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P-Glycoprotein Transport of Neurotoxic Pesticides

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ABSTRACT

P-glycoprotein (P-gp) has been associated with a number of neurodegenerative diseases, including Parkinson's disease, although the mechanisms remain unclear. Altered transport of neurotoxic pesticides has been proposed in Parkinson's disease, but it is unknown whether these pesticides are P-gp substrates. We used three in vitro transport models, stimulation of ATPase activity, xenobiotic-induced cytotoxicity, and inhibition of rhodamine-123 efflux, to evaluate P-gp transport of diazinon, dieldrin, endosulfan, ivermectin, maneb, 1-methyl-4-phenyl-4-phenylpyridinium ion (MPP^+), and rotenone. Diazinon and rotenone stimulated ATPase activity in P-gp–expressing membranes, with V_{max} values of 22.4 ± 2.1 and 16.8 ± 1.0 nmol inorganic phosphate/min per mg protein, respectively, and K_m values of 9.72 ± 3.91 and 1.62 ± 0.51 μM, respectively, compared with the P-gp substrate verapamil, with a V_{max} of 20.8 ± 0.7 nmol inorganic phosphate/min per mg protein and K_m of 0.871 ± 0.172 μM. None of the other pesticides stimulated ATPase activity. We observed an increased resistance to MPP^+ and rotenone in LLC-MDR1-WT cells compared with LLC-vector cells, with 15.4- and 2.2-fold increases in EC_{50} values, respectively. The resistance was reversed in the presence of the P-gp inhibitor verapamil. None of the other pesticides displayed differential cytotoxicity. Ivermectin was the only pesticide to inhibit P-gp transport of rhodamine-123, with an IC_{50} of 0.249 ± 0.048 μM. Our data demonstrate that dieldrin, endosulfan, and maneb are not P-gp substrates or inhibitors. We identified diazinon, MPP^+, and rotenone as P-gp substrates, although further investigation is needed to understand the role of P-gp transport in their disposition in vivo and associations with Parkinson's disease.

Introduction

P-glycoprotein (P-gp), encoded by the multidrug resistance gene *ABCB1* or *MDR1*, is a member of the ATP-binding cassette superfamily of transporters. P-gp is a transporter that effluxes a diverse array of xenobiotics in healthy tissues throughout the body, which is particularly important in limiting the distribution of xenobiotics across blood-tissue barriers, such as the blood-brain barrier (BBB) (Giacomini, 1997; Lin and Yamazaki, 2003a,b; Giacomini et al., 2010; Sharom, 2011). P-gp expression at the BBB protects the brain from toxic substances circulating in the blood. Numerous studies have suggested a role for P-gp in the development of neurodegenerative diseases, including Parkinson's disease. Potential mechanisms of these associations include changes in P-gp expression or activity with age, disease progression, and *ABCB1* pharmacogenomics (Le Couteur et al., 2001; Furuno et al., 2002; Drozdzik et al., 2003; Lee and Bendayan, 2004; Lee et al., 2004; Tan et al., 2004, 2005; Kortekaas et al., 2005; Bartels et al., 2008, 2009; Westerlund et al., 2008, 2009; Rapposelli et al., 2009; Vautier and Fernandez, 2009; Dutheil et al., 2010; Bartels, 2011; Li et al., 2014). Since P-gp is an efflux transporter, changes in P-gp function at the BBB may result in increased brain accumulation of neurotoxicants that are normally excluded from the brain. Although it has been suggested that the modulation of P-gp at the BBB in neurodegenerative diseases may not have a clinically significant effect on exposures of therapeutic compounds (<25%) (Kalvass et al., 2013), this decrease in function could be significant for neurotoxic pesticides where exposures occur chronically over decades and compounds typically have long half-lives and bioaccumulate (Hatcher et al., 2008).

We evaluated P-gp transport of the major neurotoxic pesticides that have been associated with Parkinson's disease to elucidate the role of P-gp in the development of disease. Environmental exposures to neurotoxicants are risk factors for Parkinson's disease (Langston et al., 1984; Semchuk et al., 1992; Bonnet and Houeto, 1999; Steece-Collier et al., 2002; Gatto et al., 2009; Wirdefeldt et al., 2011; Kamel, 2013; Mostafalou and Abdollahi, 2013; Pezzoli and Cereda, 2013). One of the first discoveries of an environmental risk factor was 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine is converted to

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ABBREVIATIONS: BBB, blood-brain barrier; 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; MPP^+, 1-methyl-4-phenyl-4-phenylpyridinium ion; P-gp, P-glycoprotein; P_i, inorganic phosphate; R123, rhodamine-123.

1-methyl-4-phenyl-4-phenylpyridinium ion (MPP^+) (the pesticide cyperquat) in the brain, resulting in parkinsonism (Langston et al., 1983, 1984). Several pesticides, including paraquat, diazinon, dieldrin, endosulfan, maneb, MPP^+, and rotenone, have been associated with an increased risk of Parkinson's disease in epidemiologic studies in humans, toxicity models in animals, and in vitro studies (Langston et al., 1984; Bradbury et al., 1986; Fleming et al., 1994; Liou et al., 1997; Thiffault et al., 2000; Uversky et al., 2001; Gao et al., 2002; Uversky, 2004; Firestone et al., 2005; Li et al., 2005; Richardson et al., 2006; Wang et al., 2006; Hatcher et al., 2007; Jia and Misra, 2007a,b; Dhillon et al., 2008; Kanthasamy et al., 2008; Sonsalla et al., 2008; Costello et al., 2009; Gatto et al., 2009; Sharma et al., 2010; Weisskopf et al., 2010; Slotkin and Seidler, 2011; Tanner et al., 2011). Pesticides are a diverse class of compounds with different mechanisms of action, pharmacokinetic properties, and uses, i.e., fungicides, herbicides, and insecticides (Hatcher et al., 2008; Goldman, 2014; Chin-Chan et al., 2015). We reported previously that P-gp does not transport paraquat (Lacher et al., 2014). Our current study is not an exhaustive screen of P-gp transport of pesticides, but rather a focused evaluation of pesticides most commonly associated with Parkinson's disease: diazinon, dieldrin, endosulfan, maneb, MPP^+, and rotenone (Hatcher et al., 2008; Goldman, 2014; Chin-Chan et al., 2015).

