-expressing cells. Therefore, we can conclude from the *in vitro* studies that paraquat is not a human P-gp substrate or inhibitor.

To confirm our *in vitro* studies, we next evaluated paraquat pharmacokinetics and brain accumulation following an oral dose escalation study between FVB wild-type and $mdr1a^{(-/-)}/1b^{(-/-)}$ mice. We observed subtle differences in the primary pharmacokinetic parameters between wild-type and knock-out mice. For example there was a significant increase in CL/F in $mdr1a^{(-/-)}/1b^{(-/-)}$ mice when compared to FVB wild-type. However, it is clear that this change is not due to P-gp transport because if paraquat was a P-gp substrate then the $mdr1a^{(-/-)}/1b^{(-/-)}$ mice would display decreased CL/F of paraquat as these mice are lacking P-gp not only at the BBB, but also at the intestine, kidney, and liver. Our studies indicate the opposite effect, lack of P-gp results in increased CL/F; clearly

there must be other systemic changes in the $mdr1a^{(-/-)}/1b^{(-/-)}$ mice as a result of no systemic P-gp expression. It is most notable that we did not observe differences in brain-to-plasma partitioning or total brain accumulation of paraquat between FVB wild-type and $mdr1a^{(-/-)}/1b^{(-/-)}$ mice. Although one would expect a decrease in CL/F if paraquat was a P-gp substrate, the most notable expectation would be the significant increase in brain distribution of paraquat. Drugs that are P-gp substrates, such as amprenavir, ivermectin or vinblastine, display approximately 20-80-fold increase in brain accumulation in knock-out mice relative to wild-type mice due to loss of P-gp activity at the blood-brain-barrier (Polli et al., 1999; Schinkel et al., 1994; van Asperen et al., 1996). It is known that alterations in drug-metabolizing enzymes and drug transporters occur in $mdr1a^{(-/-)}/1b^{(-/-)}$ mice that may account for the observed differences in paraquat pharmacokinetics (Schuetz et al., 2000); it is clear, however, from the lack of differential brain distribution of paraquat that these differences are not due to P-gp and that P-gp is not playing a role in paraquat disposition. The animal studies confirm our *in vitro* results that paraquat is not a human or mouse P-gp substrate.

Our study is the first comprehensive study to measure P-gp transport of paraquat both *in vitro* and *in vivo*. Previous studies in rats have found that induction of P-gp expression was protective against paraquat-induced toxicity, which would suggest that paraquat is a P-gp substrate (Dinis-Oliveira et al., 2006a; Dinis-Oliveira et al., 2006c). These studies, however, used non-selective P-gp inducers, dexamethasone and doxorubicin, which are know to be broad-spectrum inducers of not only other drug transporters but drug-metabolizing enzymes as well; therefore, several mechanisms other than P-gp may have been involved in the protection against paraquat toxicity. This same

group also examined P-gp transport of paraquat in Caco-2 cells following induction of P-gp by doxorubicin using paraquat concentrations ranging from 10-5000 μM. The researchers found less than a 2-fold increase in the reported EC_{50} values as a result of doxorubicin treatment, indicating a minimal protective effect by P-gp (Silva et al., 2011). We also observed a 3-fold increase in paraquat resistance between P-gp-expressing and non-expressing cells, but importantly observed no effect of P-gp inhibitors on the resistance, indicating that P-gp was not contributing to the observed effect. As a comparison, known P-gp substrates, doxorubicin and colchicine, exhibited marked increases in resistance, 61-fold and 48-fold, respectively, between P-gp-expressing and non-expressing cells and resistance was reversed in the presence of P-gp inhibitors. Finally, another study evaluating paraquat as a P-gp substrate in Caco-2 cells observed differential paraquat cytotoxicity between untreated and doxorubicin-induced cells, but only at paraquat concentrations of 1 mM and above (Silva et al., 2013). These concentrations far exceed reported paraquat concentrations in humans. Blood samples from patients with acute paraquat poisoning had measured plasma concentrations of paraquat that did not exceed 50,000 ng/ml paraquat, or 194 μM, and most patients' concentrations were significantly lower (Shi et al., 2012). Thus, evaluating P-gp transport of paraquat in the mM concentration range is inappropriate and not relevant to systemic paraquat exposures in humans.

While this is the first study to evaluate the role of P-gp in paraquat pharmacokinetics, there have been other studies to measure paraquat pharmacokinetics in chronic studies (Breckenridge et al., 2013; Prasad et al., 2009; Prasad et al., 2007). The paraquat plasma concentrations we observed, following an oral dose to FVB mice, were

approximately a magnitude less than what has been observed following an intraperitoneal

dose to C57BlJ/6 mice; correspondingly, the paraquat brain concentrations we observed

were also lower (Breckenridge et al., 2013; Prasad et al., 2009; Prasad et al., 2007). These

differences in paraquat pharmacokinetics may be due to the different mouse strains

studied and different dosing strategies given that paraquat has variable bioavailability

depending on the route of administration (Chui et al., 1988).

Several studies have described an association between *ABCB1* pharmacogenomics

and susceptibility to Parkinson's disease (Bartels et al., 2008a; Drozdzik et al., 2003;

Dutheil et al., 2010; Furuno et al., 2002; Le Couteur et al., 2001; Lee et al., 2004; Lee and

Bendayan, 2004; Tan et al., 2005; Tan et al., 2004; Vautier and Fernandez, 2009;

Westerlund et al., 2009; Westerlund et al., 2008). One mechanism may be that *ABCB1*

genetic variation decreases P-gp activity at the blood-brain-barrier, leading to increased

brain accumulation of neurotoxicants, and an increased incidence of Parkinson's disease.

In European populations, the 1236C>T and 3435C>T SNPs have been shown to have an

increased frequency in Parkinson's disease, while another study evaluating the

2677G>T/A and 3435C>T SNPs found no association between allele frequencies and

Parkinson's disease (Furuno *et al.*, 2002; Tan *et al.*, 2004). Studies in Asian patients

showed that the 2677T/3435T or 1236T/2677T/3435T haplotypes were significantly

associated with a reduced risk of Parkinson's disease (Lee *et al.*, 2004; Tan *et al.*, 2005).

Finally, a recent study in Europeans observed an association between *ABCB1* variation

and Parkinson's disease risk, but only after including occupational exposure to

organochlorine pesticides in the analysis; the researchers observed a higher risk of

Parkinson's disease in patients with 2677TT or TA genotypes who were also exposed to

organochlorines (Dutheil et al., 2010). Some of these conflicting associations with *ABCB1* pharmacogenomics and Parkinson's disease could be a result of small sample sizes as well as genetic heterogeneity within the study populations. In addition to pharmacogenetic evidence, another link to P-gp activity in Parkinson's disease were studies that showed increased brain accumulation of verapamil in Parkinson's disease patients compared to control subjects, indicating that P-gp activity at the blood-brain-barrier may be decreased (Bartels et al., 2008a; Kortekaas et al., 2005). While there is evidence for a role of P-gp in the development of Parkinson's disease, it is clear from our data that altered P-gp transport of paraquat is not the cause.

2.4 CONCLUSIONS

In summary, we have demonstrated in both *in vitro* and *in vivo* models that paraquat is not a substrate or inhibitor of P-gp. Namely, P-gp expression did not alter the brain distribution of paraquat in *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ mice. Therefore, the association of *ABCB1* genetic variation and the increased risk of developing Parkinson's disease is not due to alterations in P-gp efflux of the herbicide paraquat. Further research is needed to identify neurotoxicants that may play a role in the *ABCB1* pharmacogenetic associations with Parkinson's disease.

Table 2.1. Xenobiotic-induced Cytotoxicity in LLC-vector and LLC-MDR1-WT Cells

	EC$_{50}$ Values (95% Confidence Interval)	
	LLC-vector	**LLC-MDR1-WT**
Paraquat (µM)	12.8 (11.4 – 14.3)	37.0 (33.2 – 41.4)
Paraquat (µM) + GF120918	13.6 (12.4 – 15.0)	46.2 (42.5 – 50.2)
Paraquat (µM) + Verapamil	12.9 (10.9 – 15.4)	34.1 (31.2 – 37.2)
Doxorubicin (nM)	32.2 (13.7 – 85.6)	1978 (1015-3850)
Doxorubicin (nM) + GF120918	24.8 (12.0 – 51.3)	8.06 (1.32 – 49.2)[a]
Doxorubicin (nM) + Verapamil	43.4 (12.0 – 155.9)	268 (90.7 – 98.6)[a]
Colchicine (µM)	13.3 (out of range)	645 (467 – 892)
Colchicine (µM) + GF120918	12.6 (10.8 – 14.5)	17.5 (12.0 – 25.4)[a]
Colchicine (µM) + Verapamil	12.8 (out of range)	179 (117 – 273)[a]

Significant differences within a cell type between xenobiotic treatment group alone and in the presence of a P-gp inhibitor; $p < 0.0005$[a].

[*] n.d.: not determined. Nonlinear regression was unable to estimate confidence intervals.

Table 2.2. Transepithelial Permeability in LLC-vector and LLC-MDR1-WT Cells

|	$P_{app\ B \to A}/P_{app\ A \to B} \pm$ s.d.	
	LLC-vector	**LLC-MDR1-WT**
R123	1.12 ± 0.40	5.10 ± 2.19
R123 + GF120918	1.13 ± 0.29	1.33 ± 0.92
Paraquat	1.39 ± 0.43	1.55 ± 0.39
Paraquat + GF120918	1.29 ± 0.35	1.58 ± 0.36

Table 2.3. Population Pharmacokinetic Analysis of Paraquat in FVB Wild-type and *mdr1a*^(-/-)*/1b*^(-/-) Mice Following a Single Paraquat Oral Dose

	FVB wild-type (n=34)					*mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ (n=28)			
	Estimates	% RSE	95% CI	CV%		Estimates	% RSE	95% CI	CV%
CL/F (L/hr)	0.473	5.18	0.425 – 0.521	–		0.777^{a}	13.3	0.575 – 0.979	–
V/F (L)	1.77	7.91	1.50 – 2.04	–		3.36^{a}	14.8	2.39 – 4.33	–
k$_a$	1.81	16.9	1.21 – 2.41	–		5.60^{b}	–	–	–
IIV – CL/F	0.056	35.1	0.018 – 0.095	23.7		0.344	32.0	0.128 – 0.560	58.7
IIV – V/F	0.072	49.6	0.002 – 0.141	26.8		0.514	24.7	0.265 – 0.763	71.7
RUV (%)	0.118	23.3	0.064 – 0.172	34.4		0.133	22.5	0.074 – 0.192	36.5

CL/F: apparent oral clearance; V: apparent volume of distribution; k$_a$: absorption rate constant; IIV: interindividual variability; RUV: residual unexplained variability; RSE: relative standard error; 95% CI: ninety-five percent confidence interval; CV%: percent coefficient of variance; – not determined in the model

[a] Significant differences between mouse strains; $p < 0.005$.

[b] Fixed pharmacokinetic estimate from non-compartmental analysis.
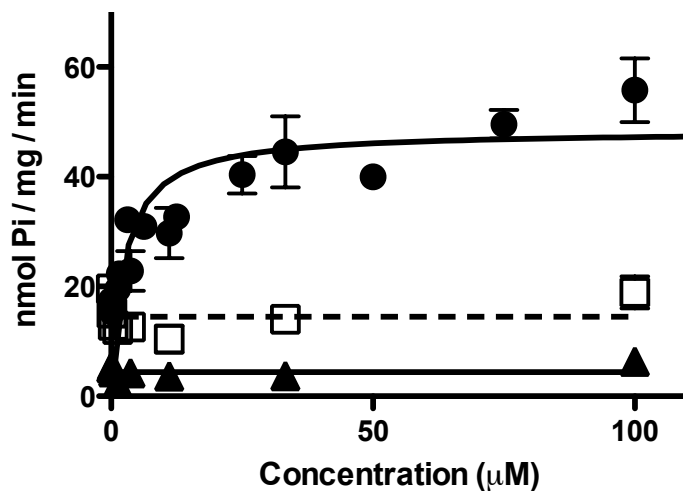
Figure 2.1. ATPase Activity in SB MDR1/P-gp Sf9 Membranes. P-gp membranes were incubated with either paraquat (closed triangle) or the known P-gp substrate verapamil (closed circle) over a concentration range of 0.05 - 100 μM. ATPase activity was determined as the difference in P_i liberation measured in the absence or presence of 1.2 mM sodium orthovanadate (verapamil + sodium orthovanadate, open square).
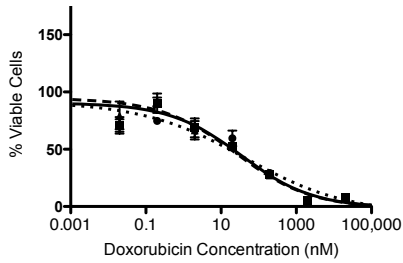
2.2.a LLC-vector　　　　　　**2.2.b LLC-MDR1-WT**
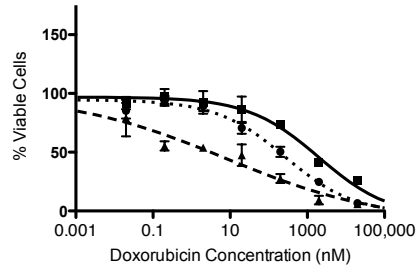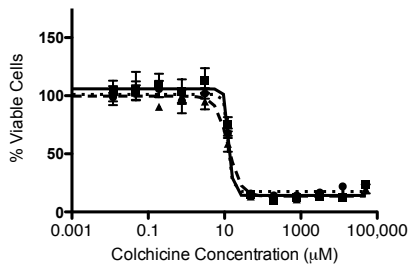
2.2.c LLC-vector　　　　　　**2.2.d LLC-MDR1-WT**
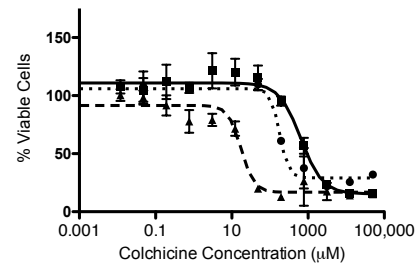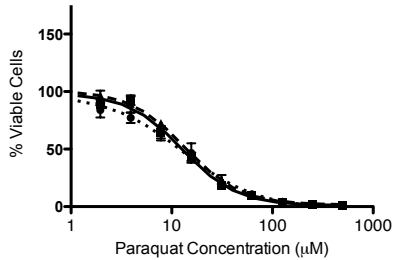
2.2.e LLC-vector　　　　　　**2.2.f LLC-MDR1-WT**
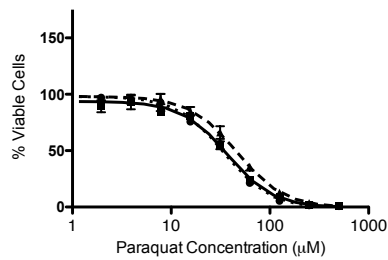
Figure 2.2. Paraquat Cytotoxic Sensitivity in LLC-vector and LLC-MDR1-WT

Cells. Cells were treated with either doxorubicin (a-b, concentration range of 0.05 nM -

250 µM), or colchicine (c-d, concentration range of 0.01-50,000 µM) paraquat (e-f,

concentration range of 15 - 250 µM), (square, solid line). Cell viability was evaluated

using the CellTiter-Glo® Cell Viability Assay. Inhibition was performed at 0.5 µM