Currently, there are sparse data to evaluate the P-gp transport of pesticides associated with Parkinson's disease (Bain and LeBlanc, 1996; Bleasby et al., 2000; Martel et al., 2001; Lecoeur et al., 2006; Pivčević and Zaja, 2006; Sreeramulu et al., 2007; Bircsak et al., 2013). Previous reports are often contradictory and many lack appropriate controls, making interpretation of the data difficult. Additionally, previous studies have often relied on a single in vitro transport model; however, it is clear in evaluating xenobiotic transporters that multiple models are necessary (Polli et al., 2001; Feng et al., 2008; Giacomini et al., 2010; Brouwer et al., 2013; Hillgren et al., 2013; Zamek-Gliszczynski et al., 2013). Therefore, classifying pesticides as P-gp substrates or inhibitors is challenging based on previous work.

To overcome some of the challenges of previous studies, we used a combination of in vitro P-gp transport models to systematically screen the major pesticides that have been linked to Parkinson's disease: diazinon, dieldrin, endosulfan, maneb, MPP^+, and rotenone. We also evaluated P-gp transport of the pesticide ivermectin. Although ivermectin has not been associated with Parkinson's, it is a pesticide that has been reported to be a P-gp substrate and was used as a comparator. We screened each compound using three models: 1) xenobiotic-induced stimulation of ATPase activity in P-gp–expressing membranes, 2) xenobiotic-induced cytotoxicity in recombinant cell lines, and 3) inhibition of intracellular rhodamine-123 (R123) efflux in recombinant cell lines. This study is the first comprehensive investigation of P-gp–mediated transport of the major pesticides associated with Parkinson's disease.

Materials and Methods

Chemicals. Diazinon, dieldrin, endosulfan, maneb, MPP^+, rotenone, doxorubicin, colchicine, ivermectin, verapamil, R123, and cyclosporine 4′,6-diamidino-2-phenylindole were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO). *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 (GF120918) was provided by GlaxoSmithKline (Research Triangle Park, NC).

ATPase Activity. Stimulation of ATPase activity by xenobiotics was measured using the SB MDR1/P-gp PREDEASY ATPase Kit (SOLVO Biotechnology, Budaörs, Hungary) according to the manufacturer's instructions. The assay utilizes the known P-gp substrate verapamil as a positive control. P-gp membranes were preincubated at 37°C for 10 minutes with diazinon, dieldrin, endosulfan, maneb, MPP^+, rotenone, ivermectin, or verapamil over a concentration range of 0.14–300 μM. The concentration range was selected based on the manufacturer's instructions. ATPase activity was measured as the rate of inorganic phosphate (P_i) liberation in the presence and absence of 1.2 mM sodium orthovanadate, a nonspecific ATPase inhibitor. The reaction was started with the addition of 2 mM MgATP and incubated at 37°C for an additional 10 minutes. The developer solution was added and incubated for 30 minutes at 37°C. Absorbance was read at 620 nm using a SpectraMax Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA). The amount of P_i was estimated from a phosphate standard curve, and orthovanadate-sensitive activity was subtracted from each value to account for background ATPase activity. Xenobiotic-stimulated ATPase activity was reported as nmol P_i liberated per minute incubation time per milligram total protein (nmol P_i/min per mg protein). Each xenobiotic was evaluated in at least triplicate and repeated once. Nonlinear regression least-squares model fit on Prism 5.0 software (GraphPad, San Diego, CA) was used to estimate Michaelis-Menten parameters (V_{max} and K_m).

Cell Culture. LLC-PK1 vector cells (LLC-vector) and recombinant *ABCB1* cells (LLC-MDR1-WT), 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 (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.

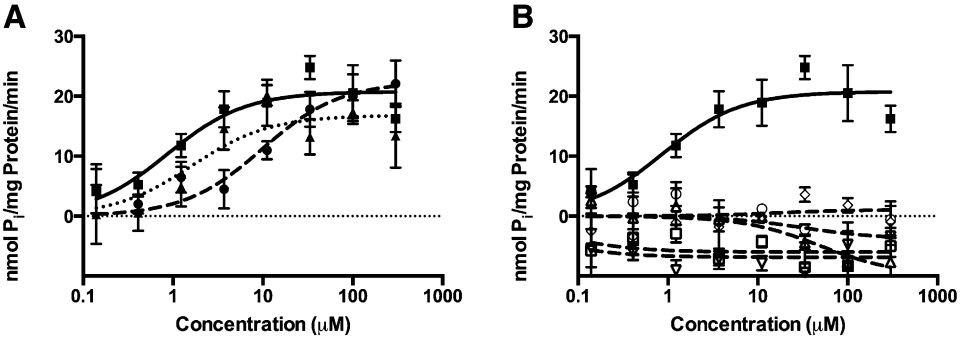

Fig. 1. Pesticide stimulation of ATPase activity in P-gp–expressing membranes. Compounds that stimulated ATPase activity: diazinon (closed circle, dashed line), rotenone (closed triangle, dotted line), or P-gp substrate verapamil (closed square, solid line) (A). Compounds that did not stimulate ATPase activity: dieldrin (open circle, dashed line), endosulfan (open triangle, dashed line), maneb (open diamond, dashed line), MPP^+ (open square, dashed line), ivermectin (open upside-down triangle, dashed line), or P-gp substrate verapamil (closed square, solid line) (B).

Xenobiotic-Induced Cytotoxicity. Sensitivity to cytotoxic agents was evaluated in LLC-vector and LLC-MDR1-WT cells as previously described (Lacher et al., 2014) using the CellTiter-Glo cell viability assay (Promega, Fitchburg, WI). Cells were plated overnight at a density of 1000 cells/well in 96-well plates and grown in the presence of test compounds for 72 hours at 37°C (Lacher et al., 2014). Cells were treated with the following pesticides over a wide concentration range: diazinon (11–1000 μM), dieldrin (4–1000 μM), endosulfan (0.4–100 μM), maneb (0.16–200 μM), MPP^+ (0.46–3000 μM), rotenone (0.76–5000 nM), and ivermectin (0.4–35 μM). The cytotoxic P-gp substrates doxorubicin (0.02–200,000 nM) and colchicine (0.01–50,000 nM) were used as positive controls. Concentration ranges were experimentally determined to capture the spectrum of the sigmoidal cell viability dose-response curve for each individual test compound. Inhibition of transport was performed with the known P-gp inhibitors GF120918 (0.5 μM) and verapamil (10 μM). Compounds were evaluated in triplicate. The final concentration of dimethylsulfoxide did not exceed 2% in cell culture experiments. A nonlinear regression log (agonist) versus response variable slope least-squares model was used to estimate cell sensitivity as the effective concentration necessary for 50% cell death (EC_{50}) with Prism 5.0 software. The fold change in cellular resistance was calculated as the ratio of EC_{50} values in LLC-MDR1-WT cells divided by the values in LLC-vector cells.

Inhibition of Intracellular Accumulation. Inhibition of R123 efflux in LLC-vector and LLC-MDR1-WT cells was performed based on a previously developed assay (Woodahl et al., 2004; Lacher et al., 2014). Concentration ranges of the following pesticides were experimentally determined based on solubility and to minimize cell death: diazinon (7–500 μM), dieldrin (3–200 μM), endosulfan (3–200 μM), maneb (1–250 μM), MPP^+ (1–1000 μM), rotenone (1–100 μM), and ivermectin (0.05–50 μM). The following known P-gp inhibitors were used as positive controls: GF120918 (1.56–1000 nM), cyclosporine (0.1–100 μM), and verapamil (0.1–500 μM). The final concentration of dimethylsulfoxide did not exceed 2% in cell culture experiments. 4′,6-Diamidino-2-phenylindole was added to the cells as a measure of cell viability immediately prior to flow cytometry, and dead cells were excluded from the analysis. A nonlinear regression log (inhibitor) versus normalized response variable slope least-squares model was used to estimate the inhibitor concentration necessary for 50% inhibition (IC_{50}) with Prism 5.0 software.

Statistical Analysis. EC_{50} estimates were compared between cell types and within a cell type using an extra sum of squares F test for the cytotoxic sensitivity assay. P values < 0.05 were considered statistically significant.

Results

Membrane-Based Analysis of P-gp Transport of Neurotoxicants. Diazinon and rotenone stimulated ATPase activity in P-gp–expressing membranes (Fig. 1A; Table 1) and were compared with the positive control verapamil. Estimates of V_{max} were similar among diazinon, rotenone, and verapamil. The K_m of diazinon is more than 10-fold higher than that of verapamil, and the K_m of rotenone is almost 2-fold higher than that of verapamil. Dieldrin, endosulfan, maneb, MPP^+, and ivermectin failed to stimulate ATPase activity (Fig. 1B; Table 1).

Cell-Based Analysis of P-gp Transport of Neurotoxicants. Cytotoxicity was measured in LLC-vector and LLC-MDR1-WT cells in the presence of neurotoxic pesticides and compared with known cytotoxic P-gp substrates as positive controls (doxorubicin and colchicine). EC_{50} values were estimated (Table 2) and dose-response curves were generated following exposure to P-gp substrates (Fig. 2) (doxorubicin, colchicine, and ivermectin) and the pesticides associated with Parkinson's disease (Fig. 3) (diazinon, dieldrin, endosulfan, maneb, MPP^+, and rotenone). Doxorubicin and colchicine data have been previously reported (Lacher et al., 2014). To confirm that differences in sensitivities between LLC-vector and LLC-MDR1-WT cells were due to P-gp, we used the P-gp inhibitors GF120918 and verapamil. Linearity in the assay was assured by optimizing the number of cells and the time course of neurotoxicant exposure. The two P-gp inhibitors did not cause cytotoxicity at the inhibitor concentrations used (data not shown).

For each compound tested, we estimated the fold change in resistance between LLC-MDR1-WT and LLC-vector

TABLE 1
ATPase activity in P-gp–expressing membranes

	Mean ± S.E.	
	V_{max}	K_m
	nmol P_i/min per mg protein	*μM*
Diazinon	22.4 ± 2.1	9.72 ± 3.91
Dieldrin	—[a]	—[a]
Endosulfan	—[a]	—[a]
Maneb	—[a]	—[a]
MPP^+	—[a]	—[a]
Rotenone	16.8 ± 1.0	1.62 ± 0.51
Ivermectin	—[a]	—[a]
Verapamil	20.8 ± 0.7	0.871 ± 0.172

[a]No ATPase activity above orthovanadate control.