GF120918 (triangle, dashed line) and 10 µM verapamil (circle, dotted line).
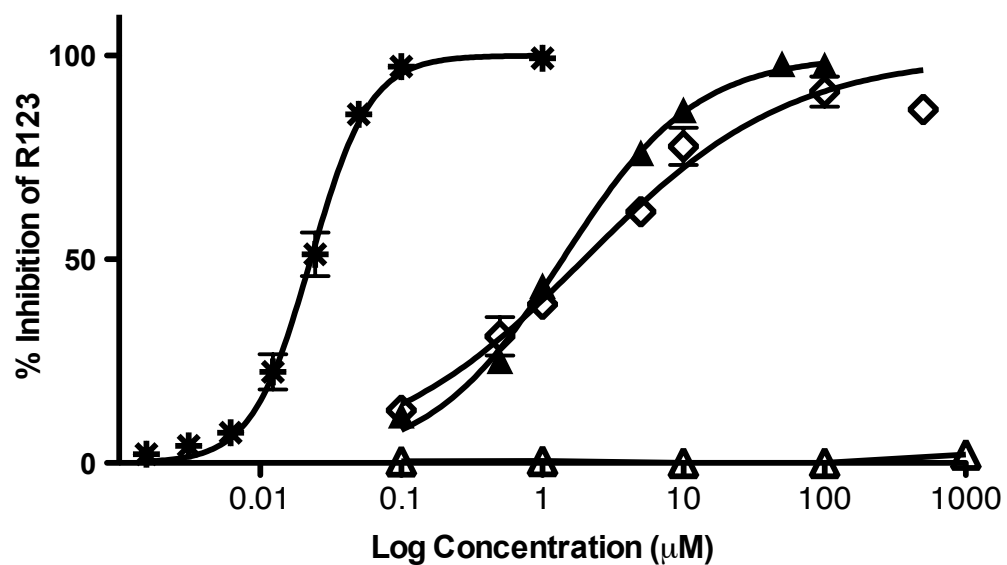
Figure 2.3. Inhibition of Rhodamine-123 Transport. Data is presented a percent of

rhodamine-123 inhibition and values are mean ± SEM. Tested compounds are paraquat

(open triangle △), GF120918 (closed star), verapamil (open diamond), and cyclosporine

(closed triangle).
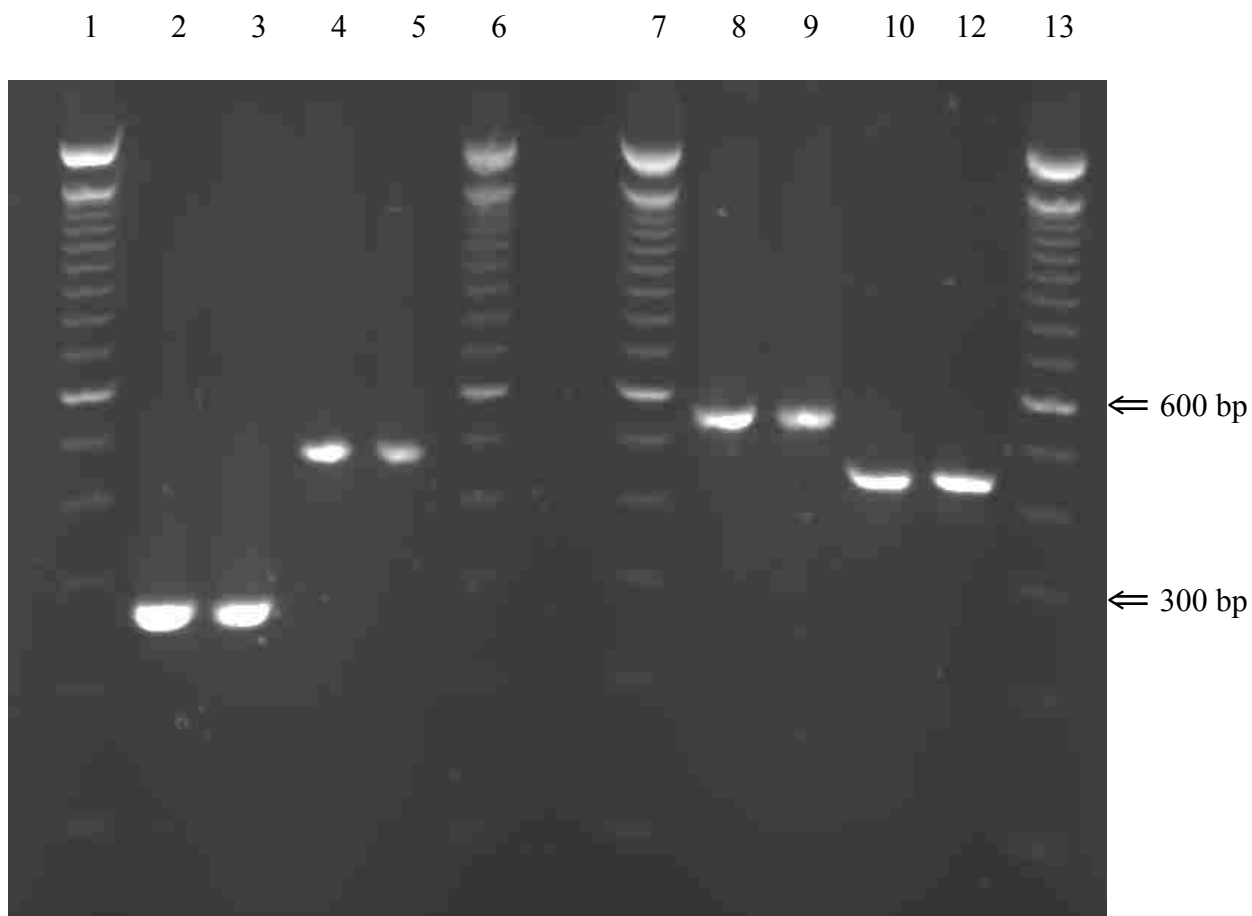
Figure 2.4. Genotype of FVB Wild-type and *mdr1a*$^{(-/-)}$*1b*$^{(-/-)}$ Mice. Lanes 1, 6, 7, and 13: DNA base pair (bp) standard; lanes 2 and 3: 50ng FVB wild-type *mdr1a*; lanes 4 and 5: 50ng *mdr1a*$^{(-/-)}$; lanes 8 and 9: 50ng FVB wild-type *mdr1b*; lanes 10 and 11: 50ng *mdr1b*$^{(-/-)}$. FVB wild-type *mdr1a* and *mdr1b* bands at 269 and 540 bp, respectively; *mdr1a*$^{(-/-)}$ and *mdr1b*$^{(-/-)}$ bands at 461 and 453 bp respectively.

2.5a
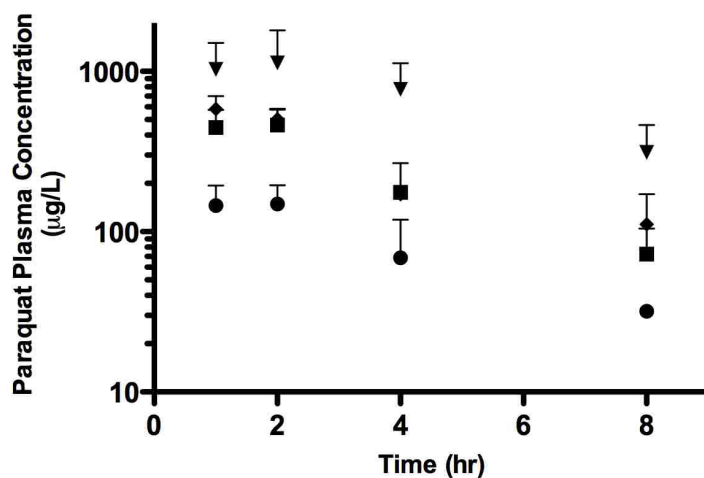
2.5b
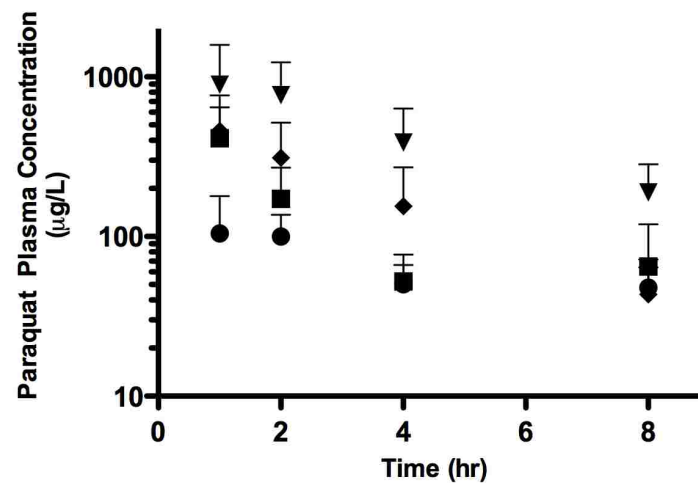
Figure 2.5. Paraquat Plasma Concentration-time Curves in FVB Wild-type and *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ Mice. Paraquat was orally administered at doses of 10 (circles), 25 (squares), 50 (diamond), or 100 mg/kg (triangle) to 2.5a) FVB wild-type and 2.5b) *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ mice. Plasma samples were collected at 1, 2, 4, and 8 hrs. Data are presented as mean paraquat plasma concentrations with error bars for standard deviation.

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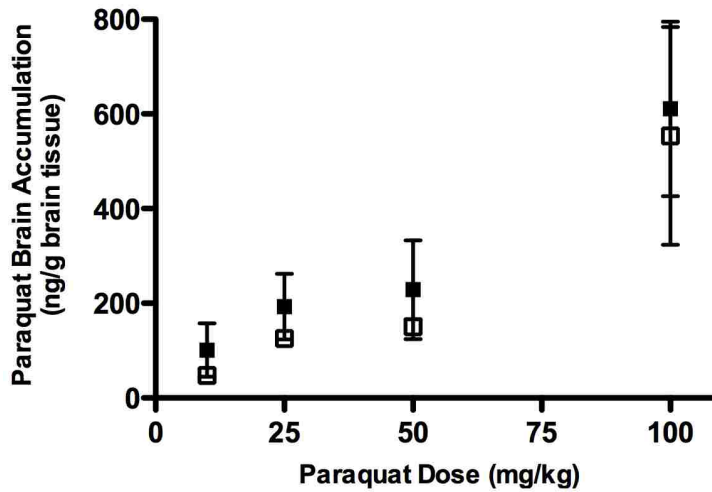
Figure 2.6. Paraquat Brain Accumulation in FVB Wild-type and *mdr1a*^(-/-)^/1b^(-/-)^
Mice. Brains were collected at 8 hrs after oral administration of paraquat at doses of 10, 25, 50, or 100 mg/kg to FVB wild-type (closed squares) or *mdr1a*^(-/-)^/1b^(-/-)^ mice (open squares). Data are presented as mean paraquat brain accumulation with error bars for standard deviation.

CHAPTER 3

P-GLYCOPROTEIN TRANSPORT OF

PARKINSON'S DISEASE-ASSOCIATED PESTICIDES

ABSTRACT

Pesticides exposure is associated with Parkinson's disease and pharmacogenomic studies have associated the gene that encodes P-glycoprotein (P-gp), *ABCB1*, with Parkinson's disease risk, however evidence is missing whether pesticides are P-gp substrates. We used three *in vitro* transport models to screen diazinon, dieldrin, endosulfan, maneb, MPP+ and rotenone as P-gp substrates and inhibitors. We also screened the pesticide ivermectin, which although not related to Parkinson's disease risk, has been reported to be a P-gp substrate. Only rotenone stimulated ATPase activity in P-gp-expressing membranes with V_{max} and K_m of 32.4 ± 1.64 nmol P_i/min/mg protein and 1.78 ± 0.55 μM, respectively, compared to the known P-gp substrate verapamil with V_{max} and K_m of 48.4 ± 2.58 nmol P_i/min/mg protein and 2.54 ± 0.68 μM, respectively. None of the other pesticides stimulated ATPase activity. We observed no differential cytotoxicity to any of the pesticides in LLC-vector and LLC-MDR1-WT cells in the absence or presence of P-gp inhibitors GF120918 or verapamil. Only ivermectin inhibited the P-gp transport of rhodamine-123 with an IC_{50} of 0.252 ± 0.048 μM; the other pesticides did not inhibit P-gp at concentrations up to 1000 μM. We have screened the primary pesticides that have been associated with Parkinson's disease as either P-gp substrates or inhibitors and found that only rotenone stimulated P-gp mediated ATPase activity. Our data suggest that diazinon, dieldrin, endosulfan, maneb, and MPP+ are not P-gp substrates or inhibitors. Further investigation is needed to understand the role of P-gp transport in rotenone disposition and whether transport at the blood-brain-barrier is altered in people with *ABCB1* genetic variants.

3.1. INTRODUCTION

P-glycoprotein (P-gp) is a drug transporter that effluxes a diverse array of xenobiotics in healthy tissues throughout the body including the intestine, liver, kidney, and the blood-brain-barrier (BBB) [as reviewed in (Cascorbi, 2011; Giacomini, 1997; Giacomini et al., 2010; Lin and Yamazaki, 2003a; Lin and Yamazaki, 2003b; Schinkel, 1997; Sharom, 2006; Sharom, 2011)]. P-gp is a member of the ATP-binding cassette (ABC) superfamily of transporters and is encoded by the multidrug resistance gene *ABCB1* (also known as *MDR1*). The role of P-gp in the disposition of drugs is well known. Little has been described, however, regarding the role of P-gp in toxicant disposition.

P-gp has also been associated with a number of diseases, including the neurodegenerative Parkinson's disease (Bartels et al., 2008a; Drozdzik et al., 2003; Dutheil et al., 2010; Furuno et al., 2002; Le Couteur et al., 2001; Lee et al., 2004; Lee and Bendayan, 2004; Tan et al., 2005; Tan et al., 2004; Vautier and Fernandez, 2009; Westerlund et al., 2009; Westerlund et al., 2008). Pharmacogenomic studies have described an association between *ABCB1* genetic variation and Parkinson's disease, specifically with three single nucleotide polymorphisms (SNPs): 1236C>T, 2677G>T/A, and 3435C>T. A mechanism for this association is not known, but one possibility is that *ABCB1* SNPs lead to decreased P-gp transport of neurotoxic xenobiotics at the blood-brain barrier and increased brain accumulation.

Environmental exposure to neurotoxicants has long been known as a risk factor for Parkinson's disease (Bonnet and Houeto, 1999; Gatto et al., 2009; Langston et al., 1984; Semchuk et al., 1992; Steece-Collier et al., 2002). One of the first discoveries of

environmental risk factor was 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

MPTP is converted to 1-methyl-4-phenyl-4-phenylpyridinium ion (MPP+) in the brain

and results in a parkinsonian state (Langston et al., 1983; Langston et al., 1984). MPP+

has been used as a pesticide under the name cyperquat. Several other pesticides have also

been associated with an increased risk to Parkinson's disease including paraquat,

diazinon, dieldrin, endosulfan, maneb, MPP+ and rotenone. Epidemiological evidence

and *in vitro* and animal toxicity models have demonstrated the association of these

pesticides with Parkinson's disease (Bradbury et al., 1986; Costello et al., 2009; Dhillon

et al., 2008; Firestone et al., 2005; Fleming et al., 1994; Gao et al., 2002; Gatto et al.,

2009; Hatcher et al., 2007; Jia and Misra, 2007a; Jia and Misra, 2007b; Kanthasamy et

al., 2008; Langston et al., 1984; Li et al., 2005a; Liou et al., 1997; Richardson et al.,

2006; Sharma et al., 2010; Slotkin and Seidler, 2011; Sonsalla et al., 2008; Tanner et al.,

2011; Thiffault et al., 2000; Uversky, 2004; Uversky et al., 2001; Wang et al., 2006;

Weisskopf et al., 2010).

P-gp plays a significant role in protecting the brain from toxic substances

circulating in the brain, but it id uncertain if this is the role for P-gp in Parkinson's

disease. While not associated with Parkinson's disease, the role of P-gp transport in the

disposition of the neurotoxic pesticide ivermectin has been shown (Edwards, 2003;

Mealey et al., 2003; Nelson et al., 2003; Rose et al., 1998; Roulet et al., 2003; Schinkel et

al., 1994). This discovery was made serendipitously in knockout *mdr1a*$^{(-/-)}$ mice that were

treated with ivermectin to combat a mite infestation. The majority of the *mdr1a*$^{(-/-)}$ mice

died of neurotoxicity and it was determined that these mice had a 88-fold increase in

ivermectin brain accumulation compared to wild-type mice (Edwards, 2003; Schinkel et

al., 1994). The case of ivermectin provides a rationale for P-gp in the transport of other pesticides. Although there is currently limited evidence for P-gp transport of the pesticides associated with Parkinson's disease.