TABLE 2
Xenobiotic-induced cytotoxicity in LLC-vector and LLC-MDR1-WT cells

LLC-PK1 Cells[a]	EC_{50} Values (95% Confidence Interval)	
	LLC-Vector Cells[b]	LLC-MDR1-WT Cells[b]
Doxorubicin (nM)[c]	32.2 (13.7–85.6)	1978 (1015–3850)***
+ GF120918[c]	24.8 (12.0–51.3)	8.06 (1.32–49.2)†††
+ Verapamil[c]	43.4 (12.0–155.9)	268 (90.7–98.6)†††
Colchicine (nM)[c]	13.3 (N.D.)[d]	645 (467–892)
+ GF120918[c]	12.6 (10.8–14.5)	17.5 (12.0–25.4)†††
+ Verapamil[c]	12.8 (N.D.)[d]	179 (117–273)†††
Ivermectin (μM)	4.86 (4.51–5.23)	4.04 (3.76–4.35)***
+ GF120918	5.76 (5.33–6.23)††	4.59 (3.99–5.28)
+ Verapamil	3.99 (3.31–4.81)†	4.25 (4.02–4.50)
Diazinon (μM)	1789 (N.D.)[d]	384 (279–528)
+ GF120918	132 (68.1–255)	414 (165–1040)
+ Verapamil	592 (50.6–6920)	373 (159–877)
Dieldrin (μM)	51.4 (41.1–64.4)	75.4 (62.9–90.4)*
+ GF120918	38.2 (28.0–52.0)	70.7 (57.0–87.6)
+ Verapamil	33.0 (27.3–39.8)†	40.0 (32.4–49.4)†††
Endosulfan (μM)	14.0 (10.4–18.8)	19.4 (12.5–30.2)
+ GF120918	17.4 (11.6–26.0)	40.7 (16.9–98.0)
+ Verapamil	8.40 (5.01–14.1)	16.8 (10.7–26.4)
Maneb (μM)	N.D.[d]	40.3 (32.7–49.6)
+ GF120918	24.6 (13.9 – 43.7)	37.7 (30.0–47.3)
+ Verapamil	N.D.[d]	39.2 (32.9–46.7)
MPP^+ (μM)	27.1 (16.7–43.9)	418 (170–1030)***
+ GF120918	84.9 (37.1–194)	566 (299–1070)
+ Verapamil	31.9 (18.1–56.2)	69.0 (39.4–121)††
Rotenone (nM)	12.5 (7.9–19.8)	27.7 (21.7–35.3)**
+ GF120918	17.4 (12.7–23.8)	35.0 (23.5–52.1)
+ Verapamil	5.89 (3.97–8.73)	7.24 (4.50–11.6)†††

N.D., not determined.
[a]Significant differences between cell types in the xenobiotic treatment alone group; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
[b]Significant differences within a cell type between the xenobiotic treatment alone group and in the presence of a P-gp inhibitor; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$.
[c]Doxorubicin and colchicine have been reported previously (Lacher et al., 2014).
[d]Nonlinear regression was unable to estimate confidence intervals.

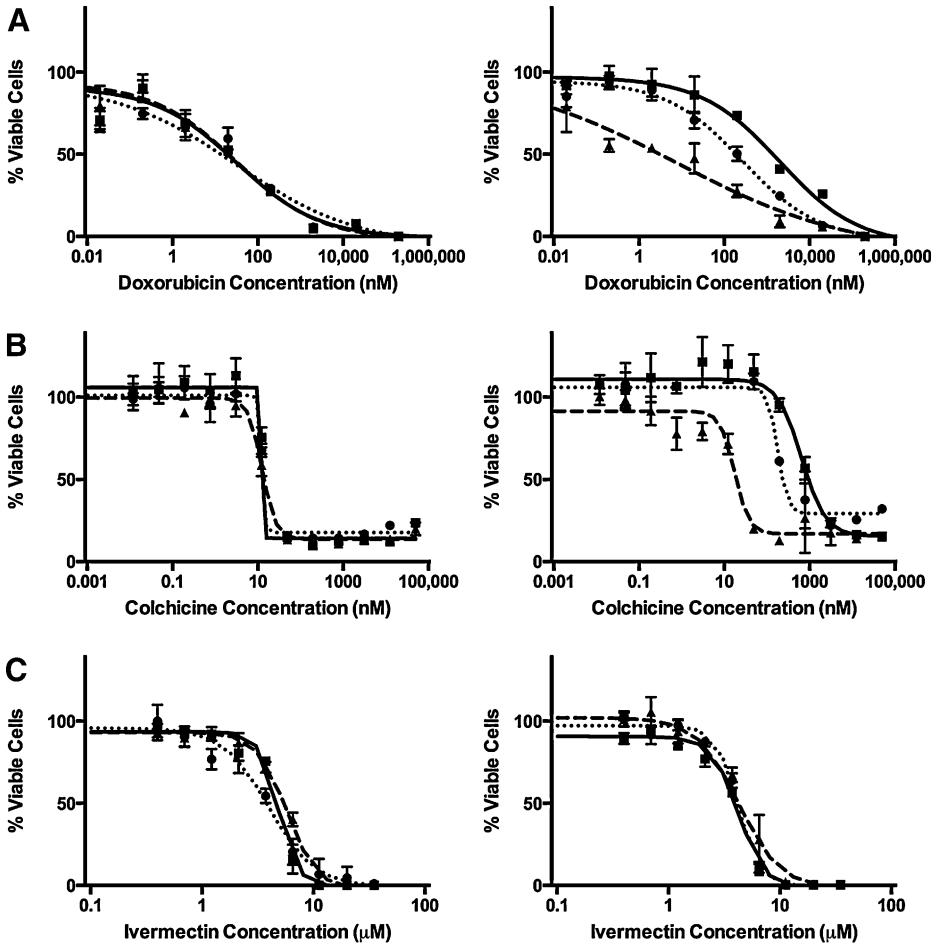

Fig. 2. P-gp substrate cytotoxicity in LLC-vector and LLC-MDR1-WT cells. Cell viability was tested in the presence of doxorubicin (A), colchicine (B), and ivermectin (C). LLC-vector (left column) and LLC-MDR1-WT cells (right column) treated over a range of substrate concentrations alone (square, solid line) or in the presence of the P-gp inhibitors GF120918 (triangle, dashed line) or verapamil (circle, dotted line). Compounds were tested in triplicate (n = 3) at each concentration point; data are represented as mean ± S.D.

control cells (Fig. 4). A fold change greater than one indicates P-gp–mediated resistance (red), and a fold change of one (white) indicates no difference in cellular resistance. To confirm that an increase in resistance was due to P-gp transport, compounds were cotreated with P-gp inhibitors, leading to a decrease in the fold change. LLC-MDR1-WT cells were significantly more resistant to doxorubicin and colchicine than LLC-vector control cells with 61.4- and 48.5-fold increases in EC_{50} values, respectively. Importantly, the P-gp inhibitors GF120918 and verapamil reversed the resistance to doxorubicin and colchicine, verifying resistance was mediated by P-gp. We observed more modest increases in resistance to MPP^+ and rotenone in LLC-MDR1-WT cells compared with LLC-vector cells (15.4- and 2.2-fold, respectively), and this resistance to MPP^+ and rotenone was reversed in the presence of verapamil, but not by GF120918, displaying an inhibitor-specific reversal of resistance. LLC-MDR1-WT cells were slightly more resistant to dieldrin than LLC-vector cells (1.5-fold); however, although verapamil had a small effect on increasing sensitivity to dieldrin, this was observed in both LLC-MDR1-WT and LLC-vector cells, indicating that the effect was not mediated by P-gp. We observed no differential resistance to diazinon, endosulfan, maneb, and ivermectin between LLC-MDR1-WT and LLC-vector cells, and GF120918 and verapamil had no effect on cytotoxicity. Together, these data indicate that P-gp mediates cellular resistance to MPP^+ and rotenone.

To evaluate if the pesticides were P-gp inhibitors, we measured their ability to inhibit R123 efflux in LLC-vector and LLC-MDR1-WT cells and compared them to the known P-gp inhibitors GF120918, verapamil, and cyclosporine (Fig. 5). The test compounds had no effect on R123 intracellular accumulation in LLC-vector cells (data not shown). Verapamil, cyclosporine, and GF120928 produced 100% inhibition of R123 transport, with IC_{50} values of 1.98 ± 0.12 μM, 1.39 ± 0.07 μM, and 22.9 ± 1.9 nM, respectively [data reported previously (Lacher et al., 2014)]. Of the pesticides tested, only ivermectin, which is not associated with Parkinson's disease, inhibited R123 efflux, with an IC_{50} value of 0.249 ± 0.048 μM. Diazinon, dieldrin, endosulfan, maneb, MPP^+, and rotenone failed to inhibit R123 accumulation in P-gp–expressing cells and are not P-gp inhibitors.

Discussion

P-gp transport may be a protective mechanism in the development of neurodegenerative diseases by effluxing neurotoxic compounds from the brain. We screened a panel of pesticides associated with Parkinson's disease, diazinon, dieldrin, endosulfan, maneb, MPP^+, and rotenone using three different P-gp transport models (summarized in Table 3). We identified diazinon, MPP^+, and rotenone as P-gp substrates. None of the pesticides were P-gp inhibitors. These findings are interesting in light of our previous work to demonstrate that