Recently, we have determined that P-gp does not mediate *in vitro* or *in vivo* paraquat disposition (Chapter 2). Several studies have also reported that MPP+ is not a P-gp substrate (Martel and Azevedo, 2003; Martel et al., 2001). Maneb and rotenone have not been evaluated as P-gp substrates or inhibitors. There are conflicting reports on the P-gp transport of the other pesticides diazinon, dieldrin, and endosulfan. (Bain and LeBlanc, 1996; Bircsak et al., 2012; Lecoeur et al., 2006; Pivcevic and Zaja, 2006). One report found that diazinon was transported by P-gp and also induced P-gp expression (Lecoeur et al., 2006), while another found only minimal P-gp inhibition relative to the known inhibitor verapamil (Pivcevic and Zaja, 2006). Other studies have found that neither dieldrin nor endosulfan were P-gp substrates, and that endosulfan, but not dieldrin, was capable of inhibiting P-gp (Bain and LeBlanc, 1996; Bircsak et al., 2012). Another study, however, observed resistance to endosulfan in P-gp expressing cells and concluded it was a P-gp substrate (Bain and LeBlanc, 1996), although this study did not confirm these findings with a known P-gp inhibitor to ensure the effect was specific to P-gp.

It is clear that there are contradictory reports in the literature regarding P-gp transport of pesticides. Currently, there is wide agreement that a reliable strategy for the evaluation of substrates and inhibitors of drug transporters should integrate multiple transport models. One challenge in interpreting previous studies that evaluated pesticides as P-gp substrates or inhibitors is that these studies relied on one i*n vitro* transport model.

Relying on only one model can lead to false negative or false positive results that may vary from model to model. (Brouwer et al., 2013; Feng et al., 2008; Giacomini et al., 2010; Polli et al., 2001). Therefore, in order to overcome some of the challenges of previous studies, we have used a combination of *in vitro* models of P-gp transport. Our goals are to systematically screen the Parkinson's disease associated pesticides using a combination of models.

The goals of this study were to systematically evaluate P-gp-mediated transport of the major pesticides that have been linked to Parkinson's disease: diazinon, dieldrin, endosulfan, maneb, MPP+, and rotenone. We have screened each compound using three models: 1) a xenobiotic-stimulated ATPase assay in P-gp expressing membranes, 2) a cytotoxic sensitivity assay in LLC-PK1 recombinant cell lines, expressing either *ABCB1/MDR1* or an empty vector, and 3) inhibition of intracellular of rhodamine-123 (R123) accumulation. This study is the first comprehensive investigation of P-gp-mediated transport of the major pesticides associated with Parkinson's disease.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

Diazinon, dieldrin, endosulfan, maneb, MPP+, rotenone, ivermectin, doxorubicin, colchicine, verapamil, R123, and cyclosporine were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). GF120918, [N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] was kindly provided by GlaxoSmithKline (Research Triangle Park, NC).

3.2.2. ATPase Activity

Stimulation of ATPase activity was measured using SB MDR1/P-gp Sf9 ATPase Membranes (SOLVO Biotechnology, Budaors, Hungary) as previously described (Chapter 2). ATPase activity in the presence of xenobiotics was estimated by measuring the release of inorganic phosphate (P_i) in a colorimetric reaction; and xenobiotic-stimulated ATPase activity was determined as the difference in P_i liberation measured in the absence or presence of 1.2 mM sodium orthovanadate (a non-specific ATPase inhibitor). P-gp membranes were incubated with either diazinon, dieldrin, endosulfan, maneb, MPP+, rotenone, ivermectin, or the known P-gp substrate verapamil over a concentration range of 0.05-100 μM. Michaelis-Menten parameters (V_{max} and K_m) were estimated using a nonlinear regression Michaelis-Menten least squares model fit on Prism 5.0 software (GraphPad, San Diego, CA).

3.2.3. Cell Culture

LLC-PK1 vector cells (LLC-vector) and recombinant *MDR1* cells (LLC-MDR1-WT), generously provided by Michael M. Gottesman in the Laboratory of Cell Biology at the National Cancer Institute (Bethesda, MD), were cultured in complete Media 199 (Mediatech, Manassas, VA) supplemented with 3% (v/v) fetal bovine serum (FBS) (Mediatech), 1% (v/v) L-glutamine (Mediatech), 1% (v/v) penicillin/streptomycin (Mediatech), and 1% (v/v) geneticin (Life Technologies, Carlsbad, CA); and grown at 37°C in the presence of 5% CO_2.

3.2.4. Cytotoxic Sensitivity

Sensitivity to cytotoxic agents was evaluated in LLC-vector and LLC-MDR1-WT cells as previously described (Chapter 2). Cells were treated with pesticides over a wide concentration range: diazinon (0.3-1000 μM), dieldrin (2.0-1000 μM), endosulfan (0.4-100 μM), maneb (0.7-100 μM), MPP+ (0.5-3000 μM), rotenone (0.7-5000 nM), and ivermectin (0.4-20 μM). P-gp substrates doxorubicin (0.05 nM - 250 μM), and colchicine (0.01-50,000 μM) were used as positive controls. Inhibition was performed at 0.5 μM GF120918 and 10 μM verapamil. Compounds were evaluated in triplicate. Viability was estimated as the effective concentration necessary for 50% cell death (EC_{50}) based on a nonlinear regression log (agonist) vs. response variable slope least squares model fit using Prism 5.0 software.

3.2.5. Intracellular Accumulation

Inhibition of intracellular accumulation of the P-gp substrate, R123, was evaluated in LLC-vector and LLC-MDR1-WT cells as previously described (Woodahl *et al.*, 2004) (Chapter 2). Cells were incubated in 5 μM R123 with and without inhibitors in serum-free Media 199 at 37°C and then allowed to efflux in the presence of inhibitor in complete Media 199. Flow cytometry was used to evaluate intracellular R123 over a range of pesticide concentrations: diazinon (7.0-500 μM), dieldrin (3.0-200 μM), endosulfan (3.0-200 μM), maneb (0.1-500 μM), MPP+ (1.0-1000 μM), rotenone (0.1-500 μM), and ivermectin (0.05-50 μM). Known P-gp inhibitors were used as positive controls: GF120918 (0.78-1000 nM), cyclosporine (0.1-100 μM), and verapamil (0.1-500 μM). Samples were run in triplicate. Immediately prior to flow cytometry, 4',6-diamidino-2-phenylindole (DAPI) was added to the cells as a measure of cell viability.

Inhibition was estimated as the inhibitor concentration necessary for 50% inhibition (IC_{50}) based on a nonlinear regression log (inhibitor) vs. normalized response variable slope least squares model fit on Prism 5.0 software.

3.2.6. Statistical Analysis

Student's one- or two-tailed t test was used to determine differences between two sets of data. An extra sum of squares F-test was utilized to compare individual parameters for the cytotoxic sensitiviy assay. The null hypothesis was that the EC_{50} estimates are the same within a cell type between xenobiotic treatment group alone and in the presence of a P-gp inhibitor, and the null hypothesis was only rejected if the inhibitor decreased cytotoxic resistance. For all analyses, p-values of < 0.05 were considered statistically significant.

3.3. RESULTS

Diazinon, dieldrin, endosulfan, maneb, MPP+, and ivermectin, failed to stimulate ATPase activity above the level of the orthovanadate-sensitive control (14.5 ± 1.0 nmol P_i/min/mg protein). This was similar to our observation that paraquat does not stimulated ATPase activity in P-gp membranes (Chapter 2). Rotenone, however, stimulated ATPase activity with V_{max} and K_m parameters of 32.4 ± 1.64 nmol P_i/min/mg protein and 1.78 ± 0.55 μM, respectively (Figure 3.1., page 78). This compared to the P-gp substrate verapamil with V_{max} and K_m parameters of 48.4 ± 2.58 nmol P_i/min/mg protein and 2.54 ± 0.68, respectively.

Dose–response curves of cytotoxicity to diazinon, dieldrin, endosulfan, maneb, MPP+, rotenone, and ivermectin for LLC-vector and LLC-MDR1-WT cells are presented in Figures 3.2.-3.10., pages 79 -87. EC_{50} values are presented in Table 3.1., page 77. When compared to LLC-vector cells, LLC-MDR1-WT cells exhibit significantly increased resistance to the positive controls doxorubicin and colchicine as expected (Figure 3.9. and 3.10., pages 86-87). Additionally, the P-gp inhibitors GF120918 and verapamil reversed cellular resistance in LLC-MDR1-WT cells. None of the tested pesticides showed differential cytotoxicity mediated by P-gp in this model that was sensitive to both P-gp inhibitors GF120918 and verapamil (Figures 3.2.-3.8., pages 79-85). While there was modest increase in resistance to dieldrin, maneb, MPP+, and rotenone in LLC-MDR1-WT cells compared to LLC-vector cells, the P-gp inhibitor GF120918 did not reverse the resistance in LLC-MDR1-WT. We have also found a similar observation in cellular sensitivity to paraquat (Chapter 2). Verapamil reduced the cytotoxic sensitivity of LLC-MDR1-WT cells to dieldrin, MPP+, and rotenone, however, GF120918 was unable to reproduce this effect. There were no cellular differences in the resistance to endosulfan and ivermectin in LLC-MDR1-WT cells. Together, these data indicate that in this model of cytotoxic sensitivity, P-gp possibly mediates cellular resistance to dieldrin, MPP+, and rotenone.

The pesticides were also evaluated as inhibitors of P-gp by their ability to inhibit R123 efflux in LLC-vector and LLC-MDR1-WT cells compared to known P-gp inhibitors GF120918, verapamil, and cyclosporine (Figure 3.11., page 88). There was no effect of the pesticides or known P-gp inhibitors had any effect on intracellular accumulation of R123 in LLC-vector cells (data not shown). Diazinon, dieldrin,

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endosulfan, maneb, MPP+, and rotenone did not inhibit R123 accumulation in P-gp-expressing cells even at the highest concentrations. We also found previously that paraquat does not inhibit R123 efflux (Chapter 2). Ivermectin inhibited R123 with an IC$_{50}$ value of 0.252 ± 0.048 μM compared to known inhibitors GF120918, verapamil, and cyclosporine with IC$_{50}$ values of 22.9 ± 1.92 nM, 1.98 ± 0.12 μM, and 1.39 ± 0.07 μM, respectively.

3.4. DISCUSSION

We have screened for P-gp transport of the pesticides diazinon, dieldrin, endosulfan, maneb, MPP+, and rotenone using a combination of *in vitro* models to investigate the relationship between *ABCB1* genetic variation and Parkinson's disease risk. Of these pesticides, we determined that only rotenone is a substrate of P-gp. We determined that diazinon, dieldrin, endosulfan, maneb, and MPP+ are not P-gp substrates or inhibitors. This is consistent with our previous work with the Parkinson's disease-associated pesticide paraquat (Chapter 2).

ABCB1 pharmacogenomics have been associated with altered susceptibility to Parkinson's disease (Bartels et al., 2008a; Drozdzik et al., 2003; Dutheil et al., 2010; Furuno et al., 2002; Le Couteur et al., 2001; Lee et al., 2004; Lee and Bendayan, 2004; Tan et al., 2005; Tan et al., 2004; Vautier and Fernandez, 2009; Westerlund et al., 2009; Westerlund et al., 2008). While these studies provide evidence for a role of P-gp in Parkinson's disease, the mechanism is unclear. One hypothesis is that decreased P-gp activity at the BBB results in increased brain accumulation of neurotoxicants and an increased incidence of Parkinson's disease. Neurotoxic pesticides are known risk factors

for the development of Parkinson's disease (Bradbury et al., 1986; Costello et al., 2009; Dhillon et al., 2008; Firestone et al., 2005; Fleming et al., 1994; Gao et al., 2002; Gatto et al., 2009; Hatcher et al., 2007; Jia and Misra, 2007a; Jia and Misra, 2007b; Kanthasamy et al., 2008; Langston et al., 1984; Li et al., 2005a; Liou et al., 1997; Richardson et al., 2006; Sharma et al., 2010; Slotkin and Seidler, 2011; Sonsalla et al., 2008; Tanner et al., 2011; Thiffault et al., 2000; Uversky, 2004; Uversky et al., 2001; Wang et al., 2006; Weisskopf et al., 2010). There has not, however, been a systematic evaluation of P-gp transport of the pesticides associated with Parkinson's disease until now.

An important consideration when evaluating compounds as substrates or inhibitors of membrane transporters is that multiple models of *in vitro* transport are required and each model has its own advantages and limitations (Brouwer et al., 2013; Feng et al., 2008; Giacomini et al., 2010; Polli et al., 2001). Unfortunately, the information that exists regarding P-gp transport of pesticides associated with Parkinson's disease is incomplete with most studies utilizing one or at most two *in vitro* models. The pesticides diazinon, dieldrin, endosulfan, maneb, MPP+, and rotenone must be evaluated as substrates or inhibitors of P-gp prior to moving forward with understanding the mechanism responsible for the relationship between genetic variation in *ABCB1* and Parkinson's disease risk. An important aspect of our work was to utilize three different *in vitro* P-gp transport assays: xenobiotic-stimulated ATPase activity, cytotoxic sensitivity, and inhibition of R123 intracellular accumulation.

The ATPase assay identified only rotenone as capable of stimulating ATPase activity in P-gp-expressing membranes. Diazinon, dieldrin, endosulfan, ivermectin, maneb, and MPP+ did not stimulate ATPase activity above the level of the

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orthovanadate-sensitive control, which is similar to our previous observations with paraquat (Chapter 2). Therefore, it is likely that rotenone is a P-gp substrate.

LLC-PK1 cells have many features that make them the gold standard cell model of transport, mainly that they form polarized monolayers, have low endogenous transporter expression, and express tight junction proteins. However, one of the drawbacks of using LLC-PK1 recombinant cell lines is that there may be other changes induced in the cells in addition to the expression of P-gp. Therefore, it is important to not only have a vector control cell line but also use a P-gp inhibitor to confirm the results. For example, a dramatic increase in resistance was observed in LLC-MDR1-WT cells compared to LLC-vector cells for known P-gp substrates, doxorubicin and colchicine, and this resistance was reversed in the presence of the P-gp inhibitors GF120918 and verapamil. When screening the pesticides diazinon, dieldrin, maneb, MPP+, and rotenone, there was a modest increase in resistance in LLC-MDR1-WT cells, however the P-gp inhibitor GF120918 did not have any effect on the resistance. The P-gp inhibitor verapamil reduced the cytotoxic sensitivity of LLC-MDR1-WT cells to dieldrin, MPP+, and rotenone, which suggests that these pesticides maybe P-gp substrates, however, GF120918 was unable to reproduce this effect. However, combining our previous observations with rotenone in the ATPase assay with the reduction in cytotoxic sensitivity in LLC-MDR1-WT cells when co-treated with verapamil makes a strong case for rotenone as a likely P-gp substrate. The results from our cytotoxic sensitivity assay clearly demonstrate the importance of using of P-gp inhibitors to interpret results in P-gp transfected cell lines.

In addition to screening the pesticides as potential substrates of P-gp, we evaluated them as potential inhibitors of P-gp. Ivermectin was able to completely inhibit R123 efflux, however, none of the pesticides associated with Parkinson's disease were able to inhibit R123 efflux in LLC-MDR1 cells, even at high concentrations.

Our study is the first to evaluate the P-gp transport of maneb and rotenone, both of which have been highly associated with Parkinson's disease risk. We observed no evidence for maneb as a P-gp substrate or inhibitor in any of the *in vitro* assays. Rotenone, however, stimulated ATPase activity with a K_m lower than that for the known P-gp substrate verapamil. This suggests that rotenone may bind with higher affinity to P-gp than verapamil. We also observed differential cytotoxicity to rotenone in LLC-MDR1-WT when co-treated with verapamil, which suggests that rotenone was a substrate in both the ATPase assay and the the cytotoxic sensitivity assay. Rotenone highlights the value in using multiple models to assess P-gp transport, and suggests a role for P-gp in rotenone transport. Further work is needed to determine if P-gp limits brain distribution of rotenone and whether alteration of P-gp transport at the BBB due to *ABCB1* genetic variation would alter rotenone brain accumulation.

Although dieldrin has not previously been evaluated as a P-gp substrate, our work is consistent with previous reports that dieldrin does not inhibit P-gp (Bain and LeBlanc, 1996). Our data conflicts, however, with previous reports for endosulfan and diazinon but such reports have been inconsistent and without appropriate controls (Bain and LeBlanc, 1996; Bircsak et al., 2012; Pivcevic and Zaja, 2006). Endosulfan was previously classified as a substrate in a study that found that B16/hMDR1 cells were 2.5-fold more resistance to endosulfan compared with B16/F10 cells (Bain and LeBlanc, 1996). This

study, however, did not use a P-gp inhibitor to confirm their findings, which we have discussed previously is critical when interpreting results from cytotoxic assays. In contrast we saw no change in resistance to endosulfan in P-gp expressing cells. Additionally, one report suggested incomplete inhibition of P-gp by endosulfan (Pivcevic and Zaja, 2006) and another reporting an IC_{50} of 33.6 µM (Bircsak et al., 2012). We did not observe inhibition of P-gp by endosulfan, however, even at concentrations up to 200 µM. Diazinon has been reported previously to be a substrate and inducer of P-gp in transepithelial studies in Caco-2 cells (Lecoeur et al., 2006), although the permeability ratios they estimated were low (<3) which suggests that while there is directional transport of diazinion in the basolateral-to-apical direction, it is much less than what is seen for canonical P-gp substrates. Diazinon was also shown to incompletely inhibit of P-gp using recombinant cells (Pivcevic and Zaja, 2006), although we found no inhibition of P-gp up to 500 µM. Since it is critical to utilize multiple models of transport to evaluate P-gp, our approach of screening pesticides with three *in vitro* models provides confidence in our observations regarding the P-gp transport of pesticides associated with Parkinson's disease.

Although not linked to Parkinson's disease, we also screened the pesticide ivermectin since its brain disposition as a result of P-gp transport has been well documented (Edwards, 2003; Mealey et al., 2003; Nelson et al., 2003; Rose et al., 1998; Roulet et al., 2003; Schinkel et al., 1994). We found that ivermectin is a good inhibitor of P-gp, but did not stimulate ATPase activity or contribute to differential cytotoxicity in P-gp expressing cells indicating that it is not a P-gp substrate. The initial studies of ivermectin as a neurotoxic P-gp substrate are from studies in *mdr1a* knock-out mice that

were much more sensitive (100-fold) and had increased brain uptake (87-fold) of ivermectin (Schinkel et al., 1994). In addition, collie dogs with a deletion of the *mdr1* gene are also extremely sensitive to the neurotoxic effects of ivermectin (Edwards, 2003; Mealey et al., 2001; Mealey et al., 2003; Nelson et al., 2003; Roulet et al., 2003). To our knowledge, there has been no direct *in vitro* investigation of ivermectin as a P-gp substrate, however, our finding was surprising given the previous *in vivo* observations in *mdr* knockout or deficient animals. Our observation reinforces the importance of using multiple models when evaluating compounds as substrates or inhibitors P-gp.

3.5. CONCLUSIONS

In summary, using three different *in vitro* transport models we have screened the primary pesticides that have been linked to Parkinson's disease as P-gp substrates and inhibitors. We have demonstrated that diazinon, endosulfan, and maneb are not substrates or inhibitors of P-gp. Interestingly, we have shown for the first time that rotenone maybe a P-gp substrate by its ability to stimulate P-gp mediated ATPase activity as well as display reduced cytotoxic sensitivity in LLC-MDR1-WT cells when co-treated with verapamil. Although dieldrin and MPP+ also displayed reduced cytotoxic sensitivity in LLC-MDR1-WT cells when co-treated with verapamil, they did not activate ATPase activity or inhibit R123 efflux, suggesting that more clarification is needed to verify the results of the cytotoxic sensitivity assay. *ABCB1* pharmacogenomic studies have shown evidence for a role of P-gp in the development of Parkinson's disease. It is clear from our data, however, that with the possible exceptions of dieldrin, MPP+, and rotenone, differential P-gp transport of many of the pesticides that have been associated with

Parkinson's disease is likely not the cause. P-gp transport of dieldrin, MPP+, and rotenone at the BBB, and alterations to transport due to *ABCB1* genetic variation, requires further investigation.

Table 3.1. Pesticide Cytotoxicity in LLC-vector and LLC-MDR1-WT Cells

LLC-PK1 Cells	EC$_{50}$ Values (95% Confidence Interval)	
	LLC-vector	LLC-MDR1-WT
Diazinon (μM)	1790 (n.d.)	384 (273 – 540)
Diazinon (μM) + GF120918	132 (67 – 261)	414 (161 – 1070)
Diazinon (μM) + Verapamil	494 (73 – 3330)	380 (157 – 922)
Dieldrin (μM)	51.4 (40.7 – 65.0)	75.4 (62.1 – 91.7)
Dieldrin (μM) + GF120918	38.2 (27.9 – 52.2)	70.7 (56.8 – 87.9)
Dieldrin (μM) + Verapamil	33.2 (27.3 – 40.2)[a]	40.0 (30.7 – 52.0)[c]
Endosulfan (μM)	14.0 (10.0 – 19.5)	19.4 (12.4 – 30.4)
Endosulfan (μM) + GF120918	17.4 (11.5 – 26.3)	40.7 (16.7 – 99.0)
Endosulfan (μM) + Verapamil	8.80 (5.33 – 14.53)	16.7 (10.4 – 27.0)
Maneb (μM)	49.1 (n.d.)	39.36 (n.d.)
Maneb (μM) + GF120918	24.6 (13.8 – 43.9)	36.4 (31.5 – 42.2)
Maneb (μM) + Verapamil	414.1 (n.d.)	37.3 (33.2 – 41.8)
MPP+ (μM)	27.1 (16.7 – 44.0)	418 (169 – 1040)
MPP+ (μM) + GF120918	84.9 (35.6 – 202.5)	566 (287 – 1120)
MPP+ (μM) + Verapamil	31.9 (18.1 – 56.4)	69.0 (39.3 – 121.1)[b]
Rotenone (nM)	12.5 (7.6 – 20.3)	27.7 (21.3 – 36.1)
Rotenone (nM) + GF120918	17.4 (11.4 -26.6)	35.0 (22.2 – 55.2)
Rotenone (nM) + Verapamil	5.89 (3.83 – 9.05)	7.25 (4.29 – 12.23)[b]
Ivermectin (μM)	4.86 (4.51 – 5.23)	4.04 (3.75 – 4.36)
Ivermectin (μM) + GF120918	5.76 (5.23 – 6.23)	4.59 (3.94 – 5.35)
Ivermectin (μM) + Verapamil	3.59 (2.66 – 4.86)[a]	4.25 (4.02 – 4.50)
Doxorubicin (nM)	34.2 (13.7 – 85.6)	1978 (1015-3850)
Doxorubicin (nM) + GF120918	24.8 (12.0 – 51.3)	8.06 (1.32 – 49.2)[c]
Doxorubicin (nM) + Verapamil	43.4 (12.0 – 155.9)	268 (90.7 – 98.6)[c]
Colchicine (μM)	13.3 (n.d.)	645 (467 – 892)
Colchicine (μM) + GF120918	12.6 (10.8 – 14.5)	17.5 (12.0 – 25.4)[c]
Colchicine (μM) + Verapamil	12.8 (n.d.)	179 (117 – 273)[c]

Significant differences within a cell type between xenobiotic treatment group alone and in the presence of a P-gp inhibitor; $p < 0.05$[a], $p < 0.005$[b], $p < 0.0005$[c].

[*] n.d.: not determined. Nonlinear regression was unable to estimate confidence intervals.
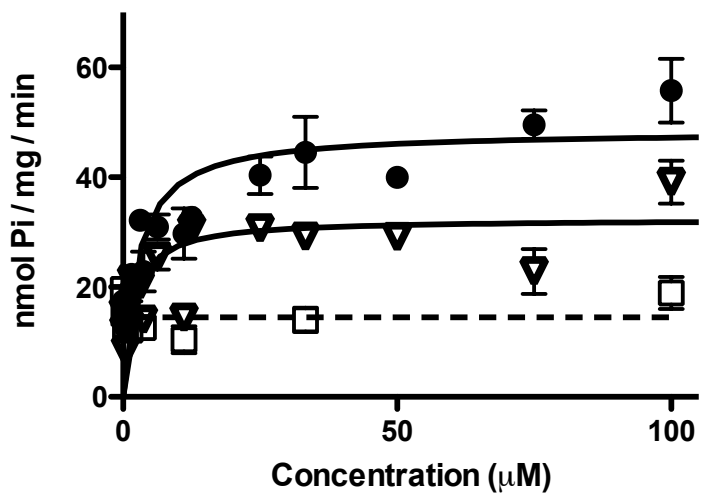
Figure 3.1. Rotenone Stimulated ATPase Activity in SB MDR1/P-gp Sf9 Membranes. P-gp membranes were incubated with either rotenone (open triangle) or the known P-gp substrate verapamil (closed circle) over a concentration range of 0.05 - 100 μM. ATPase activity was determined as the difference in P_i liberation measured in the absence or presence of 1.2 mM sodium orthovanadate (verapamil + sodium orthovanadate (open square).

3.2.a LLC-vector
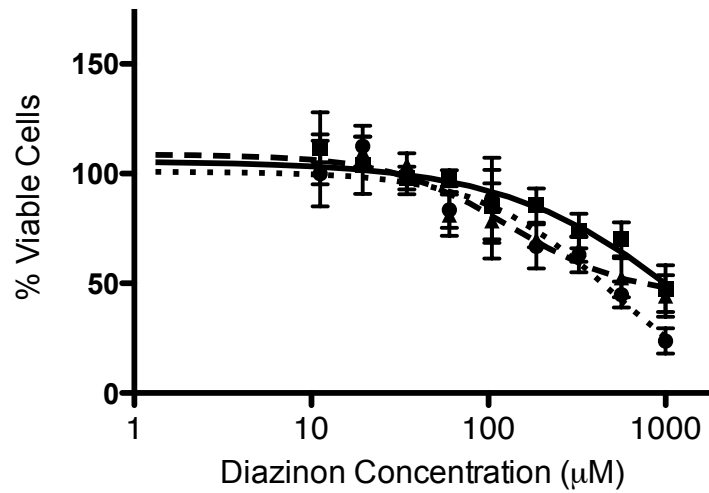
3.2.b LLC-MDR1-WT
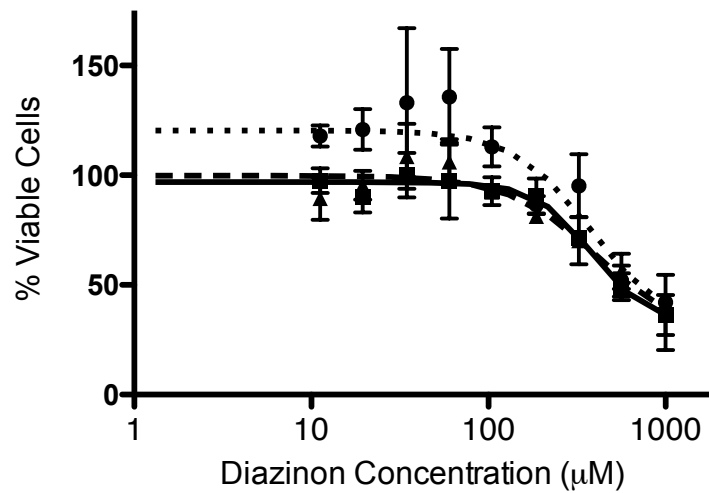
Figure 3.2. Cytotoxic Sensitivity to Diazinon in LLC-vector and LLC-MDR1-WT.
Cells were treated with diazinon (a-b, concentration range of 0.3-1000 μM), (square,
solid line). Cell viability was evaluated using the CellTiter-Glo® Cell Viability Assay.
Inhibition was performed at 0.5 μM GF120918 (triangle, dashed line) and 10 μM
verapamil (circle, dotted line).

3.3.a LLC-vector

3.3.b LLC-MDR1-WT

Figure 3.3. Cytotoxic Sensitivity to Dieldrin in LLC-vector and LLC-MDR1-WT.

Cells were treated with dieldrin (a-b, concentration range of 2.0-1000 µM), (square, solid

line). Cell viability was evaluated using the CellTiter-Glo® Cell Viability Assay.

Inhibition was performed at 0.5 µM GF120918 (triangle, dashed line) and 10 µM

verapamil (circle, dotted line).

3.4.a LLC-vector

3.4.b LLC-MDR1-WT

Figure 3.4. Cytotoxic Sensitivity to Endosulfan in LLC-vector and LLC-MDR1-WT.

Cells were treated with endosulfan (a-b, concentration range of 0.4-100 μM), (square,

solid line). Cell viability was evaluated using the CellTiter-Glo® Cell Viability Assay.

Inhibition was performed at 0.5 μM GF120918 (triangle, dashed line) and 10 μM

verapamil (circle, dotted line).

3.5.a LLC-vector

3.5.b LLC-MDR1-WT

Figure 3.5. Cytotoxic Sensitivity to Maneb in LLC-vector and LLC-MDR1-WT.
Cells were treated with maneb (a-b, concentration range of 0.7-100 µM), (square, solid line). Cell viability was evaluated using the CellTiter-Glo® Cell Viability Assay. Inhibition was performed at 0.5 µM GF120918 (triangle, dashed line) and 10 µM verapamil (circle, dotted line).

3.6.a LLC-vector
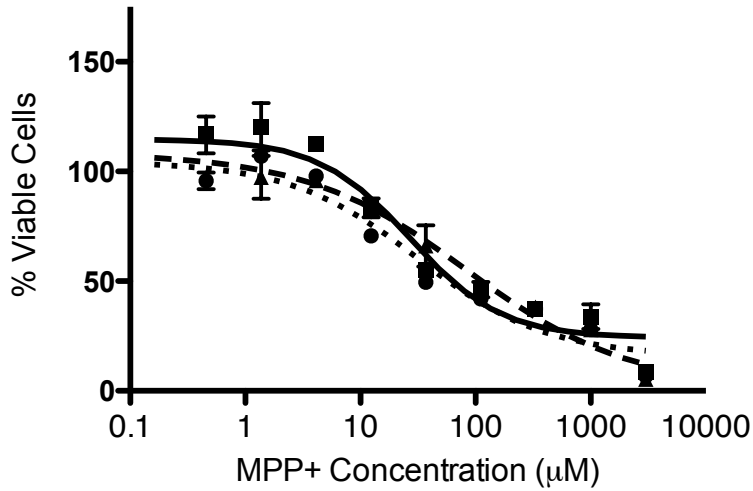
3.6.b LLC-MDR1-WT
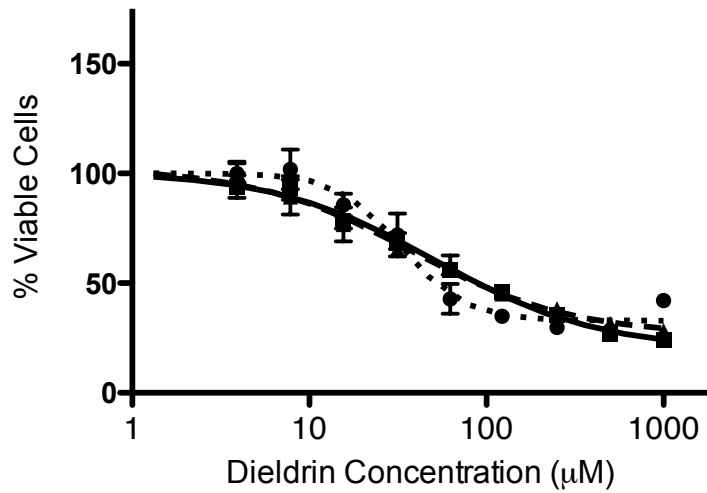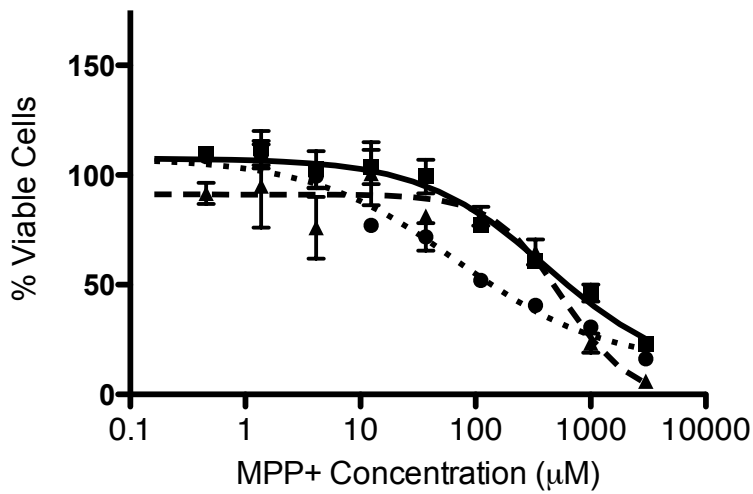

Figure 3.6. Cytotoxic Sensitivity to MPP+ in LLC-vector and LLC-MDR1-WT.