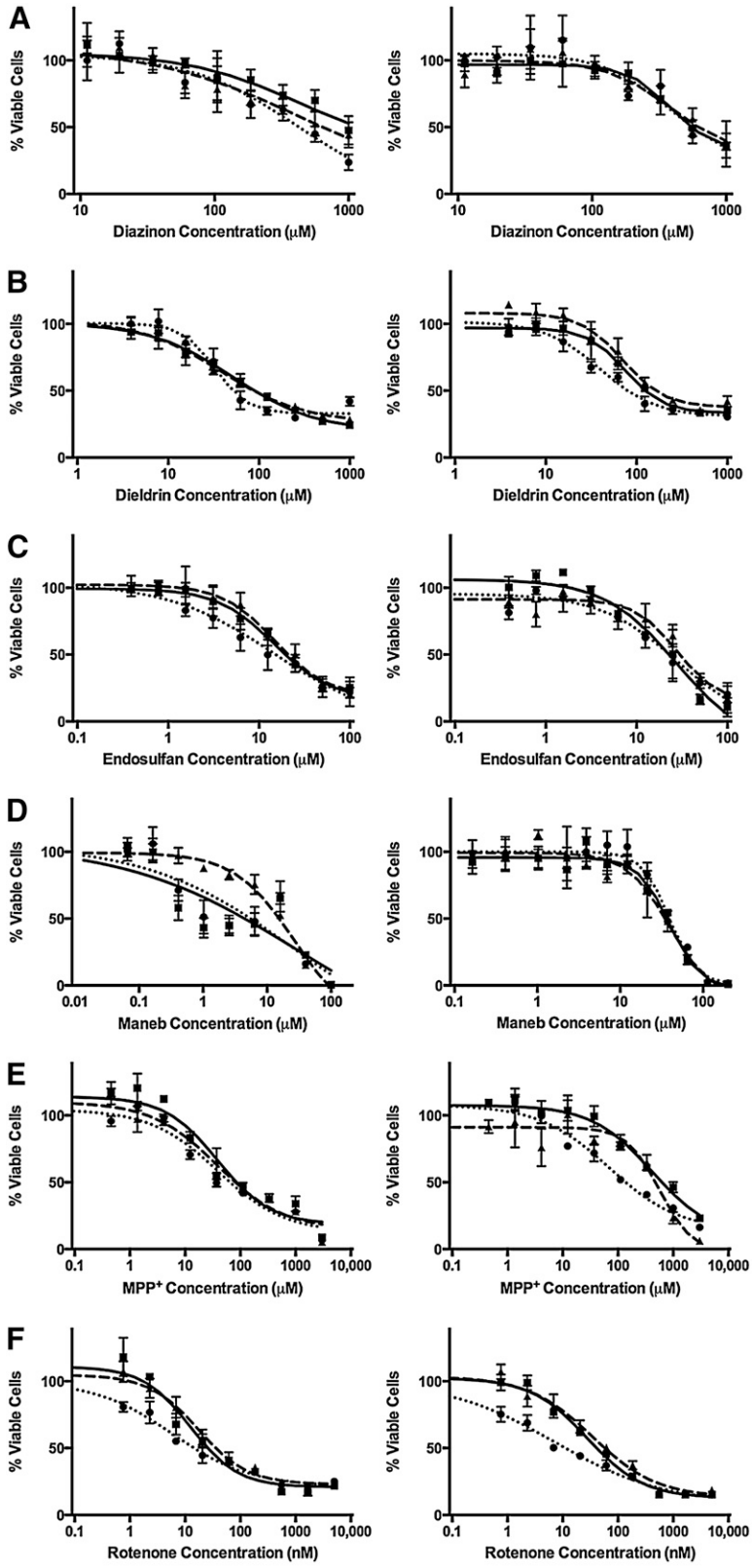

Fig. 3. Parkinson's disease–associated pesticide cytotoxicity in LLC-vector and LLC-MDR1-WT cells. Cell viability was tested in the presence of diazinon (A), dieldrin (B), endosulfan (C), maneb (D), MPP^+ (E), and rotenone (F). LLC-vector (left column) and LLC-MDR1-WT cells (right column) treated over a range of pesticide concentrations alone (square, solid line) or in the presence of the P-gp inhibitors GF120918 (triangle, dashed line) or verapamil (circle, dotted line). Compounds were tested in triplicate ($n = 3$) at each concentration point; data are represented as mean ± S.D.

paraquat, also a risk factor in Parkinson's disease, is not a P-gp substrate or inhibitor (Lacher et al., 2014).

Pesticide stimulation of P-gp ATPase activity was measured in a membrane-based system. Diazinon and rotenone both stimulated ATPase activity, indicating that they are P-gp substrates. Dieldrin, endosulfan, maneb, and MPP^+ did not stimulate ATPase activity. Although the estimates of V_{max} were similar between diazinon and rotenone, the K_m values

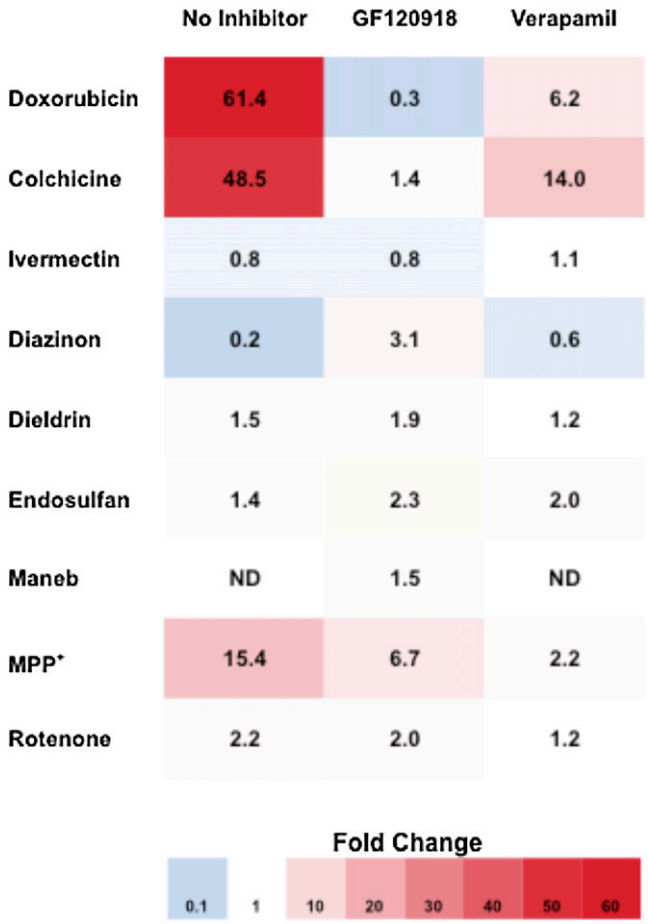

Fig. 4. Visual representation of the fold change in cellular resistance to cytotoxic agents in LLC-MDR1-WT cells compared with LLC-vector cells. LLC-MDR1-WT cells display increased cellular resistance to P-gp substrates compared with LLC-vector cells, leading to fold changes greater than one (red). No difference between cellular sensitivities results in a fold change of one (white). A fold change of less than one (blue) indicates a result that is not P-gp mediated. Compounds were tested alone or in the presence of P-gp inhibitors (GF120918 and verapamil).