Cells were treated with MPP+ (a-b, concentration range of 0.5-3000 µM), (square, solid

line). Cell viability was evaluated using the CellTiter-Glo® Cell Viability Assay.

Inhibition was performed at 0.5 µM GF120918 (triangle, dashed line) and 10 µM

verapamil (circle, dotted line).

3.7.a LLC-vector
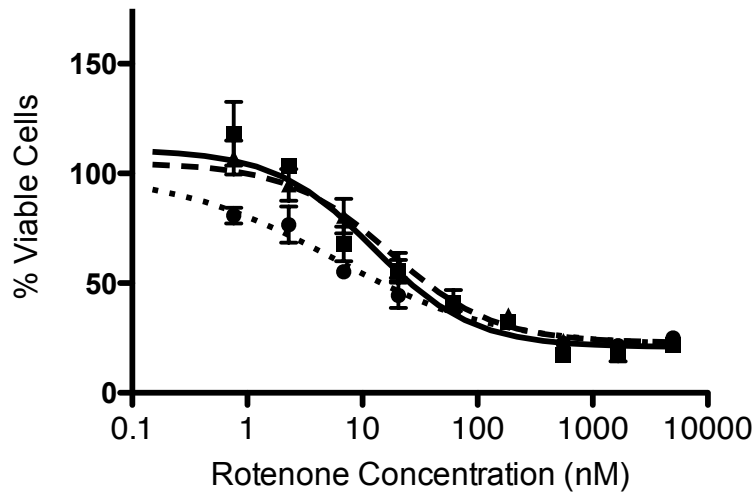
3.7.b LLC-MDR1-WT
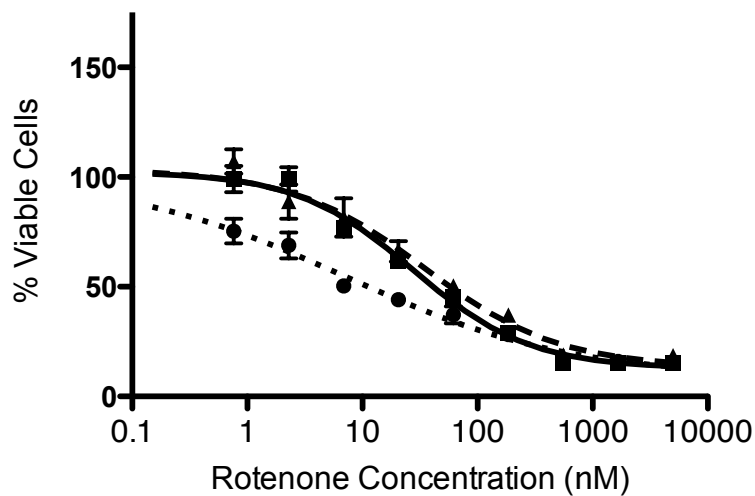
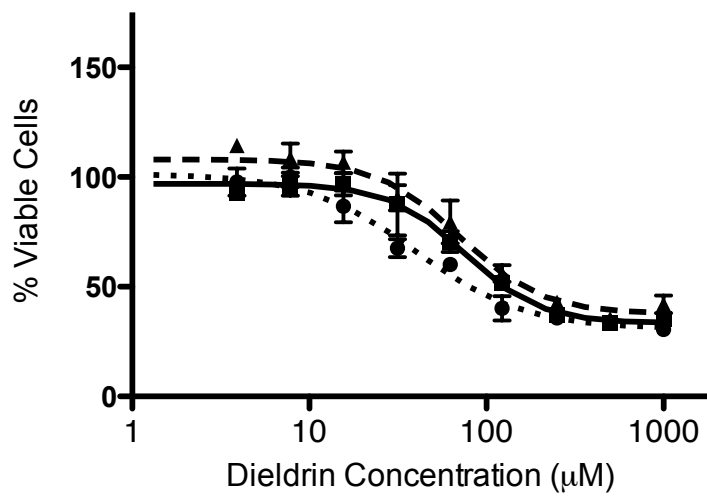**Figure 3.7. Cytotoxic Sensitivity to Rotenone in LLC-vector and LLC-MDR1-WT**.

Cells were treated with rotenone (a-b, concentration range of 0.7-5000 nM), (square,

solid line). Cell viability was evaluated using the CellTiter-Glo® Cell Viability Assay.

Inhibition was performed at 0.5 μM GF120918 (triangle, dashed line) and 10 μM

verapamil (circle, dotted line).
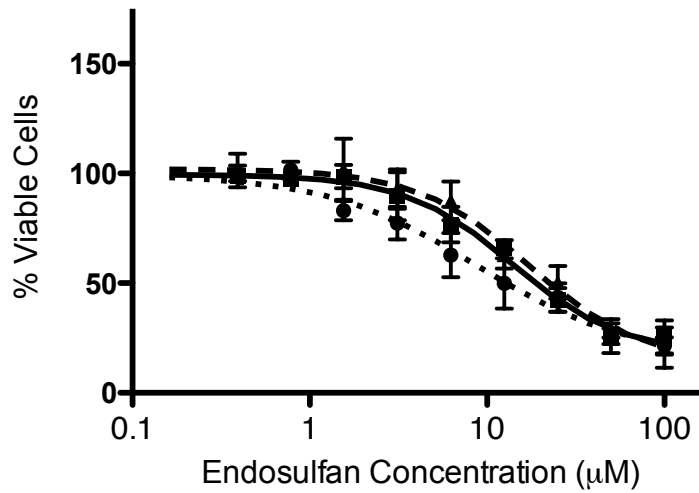
3.8.a LLC-vector
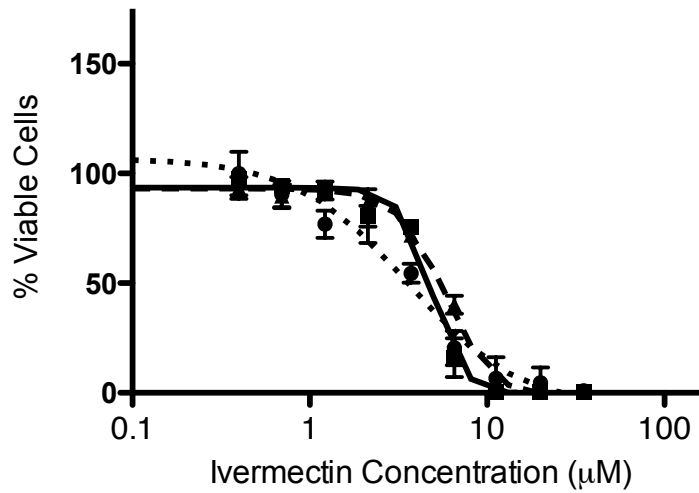
3.8.b LLC-MDR1-WT
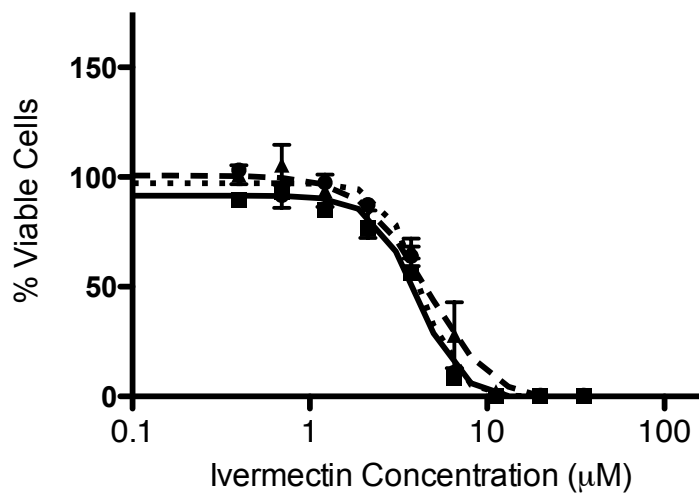
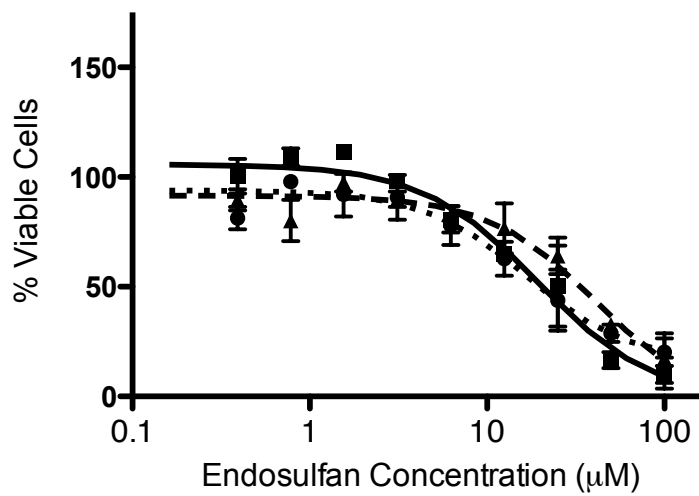**Figure 3.8. Cytotoxic Sensitivity to Ivermectin in LLC-vector and LLC-MDR1-WT**.

Cells were treated with ivermectin (a-b, concentration range of 0.4-20 µM), (square, solid

line). Cell viability was evaluated using the CellTiter-Glo[®] Cell Viability Assay.

Inhibition was performed at 0.5 µM GF120918 (triangle, dashed line) and 10 µM
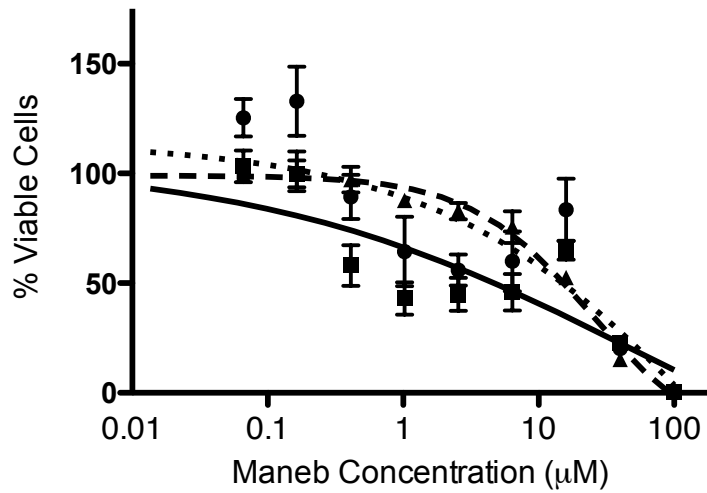
verapamil (circle, dotted line).
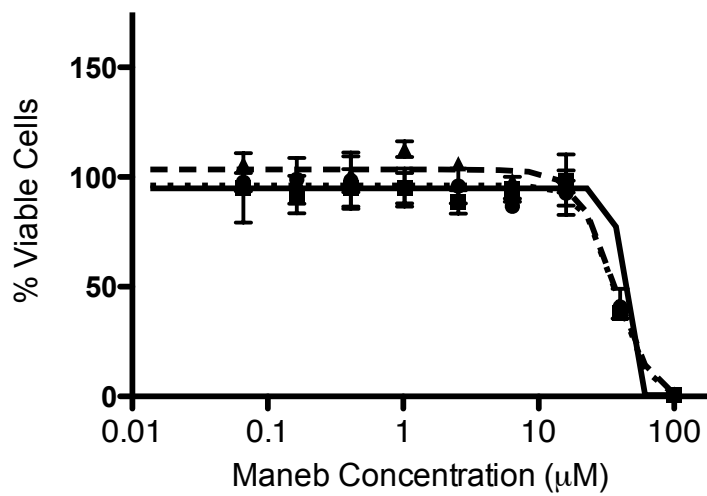
3.9.a LLC-vector
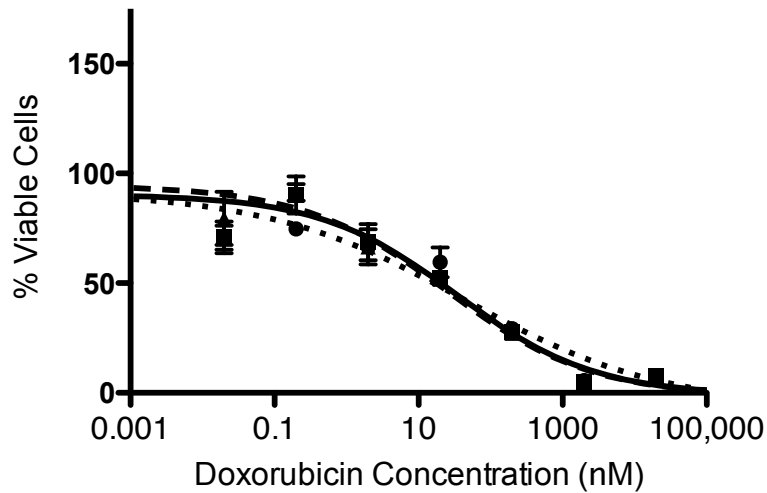
3.9.b LLC-MDR1-WT
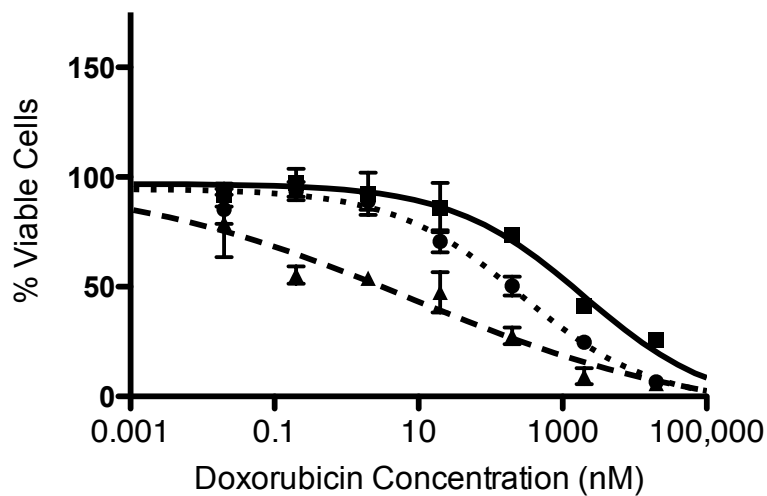
Figure 3.9. Cytotoxic Sensitivity to Doxorubicin in LLC-vector and LLC-MDR1-WT. Cells were treated with doxorubicin (a-b, concentration range of 0.05 nM - 250 µM), (square, solid line). Cell viability was evaluated using the CellTiter-Glo® Cell Viability Assay. Inhibition was performed at 0.5 µM GF120918 (triangle, dashed line) and 10 µM verapamil (circle, dotted line). Doxorubicin has been reported previously (Chapter 2).

3.10.a LLC-vector
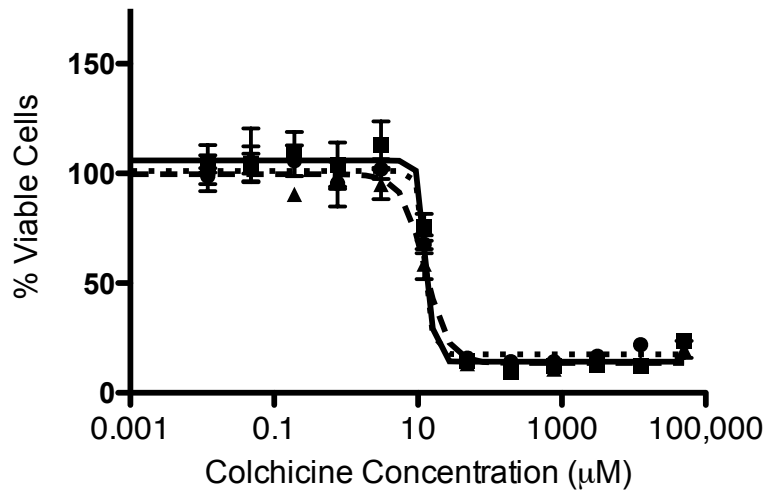
3.10.b LLC-MDR1-WT
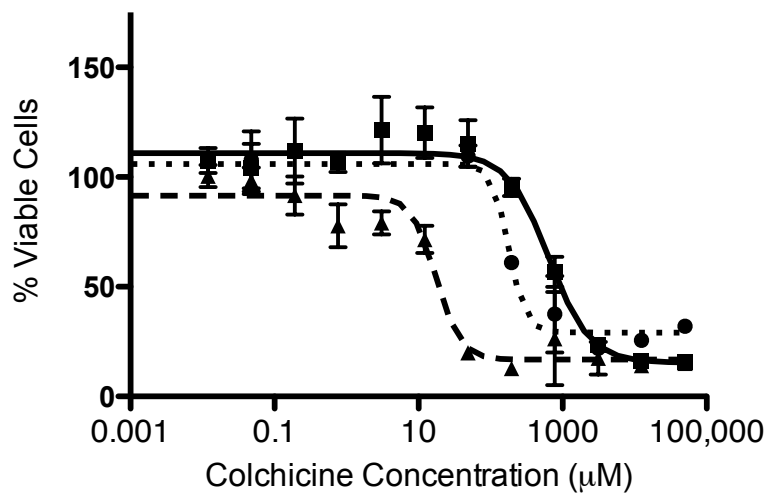
Figure 3.10. Cytotoxic Sensitivity to Colchicine in LLC-vector and LLC-MDR1-WT.

Cells were treated with colchicine (a-b, concentration range of 0.01-50,000 μM), (square,

solid line). Cell viability was evaluated using the CellTiter-Glo® Cell Viability Assay.

Inhibition was performed at 0.5 μM GF120918 (triangle, dashed line) and 10 μM

verapamil (circle, dotted line). Colchicine has been reported previously (Chapter 2).
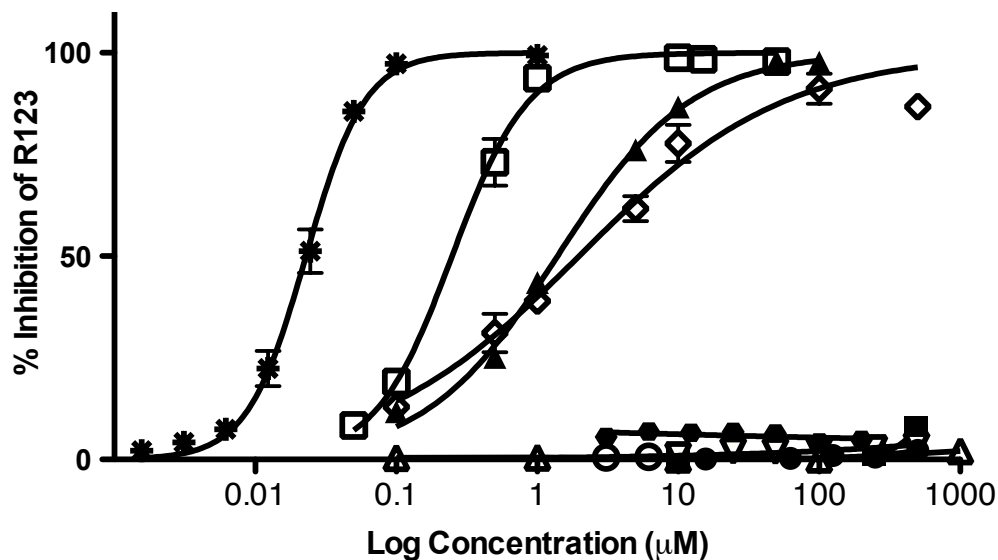
Figure 3.11. Inhibition of P-gp Transport of Rhodamine-123 by Pesticides. Data are presented as a percent of rhodamine-123 inhibition and values are mean ± SEM. Tested compounds are diazinon (closed circle), dieldrin (closed hexagon), endosulfan (open circle), maneb (closed square), MPP+ (closed diamond), rotenone (open triangle), paraquat (open triangle), ivermectin (open square), GF120918 (closed star), verapamil (open diamond), and cyclosporine (closed triangle). Paraquat, GF120918, verapamil, and cyclosporine data has been reported previously (Chapter 2).

CHAPTER 4

PARAQUAT EXPOSURE DOES NOT ALTER DOPAMINE

CONCENTRATIONS OR SPONTANEOUS MOTOR FUNCTION

IN C57BL/6J OR FVB MICE

ABSTRACT

Paraquat is an herbicide that has been studied as a risk factor for Parkinson's disease. Although some evidence suggests that paraquat exposure in mice results in death to the dopaminergic neurons in the substantia nigra and impaired motor function, there is also a growing body of evidence suggesting no relationship between paraquat and nigral damage. In an attempt to elucidate the discrepancies in these reports, the effects of chronic paraquat exposure on spontaneous motor activity, oxidative stress in the striatum, and concentrations of dopamine (DA) and its primary metabolites in the striatum, were evaluated following administration of the most common sub-lethal dosing regimen of 10 mg/kg paraquat twice a week for 3 or 4 weeks in C57BL/6J and FVB male mice. Spontaneous motor activity was evaluated using the open-field test before and after treatment with saline or paraquat. Oxidative stress was measured using the OxyBlot[TM] Protein Oxidation detection kit. DA, and its metabolites were measured using reverse-phase high performance liquid chromatography with electrochemical detection. Chronic paraquat treatment for 3 or 4 weeks did not result in any change in spontaneous motor activity or oxidative stress between paraquat and saline treated controls. In addition, paraquat failed to decrease DA or increase DA turnover in the striatum. Our data demonstrate that chronic administration of paraquat does not induce motor impairment, oxidative stress, or dopaminergic loss in the striatum in either the classic C57BL/6J mouse model for Parkinson's disease or FVB mice using the current exposure paradigm. A reliable and consistent mouse model for induction of neurotoxicity following paraquat exposure is needed to evaluate paraquat as a risk factor for Parkinson's disease in humans.

4.1. INTRODUCTION

Paraquat dichloride, or methyl viologen, is an herbicide that has been studied as a Parkinson's disease-inducing neurotoxicant (Costello et al., 2009; Dinis-Oliveira et al., 2006b; Liou et al., 1997; Tanner et al., 2011; Tomenson and Campbell, 2011). The degree of dopaminergic cell death following paraquat exposure is contradictory, however, in published reports in the literature. Conversely, the parkinsonian neurotoxicant 1-methyl-4-phenyl-1,2,3,5-tetrahydropyridine (MPTP), which is chemically similar to paraquat, consistently reduces levels of dopamine (DA) and its primary metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and induces motor dysfunction in C57BL/6J mice (Bradbury et al., 1986; Breckenridge et al., 2013; Cardozo-Pelaez et al., 2012; Gibrat et al., 2009; Langston et al., 1984; Luchtman et al., 2009; Przedborski et al., 1996). Criticisms regarding the ability of paraquat to induce Parkinson's disease include its polar charge, limited absorption, and poor BBB penetration (Koller, 1986). Others have questioned the methods researchers have used to study the mechanisms of paraquat-induced dopaminergic toxicity as well as questioned the rationale of the chemical similarity between paraquat and MPTP as a justification for paraquat being a Parkinson's disease-related neurotoxicant (Miller, 2007; Ramachandiran et al., 2007).

Epidemiological evidence has associated paraquat exposure with Parkinson's disease risk (Hertzman et al., 1990; Liou et al., 1997; Semchuk et al., 1992; Tanner et al., 2011; Tanner et al., 2009; Wang et al., 2011). Researchers have shown that paraquat induces toxicity associated with the progression of Parkinson's disease due to increased oxidative stress and a loss of dopaminergic neurons in the substantia nigra in rodents.

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Specifically, researchers have described a reduction in the number of tyrosine hydroxylase positive (TH^{+}) neurons in the substantia nigra pars compacta (SNpc) following intraperitoneally (i.p.) administered paraquat to C57BL/6J mice (Brown et al., 2006; Jiao et al., 2012; McCormack et al., 2006). The TH enzyme catalyzes the rate-limiting step in the synthesis of DA, and thus the amount of tyrosine-hydroxylase in a cell is an indirect measure of the amount of DA. Other reports suggest that DA, DOPAC, and HVA were decreased and DA turnover was increased in the striatum in mice following paraquat exposure (Li et al., 2005b; Prasad et al., 2009; Ren et al., 2009; Shepherd et al., 2006). Measures of motor behavior have also suggested that locomotor activity is affected by chronic paraquat administration (Brooks et al., 1999; Li et al., 2005b; Ren et al., 2009).

There is a growing body of evidence, however, that does not support previous findings of dopaminergic death following treatment with paraquat. The concentrations of DA, HVA, and DOPAC, as well as DA turnover in the striatum of C57BL/6J mice have been reported to be either unaffected or only marginally affected following paraquat exposure (Breckenridge et al., 2013; McCormack et al., 2002; Woolley et al., 1989). Locomotor activity has also shown to be unaffected or marginally affected following paraquat administration (Shepherd et al., 2006; Yang et al., 2007). Another important finding is that paraquat-induced SNpc neuron DA loss is highly dependent on genetic background as C57BL/6J mice, but not Swiss-Webster mice, are susceptible to dopaminergic toxicity following chronic paraquat administration (Jiao et al., 2012).

The neurotoxicity induced by paraquat exposure as a model for Parkinson's disease mice is controversial. The goal of our study was to investigate the contradictory

evidence regarding the death of dopaminergic neurons following chronic i.p. paraquat exposure following the most common non-lethal exposure paradigm. We evaluated neurotoxicity in two difference mouse strains: 1) in C57BL/6J mice, which are the standard mouse used to model Parkinson's disease and 2) FVB mice, which have not previously been used as a model of Parkinson's disease.

4.2. MATERIAL AND METHODS

4.2.1. Paraquat Exposure in Mice

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) and FVB mice (Taconic Farms, Germantown, NY) between 5-7 months of age were used for all studies. Mice were maintained on a 12 hr light-dark cycle, housed in microisolators, and were given food and deionized water *ad libitum*. All procedures were approved by the University of Montana Institutional Animal Care and Use Committee. Paraquat (Sigma-Aldrich Chemical Company, St. Louis, MO) was prepared at a concentration of 100 mg/ml and injected at a volume based on individual mouse weight (<100 μl). The most commonly reported and sub-lethal paraquat dosing regimen was selected. Two exposures were given i.p. for each mouse strain: 1) 10 mg/kg, 2 times per week for 3 weeks; and 2) 10 mg/kg, 2 times per week for 4 weeks. Control mice were treated with saline on the same schedule. All mice were euthanized via cervical dislocation followed by rapid decapitation 7 days post-injection of the final dose. During the 4 or 5 week experimental period, each animal was observed twice daily for mortality as well as for changes in appearance or behavior.

4.2.2. Motor Assessment

Motor behavior was evaluated one day prior to the first paraquat or saline treatment and again 1 week following the last treatment using the open field test. The open field test is a common measure of behavior and activity in rodents, where both the quality and quantity of the activity is evaluated (Hutter-Saunders et al., 2012). The tests were performed in a 30 cm square box, under 20 lux light intensity, and during the light hours of the day. The field is marked with a grid and infrared beams and video cameras were utilized to assess and record activity. All animals were evaluated during the same time frame and after a 1 hr conditioning period in which the mice were allowed to acclimate to the light and temperature levels in the room where the open field test is completed. Spontaneous motor activity was recorded using ANY-maze software (Stoelting, Wood Dale, IL). We measured total distance traveled, speed, time spent immobile, number of line crossings, number of rearings, and distance traveled from center over a 10 min period. Data are represented as mean ± standard deviation.

4.2.3. Oxidative Stress

Carbonylated protein abundance was evaluated by Western immunoblotting of striatum isolated following chronic paraquat administration to either C57Bl/6J or FVB mice. Frozen striatum was sonicated in 10 μl PBS followed by 90μl RIPA lysis buffer (Millipore, Billerica, MA). Following lysis samples were centrifuged for 15 min at 10,000 g and 4°C, pellet was discarded, and the cytosolic proteins in the supernatant were used. Protein carbonylation was assessed by measuring the levels of carbonyl groups using the OxyBlot protein oxidataion detection kit (Millipore, Billerica, MA). Carbonyl

groups at the protein side chains were derivatized from 2,4-dinitrophenylhydrazone by reactions with 2,4-dinitrophenylhydrazine. Derivatized proteins were subjected to SDS-PAGE and electro transferred to nitrocellulose membranes. Quantification analysis of blots was performed using Image Gauge Software (Fujifilm, Tokyo, Japan).

4.2.4. Neurochemical Analysis

Levels of DA, DOPAC, and HVA in the striatum were measured using reverse phase high performance liquid chromatography (RP-HPLC) with electrochemical detection as previously described (Cardozo-Pelaez et al., 2005; Cardozo-Pelaez et al., 2012; Cardozo-Pelaez et al., 1999). The index of DA turnover was calculated as ([DOPAC + HVA]/DA).

4.2.5. Statistical Analysis

Student's two-tailed *t* test was used to determine differences between two sets of data. For all analyses, *p*-values of < 0.05 were considered statistically significant. Statistical tests were performed with Graphpad Prism® statistical software (Sorrento, CA).

4.3. RESULTS

Administration of 10mg/kg paraquat i.p. 2 times a week for 3 weeks failed to induce oxidative stress in the striatum of either C57BL/6J or FVB mice (Figure 4.1., page 103). In addition this dosing regimen failed to decrease concentrations of DA in the striatum of both the C57BL/6J and FVB mice (Figure 4.2a, page 104), and also failed to

increase DA turnover in the striatum of C57BL/6J mice and only modestly (1.2-fold) increased turnover in FVB mice (Figure 4.2b, page 104). There were differences between pre- and post-treatment in both the C57BL/6J and FVB mice, but the differences were present in both saline and paraquat treated mice. When we compare saline to paraquat treated animals we observe no significant differences indication to effect of paraquat exposure (Table 4.1., page 101). Three week chronic treatment of paraquat failed to induce marked neurotoxicity either by biochemical or motor behavioral measurements.