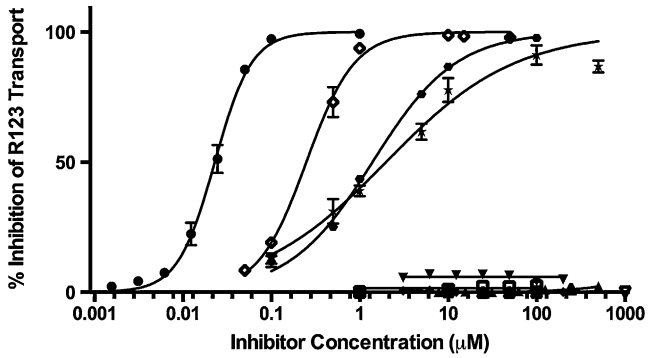

Fig. 5. Inhibition of rhodamine-123 transport by pesticides. Percentage inhibition of rhodamine-123 transport was evaluated in LLC-MDR1-WT cells in the presence of diazinon (closed triangle), dieldrin (closed upside down triangle), endosulfan (closed diamond), maneb (open triangle), MPP^+ (open upside down triangle), rotenone (open square), ivermectin (open diamond), GF120918 (closed circle), verapamil (closed star), and cyclosporine (closed hexagon). GF120918, verapamil, and cyclosporine have been previously reported (Lacher et al., 2014). Compounds were tested in triplicate (n = 3) at each concentration point; data are presented as mean ± S.D.

differed significantly. Diazinon has a K_m more than 10-fold higher verapamil, indicating a weak binding affinity for P-gp. The K_m for rotenone was comparable to verapamil, suggesting that rotenone binds P-gp with a similar affinity to a known substrate.

One limitation of ATPase assays is that slowly transported substrates may not stimulate ATPase activity (Giacomini et al., 2010; Bircsak et al., 2013); therefore, we also used a cell-based model to screen pesticides as P-gp substrates. We evaluated P-gp transport of pesticides using recombinant LLC-vector and LLC-MDR1-WT cells. LLC-PK1 cells are commonly used to study P-gp transport (Giacomini et al., 2010; Brouwer et al., 2013; Hillgren et al., 2013; Zamek-Gliszczynski et al., 2013). We used known P-gp substrates and inhibitors as positive controls to validate the cellular models. As expected, a dramatic increase in resistance was observed in LLC-MDR1-WT cells compared with LLC-vector cells for the classic P-gp cytotoxic substrates doxorubicin and colchicine (61.4- and 48.5-fold, respectively), and resistance was reversed in the presence of the P-gp inhibitors GF120918 and verapamil. Increased resistance was also observed in LLC-MDR1-WT cells to the pesticides MPP^+ and rotenone (15.4- and 2.2-fold, respectively). Interestingly, there was an inhibitor-specific ability to reverse the resistance. GF120918 did not have any effect on the resistance, whereas verapamil increased the sensitivity to both pesticides in LLC-MDR1-WT cells. Therefore, MPP^+ and rotenone were classified as P-gp substrates from the cytotoxicity data. Although diazinon stimulated ATPase activity, we did not observe an increased resistance in LLC-MDR1-WT cells or alterations in cytotoxicity by P-gp inhibitors. In fact, the LLC-vector cells appear to be slightly more resistant to diazinon than LLC-MDR1-WT cells, which could be due to compensatory alterations in the LLC-MDR1-WT cells, such as upregulation of an uptake transporter for diazinon. There was a slight increase in resistance to dieldrin (1.5-fold); however, while verapamil modestly increased sensitivity in LLC-MDR1-WT cells, it also increased the sensitivity in LLC-vector cells, indicating that dieldrin is not a P-gp substrate. We observed no significant differences in cellular sensitivities to endosulfan, and neither P-gp inhibitor had an effect on cytotoxicity in the LLC-MDR1-WT cells. Although LLC-MDR1-WT cells appear slightly more resistant to maneb, the P-gp inhibitors had no effect on cytotoxicity. Together with the results of the ATPase assay, these data confirm endosulfan and maneb are not P-gp substrates. We also screened the pesticides associated with Parkinson's disease as potential inhibitors of P-gp efflux of R123. None of the pesticides were

TABLE 3
Summary of membrane-based and cell-based assays to evaluate Parkinson's disease–associated pesticides as P-gp substrates and inhibitors

	Membrane-Based Assay	Cell-Based Assay	
	ATPase	Cytotoxicity	R123 Inhibition
Diazinon	✓	—	—
Dieldrin	—	—	—
Endosulfan	—	—	—
Maneb	—	—	—
MPP^+	—	✓	—
Rotenone	✓	✓	—

able to inhibit R123 efflux in LLC-MDR1-WT cells, even at high concentrations.

We identified rotenone as a P-gp substrate in both membrane- and cell-based models, which strongly suggests that rotenone is a P-gp substrate and may provide a mechanistic link between P-gp transport at the BBB and Parkinson's disease risk. Rotenone stimulated ATPase activity with a K_m similar to that of the known P-gp substrate verapamil. We also observed differential cytotoxicity to rotenone in LLC-MDR1-WT cells compared with LLC-vector cells, and this resistance was reversed in the presence of verapamil, further suggesting that rotenone is a P-gp substrate. MPP^+ did not stimulate ATPase activity, but we did observe differential cytotoxicity to MPP^+ between LLC-MDR1-WT and LLC-vector cells that was reversed by verapamil, suggesting that it is a P-gp substrate. ATPase methods can give false negatives (Giacomini et al., 2010; Bircsak et al., 2013), especially with slowly transported substrates, which may explain why we did not detect ATPase stimulation by MPP^+. Diazinon, on the other hand, stimulated ATPase activity, but did not lead to altered cytotoxicity. The observed K_m for diazinon in the ATPase assay was approximately 40-fold lower than the diazinon concentrations used in the cytotoxicity assay. This suggests that diazinon concentrations necessary to induce cytotoxicity in LLC-PK1 cells may be in the nonlinear range of P-gp transport and further cell-based studies may be needed.

Our study is the first to evaluate the P-gp transport of rotenone and maneb. Although there have been sparse reports about P-gp transport of the other pesticides under study, results have been mixed and often contradictory (Bain and LeBlanc, 1996; Bleasby et al., 2000; Martel et al., 2001; Lecoeur et al., 2006; Pivčević and Zaja, 2006; Sreeramulu et al., 2007; Bircsak et al., 2013). Therefore, our study is important to clarify the role of P-gp in the transport of these pesticides. An example of a lack of consistency in the literature includes the results for endosulfan (Bain and LeBlanc, 1996; Sreeramulu et al., 2007; Bircsak et al., 2013). Another issue in interpreting previous studies is the lack of appropriate controls. For example, previous reports have identified pesticides as modest substrates in P-gp–expressing cells lines but did not use P-gp inhibitors to confirm the effects were P-gp mediated (Bain and LeBlanc, 1996; Lecoeur et al., 2006). Our study clearly demonstrates the importance of using P-gp inhibitors to interpret results in recombinant cell lines. We showed that LLC-MDR1-WT cells are more resistant to maneb; however, since P-gp inhibitors do not reverse the resistance, we can disregard maneb as a P-gp substrate. Additionally, the use of a vector control cell line is critical. For example, verapamil appears to slightly reverse resistance to dieldrin in LLC-MDR1-WT cells, but we observed the same phenomenon in LLC-vector cells, which also eliminates dieldrin as a P-gp substrate. Our approach of screening pesticides with three in vitro models and proper controls provides confidence in our observations regarding the P-gp transport of pesticides.

Although not linked to Parkinson's disease, we also screened the neurotoxic pesticide ivermectin since P-gp is thought to play a role in its in vivo brain disposition. Studies that support ivermectin as a P-gp substrate are in *mdr1a* knockout mice (Schinkel et al., 1994) and collie dogs with a natural deletion of the *mdr1* gene (Mealey et al., 2001, 2003; Edwards, 2003; Nelson et al., 2003; Roulet et al., 2003) that were more sensitive to ivermectin. Interestingly, there has been little direct in vitro investigation of ivermectin as a P-gp substrate other than a previous study that identified ivermectin as an inhibitor, not a substrate, of P-gp (Bain and LeBlanc, 1996). Our results support these findings. We found that ivermectin is a good inhibitor of P-gp, but that it did not stimulate ATPase activity or contribute to differential cytotoxicity in P-gp–expressing cells, indicating that it was not transported by P-gp. Therefore, the role of P-gp in ivermectin neurotoxicity is likely more complicated than what has been previously predicted and may merit further study.

Our goal was to comprehensively study the P-gp transport of the key pesticides associated with Parkinson's disease to provide a potential mechanism for the role of P-gp in neurodegenerative diseases. An important aspect of our work was to use a combination of in vitro P-gp transport models, which is important in studying xenobiotic interactions with membrane transporters (Polli et al., 2001; Feng et al., 2008; Giacomini et al., 2010; Brouwer et al., 2013; Hillgren et al., 2013; Zamek-Gliszczynski et al., 2013). Our strategy also allowed us to prioritize which pesticides require further study to understand the role of P-gp in their brain accumulation. Further investigation of diazinon, MPP^+, and rotenone using in vitro transepithelial transport models and in vivo P-gp–deficient mice models, similar to our work with paraquat (Lacher et al., 2014), will be valuable. One of the limitations of in vitro transport models using recombinant cells is the possibility that endogenous uptake transporters may complicate data interpretation. Little is known about the uptake transporters of these pesticides. Organic cation transporters are thought to mediate MPP^+ uptake in humans and rodents (Ahmadimoghaddam et al., 2012; Belzer et al., 2013; Ingoglia et al., 2015; Wu et al., 2015), and a organic anion transporting polypeptide may be involved in diazinon uptake in zebrafish (Popovic et al., 2014). However, it is likely that other uptake transporters of pesticides will be identified in the future.

In summary, we have screened for P-gp transport of the key neurotoxic pesticides linked to Parkinson's disease using three different in vitro transport models and identified diazinon, MPP^+, and rotenone as P-gp substrates. Our data also highlight the value in using multiple models to assess P-gp transport. Further work is needed to determine if P-gp limits the brain distribution of diazinon, MPP^+, and rotenone in vivo. Although we selected these pesticides because of their link to Parkinson's disease, the relevance of P-gp in pesticide-associated toxicity may have a much broader impact because of the wide distribution of P-gp in tissues important in xenobiotic disposition. Therefore, it is important to characterize the P-gp transport of pesticides associated with a spectrum of human diseases.

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Authorship Contributions

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Performed data analysis: Lacher, Skagen, Veit, Dalton, Woodahl.

Wrote or contributed to the writing of the manuscript: Lacher, Skagen, Veit, Dalton, Woodahl.

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