In an attempt to replicate the reported dopaminergic toxicity in C57BL/6J mice, we repeated the paraquat exposure of 10 mg/kg i.p. 2 times a week and extended it for an additional week, for a 4 week total exposure. Administration of 10mg/kg paraquat i.p. 2 times a week for 4 weeks failed to induce oxidative stress in the striatum of either C57BL/6J or FVB mice (Figure 4.3., page 105). We again did not observe any significant differences in the DA concentrations (Figure 4.4a, page 106) or the DA turnover (Figure 4.4b, page 106) in either strain of mice. Similar to the 3 week exposure, there were differences between pre- and post-treatment in both the C57BL/6J and FVB mice in both saline and paraquat treated mice, but almost all differences disappeared when comparing saline and paraquat treatment indicating no effect on motor function following paraquat exposure (Table 4.2., page 102). The only significant difference between saline and paraquat treated mice was in time spent immobile in the FVB mice, but it was only observed in the pre-treatment group, indicating it was not related to paraquat exposure.

4.4. DISCUSSION

We failed to induce neurotoxicity in C57BL/6J mice via chronic administration of paraquat using a previously published sub-lethal exposure paradigm. We also could not induce neurotoxicity in FVB mice using this method. While C57BL/6J mice are routinely used as a model for neurodegeneration in Parkinson's disease, FVB mice have not been used in such models. Neither a 3 nor 4 week treatment with 10 mg/kg i.p. paraquat 2 times a week induced significant changes indicative of paraquat-induced dopaminergic dysfunction including changes in motor function, oxidative stress, DA concentrations, or DA turnover. The assessment and neurochemical analysis methods to test neurodegeneration have been validated previously with the known Parkinsonian neurotoxicant, MPTP (Cardozo-Pelaez et al., 2012). If paraquat caused toxicity to the SNpc neurons then we would expect motor dysfunction, and increase in oxidative stress, a decrease in DA, and an increase in DA turnover. Current literature suggests this is precisely the case for the known Parkinson's disease neurotoxicant MPTP (Bradbury et al., 1986; Breckenridge et al., 2013; Cardozo-Pelaez et al., 2012; Gibrat et al., 2009; Langston et al., 1984; Luchtman et al., 2009; Przedborski et al., 1996). We did not observe any changes that would be associated with paraquat-induced neurodegeneration. Our data suggest that paraquat is not inducing neurodegeneration following chronic sub-lethal doses of paraquat.

Our results are consistent with previous reports of a lack of alterations in DA levels or DA turnover in the striatum in C57BL/6J mice (Breckenridge et al., 2013; McCormack et al., 2002; Woolley et al., 1989). These results are especially interesting in light of a recent study using a similar paraquat exposure model (10, 15, or 25

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mg/kg/week for 1, 2, or 3 weeks) in C57BL/6J mice where they showed no difference in TH^{+} neurons, DA levels, DA turnover, or apoptotic cell death (Breckenridge et al., 2013).

The growing body of evidence against paraquat-induced neurotoxicity is in contrast with other researchers who have demonstrated dopaminergic toxicity following chronic paraquat administration in C57BL/6J mice. Following 5 or 10 mg/kg i.p. administration of paraquat 2 times a week for 3 weeks in C57BL/6J mice, researchers observed a reduction in the number of TH^{+} neurons (Jiao et al., 2012; McCormack et al., 2006). Conversely, neurotoxicity could not be induced in Swiss-Webster mice (Jiao et al., 2012). Another study also found a reduction in the TH^{+} neurons following 10 mg/kg i.p. administration of paraquat every 2 days for a total of 10 doses, however, one of the mice died during the treatment (Li et al., 2005b). Staining for TH^{+} neurons is only an indirect approximate of the amount of DA in the striatum, but other researchers have also directly measured the loss of DA, HVA, and DOPAC in the SNpc following administration of paraquat (Li et al., 2005b; Prasad et al., 2009; Ren et al., 2009). However, dosing regimens in those studies varied widely from daily oral doses for 4 months to i.p. administration 2 or 3 times a week for up to 36 total doses. Motor behavior assessments following 10 mg/kg i.p. administration of paraquat have also suggested that spontaneous locomotor activity is affected (Brooks et al., 1999; Li et al., 2005b; Ren et al., 2009), but again these studies varied in dosing regimen ranging from daily oral doses for 4 months to i.p. administration once a week for 3 weeks. The lack of consistency in routes of administration and exposure regimens could be a possible cause of many of the discrepancies when comparing data.

Finally, to add to the complexity of chronic paraquat administration in mice, the Office of Research Integrity (ORI) recently reported that a former assistant professor committed research misconduct by fabricating stereological cell count data in a study regarding how pesticides, including paraquat, influence neuronal mechanisms involved in Parkinson's disease (Dahlberg; Dunning, 2012). The ORI found that the fabrication had been intentional and two papers were recalled, including one specifically regarding paraquat exposure (Rodriguez et al., 2005; Thiruchelvam et al., 2005). In addition to the recalled papers, the author who was cited for research misconduct has also published numerous papers regarding chronic paraquat administration and neurotoxicity (McCormack et al., 2002; Prasad et al., 2007; Thiruchelvam et al., 2000a; Thiruchelvam et al., 2003; Thiruchelvam et al., 2000b; Thiruchelvam et al., 2002; Winnik et al., 2009). This issue most certainly casts doubt on all of the work generated by this researcher regarding paraquat-induced neurodegeneration and highlights the need for a reliable parquat-exposure model.

In addition to not inducing dopaminergic loss in the SNpc in the Parkinson's disease model organism C57BL/6J mice, we were also not able to induce toxicity in FVB mice, which have not been studied previously. In light of our observations and previous reports, it is possible that paraquat induced dopaminergic dysfunction is highly dependent on mouse strain. In order to fully understand the role of paraquat in human toxicity and Parkinson's disease risk, a standardized and reproducible model of paraquat induced neurotoxicity in crucial and may need to be tailored accordingly in different mouse strains.

4.4. CONCLUSIONS

In conclusion, chronic i.p. paraquat administration, using the most common sub-lethal exposure paradigm, did not cause alterations in the spontaneous motor activity, oxidative stress, or changes to DA or DA turnover in either C57BL/6J or FVB mice. There is conflicting and contradictory reports regarding neurodegeneration induced by paraquat, and it is unclear whether paraquat can be used as a Parkinson's disease model in mice. This study highlights the need to verify and confirm which strains of mice and which exposure regimens consistently produce neurotoxicity to study paraquat induced Parkinson's disease models. Until a reliable model of the chronic effects of paraquat on the dopaminergic neurons can be agreed upon, we will be unable to move forward with understanding the role of paraquat exposure and Parkinson's disease risk in humans.

Table 4.1. Motor Assessment Pre- and Post-treatment of 10mg/kg i.p. Paraquat Exposure Twice a Week for 3 Weeks in C57BL/6J and FVB Mice.

	Saline		Paraquat	
	Pre-Treatment	Post-Treatment[a]	Pre-Treatment	Post-Treatment[a]
C57BL/6J Mice				
Total distance traveled (m)	28.3 ± 3.3	24.1 ± 12.3	29.1 ± 7.4	33.0 ± 10.0
Speed (m/sec)	0.047 ± 0.005	0.040 ± 0.021	0.049 ± 0.012	0.043 ± 0.017
Time spent immobile (sec)	136 ± 50	269 ± 77[*]	135 ± 74	260 ± 92[*]
Number of line crossings	554 ± 65	473 ± 222	560 ± 151	486 ± 185
Number of rearings	131 ± 40	133 ± 51	127 ± 43	134 ± 57
Distance traveled from center over 10 min (m)	0.178 ± 0.006	0.177 ± 0.007	0.181 ± 0.008	0.180 ± 0.013
FVB Mice				
Total distance traveled (m)	38.6 ± 8.9	38.5 ± 13.0	42.4 ± 7.7	28.4 ± 5.6[**]
Speed (m/sec)	0.064 ± 0.015	0.043 ± 0.022	0.071 ± 0.013	0.047 ± 0.009[**]
Time spent immobile (sec)	137 ± 40	268 ± 87[*]	139 ± 31	218 ± 44[**]
Number of line crossings	781 ± 148	479 ± 204[*]	811 ± 147	555 ± 100[*]
Number of rearings	237 ± 37	185 ± 112	190 ± 47	134 ± 20[*]
Distance traveled from center over 10 min (m)	0.152 ± 0.016	0.178 ± 0.009[*]	0.160 ± 0.007	0.174 ± 0.008[*]

[a]Significant difference between pre- and post-treatment; [*]$p < 0.05$, [**]$p < 0.005$.

Table 4.2. Motor Assessment Pre- and Post-treatment of 10mg/kg i.p. Paraquat Exposure Twice a Week for 4 Weeks in C57BL/6J and FVB Mice.

	Saline		Paraquat[b]	
	Pre-Treatment	Post-Treatment[a]	Pre-Treatment	Post-Treatment[a]
C57BL/6J Mice				
Total distance traveled (m)	30.4 ± 7.6	$20.4 \pm 5.5^{**}$	27.0 ± 5.4	$18.0 \pm 4.9^{**}$
Speed (m/sec)	0.051 ± 0.013	$0.034 \pm 0.009^{**}$	0.045 ± 0.009	$0.030 \pm 0.008^{**}$
Time spent immobile (sec)	124 ± 66	191 ± 85	131 ± 47	$241 \pm 77^{**}$
Number of line crossings	592 ± 129	$457 \pm 102^{*}$	528 ± 117	$422 \pm 120^{*}$
Number of rearings	139 ± 45	$60.3 \pm 15.7^{*}$	155 ± 72	123 ± 104
Distance traveled from center over 10 min (m)	0.172 ± 0.014	0.166 ± 0.008	0.172 ± 0.008	0.174 ± 0.018
FVB Mice				
Total distance traveled (m)	23.2 ± 11.9	24.1 ± 7.2	32.0 ± 6.6	24.9 ± 3.8
Speed (m/sec)	0.039 ± 0.019	0.040 ± 0.012	0.053 ± 0.011	0.041 ± 0.006
Time spent immobile (sec)	274 ± 74	235 ± 25	$191 \pm 32^{\dagger}$	241 ± 40
Number of line crossings	458 ± 242	502 ± 127	615 ± 103	507 ± 75
Number of rearings	141 ± 62	108 ± 48	184 ± 53	148 ± 44
Distance traveled from center over 10 min (m)	0.169 ± 0.013	0.163 ± 0.008	0.171 ± 0.007	0.159 ± 0.015

[a]Significant difference between pre- and post-treatment; $^{*}p < 0.05$, $^{**}p < 0.005$
[b]Significant difference between saline and paraquat treatment; $^{\dagger}p < 0.05$

4.1.
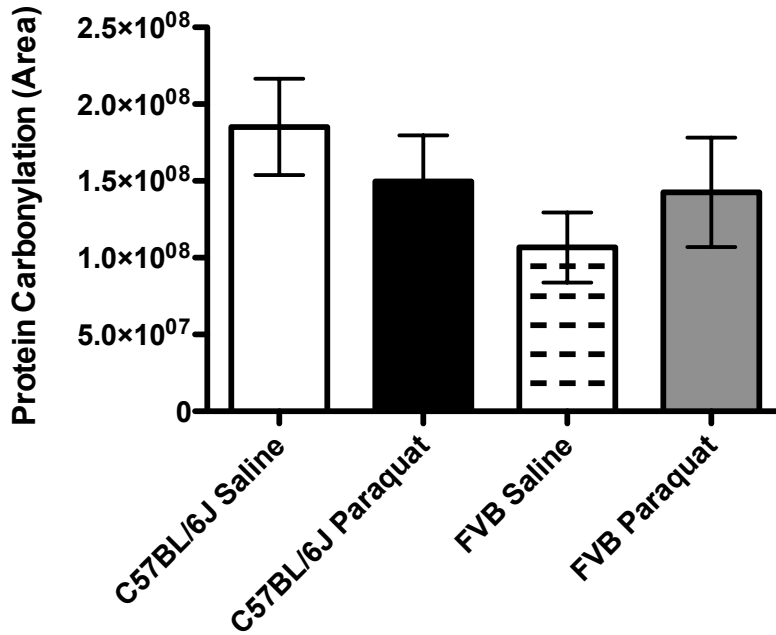
Figure 4.1. Striatal Oxidative Stress in C57BL/6J and FVB Mice Administered Either Saline or Paraquat for 3 Weeks. Saline or paraquat was administered i.p. at 10 mg/kg twice a week for 3 weeks. Brains were collected 1 week following the final dose and protein carbonylation was assessed by measuring the levels of carbonyl groups using the OxyBlot protein oxidataion detection kit (Millipore, Billerica, MA). Protein carbonylation was quantified and are shown as mean area ± SEM.

4.2a
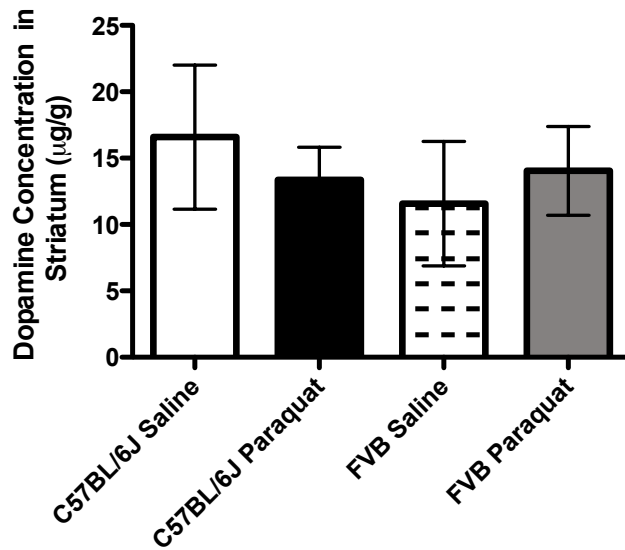
4.2b
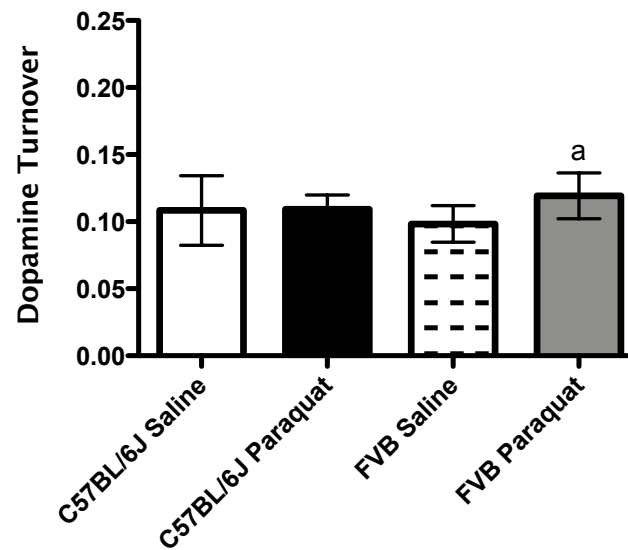
Figure 4.2. Striatal DA (4.2a) and DA Turnover (4.2b) in C57BL/6J and FVB Mice Administered Either Saline or Paraquat for 3 Weeks. Saline or paraquat was administered i.p. at 10 mg/kg twice a week for 3 weeks. Brains were collected 1 week following the final dose and evaluated using electrochemical RP-HPLC detection for concentrations of DA, HVA, and DOPAC. DA concentrations are shown in μg/g brain tissue as mean ± s.d. (4.2a) and DA turnover (4.2b) is presented as a ratio of [DOPAC + HVA]/DA. Significant difference within a mouse strain between treatment groups; [a]$p < 0.05$

4.3.
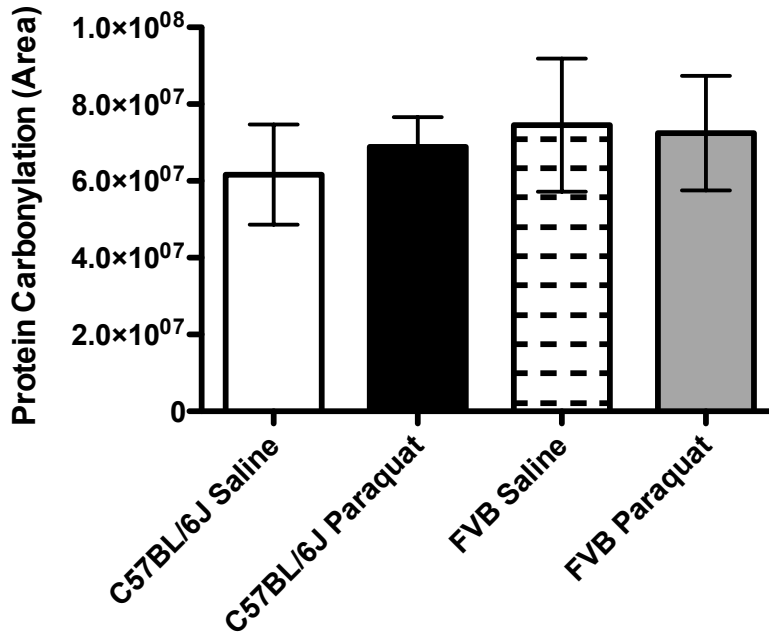
Figure 4.3. Striatal Oxidative Stress in C57BL/6J and FVB Mice Administered Either Saline or Paraquat for 4 Weeks. Saline or paraquat was administered i.p. at 10 mg/kg twice a week for 4 weeks. Brains were collected 1 week following the final dose and protein carbonylation was assessed by measuring the levels of carbonyl groups using the OxyBlot protein oxidataion detection kit (Millipore, Billerica, MA). Protein carbonylation was quantified and are shown as mean area ± SEM.

4.4a
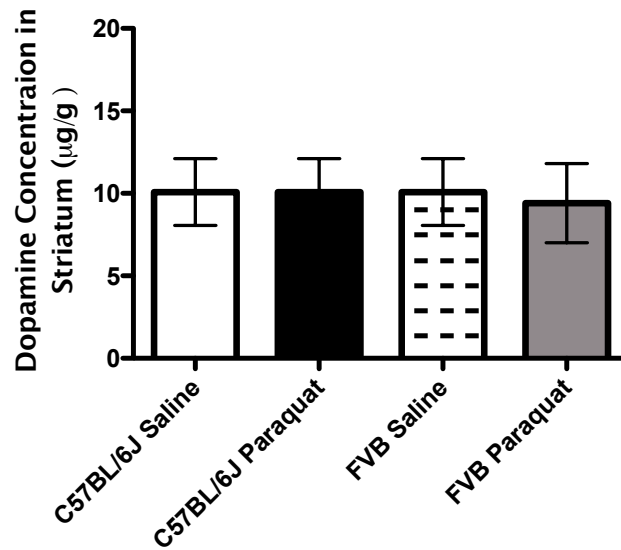
4.4b
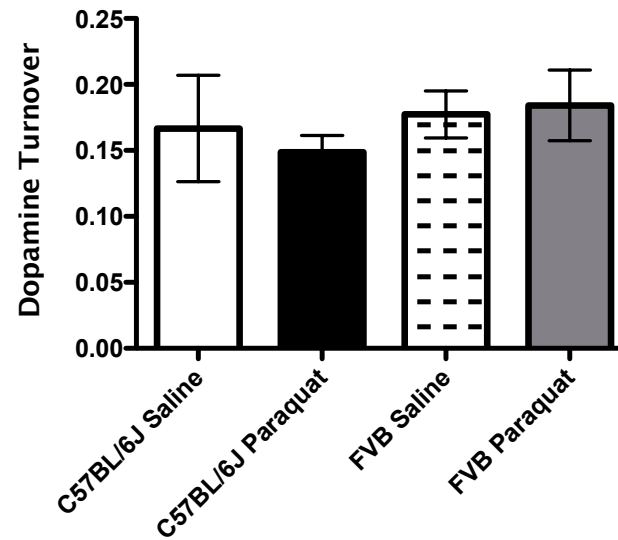
Figure 4.4. Striatal DA (4.4a) and DA Turnover (4.4b) in C57BL/6J and FVB Mice Administered Either Saline or Paraquat for 4 Weeks. Saline or paraquat was administered i.p. at 10 mg/kg twice a week for 4 weeks. Brains were collected 1 week following the final dose and evaluated using electrochemical RP-HPLC detection for concentrations of DA, HVA, and DOPAC. DA concentrations are shown in µg/g brain tissue as mean ± s.d. (4.4a) and DA turnover (4.4b) is presented as a ratio of [DOPAC + HVA]/DA.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The central goal of my research was to evaluate P-gp transport of pesticides associated with Parkinson's disease to gain knowledge regarding the contribution of P-gp in Parkinson's disease risk. To accomplish this goal, I utilized a variety of models that allowed me to determine if these pesticides are substrates or inhibitors of P-gp, and to evaluate the use of chronic paraquat treatment in mice as a reproducible model of Parkinson's disease. These experiments were pivotal to evaluating the significance of pesticide brain distribution, P-gp transport of pesticides, and potential Parkinson's disease risk. Results obtained from this research will be vital in understanding mechanisms of pesticide transport at the BBB and accumulation in the CNS. Understanding how P-gp transport is important in p[pesticide disposition will allow us to move closer to better diagnostic tools, or preventative mechanism for Parkinson's disease.

Genetic variation in the *ABCB1* gene results in altered P-gp function and expression. Several epidemiological studies have described an association between *ABCB1* pharmacogenetics and susceptibility to Parkinson's disease (Bartels et al., 2008a; Drozdzik et al., 2003; Dutheil et al., 2010; Furuno et al., 2002; Le Couteur et al., 2001; Lee et al., 2004; Lee and Bendayan, 2004; Tan et al., 2005; Tan et al., 2004; Vautier and Fernandez, 2009; Westerlund et al., 2009; Westerlund et al., 2008). One possible mechanism to support this association is that *ABCB1* genetic variation decreases P-gp activity at the blood-brain-barrier, leading to increased brain accumulation of neurotoxicants and an increased incidence of Parkinson's disease. Therefore, the goal of this research was to evaluate the association of *ABCB1* with Parkinson's disease by evaluating the pesticides paraquat, rotenone, maneb, endosulfan, dieldrin, and diazinon as potential P-gp substrates. If P-gp transports pesticides at the BBB and is responsible for

maintaining low pesticide brain distribution, then a decrease in P-gp activity due to genetic variation in *ABCB1* could significantly increase the pesticide brain concentrations and potential Parkinson's disease risk. Identifying which pesticides are P-gp substrates provides insight into the relationship between *ABCB1* and Parkinson's disease.

The most frequently associated pesticide with Parkinson's disease risk is the herbicide paraquat. In Specific Aim 1, I evaluated the P-gp-mediated transport of paraquat using a combination of *in vitro* and *in vivo* models in which I determined that paraquat is neither a substrate nor an inhibitor of P-gp. Paraquat was screened as a substrate for P-gp in three *in vitro* experimental models: ATPase activity, xenobiotic-induced cytotoxicity, and transepithelial permeability. Paraquat did not stimulate the P-gp mediated hydrolysis of ATP in P-gp expressing membranes, P-gp expression did not alter sensitivity to paraquat-induced cytotoxicity in cells, and there was no directional transepithelial transport of paraquat mediated by P-gp. In addition, paraquat did not inhibit R123 efflux in P-gp-expressing cells and therefore is not a P-gp inhibitor. This work indicates that paraquat is not a P-gp substrate or inhibitor.

In addition to the *in vitro* models, paraquat was evaluated as a P-gp substrate *in vivo* in Specific Aim 1. While there were subtle differences in the systemic pharmacokinetic parameters between wild-type and knock-out mice, we did not observe differences in brain-to-plasma partitioning or total brain accumulation of paraquat between FVB wild-type and *mdr1a*$^{(-/-)}$*/1b*$^{(-/-)}$ mice, which was the most important indicator that paraquat is not a P-gp substrate. Therapeutic agents that are P-gp substrates such as amprenavir, ivermectin or vinblastine, display approximately 20-80-fold increase in brain accumulation in knock-out mice relative to wild-type mice due to loss of P-gp

activity at the blood-brain-barrier (Polli et al., 1999; Schinkel et al., 1994; van Asperen et al., 1996). Alterations in drug-metabolizing enzymes and drug transporters occur in $mdr1a^{(-/-)}/1b^{(-/-)}$ mice and may account for the differences in paraquat pharmacokinetics I observed (Schuetz et al., 2000). However, the lack of differences in brain concentrations between wild-type and knockout mice imply that P-gp is not involved in paraquat efflux in the BBB. Therefore, our *in vivo* models confirms our *in vitro* results that paraquat is not a P-gp substrate. My research with paraquat in Specific Aim 1 was the first comprehensive study designed to measure P-gp transport of paraquat in a variety of *in vitro* and *in vivo* models.

We found in Specific Aim 1 that paraquat, the pesticide most commonly associated with Parkinson's disease, was not a substrate or inhibitor of P-gp, and thus the association between genetic variation in *ABCB1* and Parkinson's disease risk was not due to altered paraquat exposure. However, exposure to other pesticides has also been linked with an increased risk of developing Parkinson's disease. It was important to further investigate the relationship between *ABCB1* and Parkinson's disease by evaluating other Parkinson's disease associated pesticides as P-gp substrates. Thus, in Specific Aim 2, I systematically evaluated the P-gp mediated transport of diazinon, dieldrin, endosulfan, maneb, MPP+, rotenone, or ivermectin using a combination of *in vitro* models. All of the above mentioned pesticides besides ivermectin have been associated with Parkinson's disease risk. I observed no role for P-gp in the disposition of diazinon, dieldrin, endosulfan, maneb, and MPP+, however I observed that rotenone maybe a substrate of P-gp. Rotenone stimulated ATPase activity with a lower K_m than the known P-gp substrate, verapamil, suggesting that rotenone binds tightly to P-gp. These data demonstrate the first

piece of evidence to provide a mechanism for the causal relationship between P-gp and Parkinson's disease, possibly due to P-gp-mediated disposition of the pesticide rotenone.

To summarize the implications of the findings from Specific Aim 2, I discovered that the pesticide rotenone is a substrate of P-gp, which provides a possible explanation for the epidemiological evidence that has linked genetic variation in *ABCB1* with Parkinson's disease risk. In order to move forward with this exciting observation further research regarding the relationship between P-gp and rotenone is needed. The next step is to utilize the models described herein to further confirm rotenone as a P-gp substrate to validate the hypothesis that rotenone is the environmental neurotoxicant that links genetic variation in *ABCB1* to Parkinson's disease. Rotenone transepithelial transport in LLC-MDR1-WT and LLC-vector cells as well as an *in vivo* evaluation in FVB wild-type and $mdr1a^{(-/-)}/1b^{(-/-)}$ mice should be utilized to more completely understand P-gp transport of rotenone at the BBB.

A future direction of this research is to evaluate the pesticide rotenone using LLC-MDR1-variant cells. The functional *ABCB1* SNPs 1236C>T, 2677G>T/A, and 3435C>T have all been shown to alter P-gp expression or function, and have been associated with an altered risk of developing Parkinson's disease. Transepithelial transport, intracellular accumulation, and cytotoxic sensitivity assays using recombinant LLC-PK1 cells lines expressing either wild-type or a variant *ABCB1* will investigate if genetic variation in *ABCB1* results in altered P-gp transport of rotenone.

A current controversial topic is the reproducibility of the mouse model of Parkinson's disease induced by chronic paraquat exposure. Several groups have reported a reduction in the number of TH^+ neurons and loss of DA, DOPAC, and HVA in the

SNpc following i.p. administered paraquat to C57BL/6J mice (Brown et al., 2006; Jiao et al., 2012; Li et al., 2005b; McCormack et al., 2006; Prasad et al., 2009; Ren et al., 2009; Shepherd et al., 2006). Motor behavior has been reported to be affected by chronic paraquat administration (Brooks et al., 1999; Li et al., 2005b; Ren et al., 2009). However, there is growing evidence that suggests chronic exposure to paraquat in rodents does not result in Parkinson's disease phenotypes. Locomotor activity as well as the concentrations of DA, HVA, and DOPAC, as well as DA turnover in the striatum of C57BL/6J mice have been reported to be either unaffected or only marginally affected following paraquat exposure (Breckenridge et al., 2013; McCormack et al., 2002; Shepherd et al., 2006; Woolley et al., 1989; Yang et al., 2007). Another important finding is that paraquat-induced SNpc neuron DA loss is highly dependent on genetic background. C57BL/6J but not Swiss-Webster mice are susceptible to dopaminergic toxicity following chronic paraquat administration (Jiao et al., 2012). Thus, the goal of Specific Aim 3 was to investigate the contradictory evidence regarding the death of dopaminergic neurons following chronic i.p. paraquat exposure. Paraquat neurotoxicity was evaluated in two different mouse strains: 1) the standard mouse strain used as a model of paraquat-induced Parkinson's disease, C57BL/6J mice, and 2) FVB mice, which have not previously been used as a model of neurotoxicant-induced Parkinson's disease.

In my evaluation of chronic paraquat exposure in mice and validation of the paraquat model of Parkinson's disease I replicated the most common sub-lethal exposure paradigm. Following administration of 10mg/kg paraquat i.p. either twice a week for 3 weeks, or for 4 weeks, I observed no differences in spontaneous locomotor activity,

oxidative stress, or DA concentration between saline and paraquat treatment in either C57BL/6J or FVB mice. The results gained from my research regarding the effect of chronic i.p. paraquat administration in mice are supported by other reports of a lack of change in DA levels or DA turnover in the striatum in C57BL/6J mice (Breckenridge et al., 2013; McCormack et al., 2002; Woolley et al., 1989). Combining our observations with previous evidence it is clear that paraquat-induced dopaminergic dysfunction is highly dependent on mouse strain. Thus, administering paraquat chronically to induce motor dysfunction, oxidative stress and dopamine loss in the striatum of C57BL/6J mice is not possible using the regimen that is currently described; it remains uncertain whether that paraquat can induce Parkinson's disease in mice. Future work in this area should focus on developing a consistently reproducible mouse model of paraquat-induced Parkinson's disease in which the route of administration, dosing regimen, and strain of mouse are standardized and reproducible. In order to better understand Parkinson's disease and paraquat exposure in humans it is vital that this model be further validated and reported.

In addition to Parkinson's disease, *ABCB1* has been linked to susceptibility to other diseases such as childhood acute lymphoblastic leukemia, renal cancer, multiple myeloma, irritable bowl syndrome, Alzheimer's disease, progressive supranuclear palsy, and multiple system atrophy (Bartels et al., 2008b; Blokzijl et al., 2007; Jamroziak et al., 2004; Kuhnke et al., 2007; Maggini et al., 2008; Semsei et al., 2008; Siegsmund et al., 2002; Urayama et al., 2007; van Assema et al., 2012). The results from these future studies with recombinant *ABCB1* variant cells will likely have application in these

diseases as well. Understanding the complex interplay between genes and environmental exposure may lead to novel methods of disease detection, prevention, and treatment.

In conclusion, pesticides threaten the safety of drinking water in rural communities, impose a risk to applicators and manufacturers in occupational settings and have been highly linked to the development of Parkinson's disease. It is clear from my research that while the pesticide paraquat is not a P-gp substrate, further validation of paraquat as a Parkinsonian neurotoxicant in animals models is desperately needed before we can further link paraquat exposure to Parkinson's disease. The information gained from this research regarding the pesticide rotenone as a P-gp substrate could result in new standards and regulatory policies for drinking water in rural communities. People residing in rural communities should be evaluated for their *ABCB1* genotype and advised accordingly for the safety of consumption of well water. In addition, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) under the sections "Restricted-Use Classification and Certification of Applicators" and "Worker Protection Standard for Agricultural Pesticides", suggest that the use of some pesticides be limited if they cause adverse effects even when used as directed. The FIFRA may be improved by incorporating data gained from this research to require that people working with rotenone be assessed for individual risk based on their *ABCB1* genotype. This research is unique because it linked together pharmacokinetics and toxicology to evaluate the result of pesticide exposure on human health. This research has the potential to be applied translationally in the promotion of individualized risk assessment in people who are exposed to disease-causing toxicants in their environment and could impact large numbers of people worldwide.

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