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Evidence Project Final Report

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Project identification

1. Defra Project code: PS2608

2. Project title: An Examination and Analysis of the Role of Paraquat and Maneb in Induced Neurodegeneration of the Substantia Nigra at Representative Human Exposure Doses

3. Contractor organisation(s): Exponent, Inc.

4. Total Defra project costs (agreed fixed price): £906552.00

5. Project: start date ..... March 2006
   end date ..... November 2012

6. It is Defra's intention to publish this form.

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Objectives: The aim of this project is to determine whether developmental and/or adult exposure to a combination of the pesticides paraquat (PQ) and maneb (MB) results in movement abnormalities and fewer tyrosine hydroxylase (TH) staining neurons within the substantia nigra (SN), a brain region that shows loss of TH+ neurons in human Parkinson's Disease. A proposed mouse model reported motor effects and fewer TH+ neurons in the SN after combined early postnatal (pup) exposure plus adult exposure to combined PQ and MB (Thiruchelvam *et al*. 2002). However, the dose administered in this previous study is close to the maximum tolerated dose, and much greater than human occupational exposures, so the relevance of that high dose to humans should be examined. Also, the mouse model protocol suggests evaluation at age 8 months when the mice are still young adults, whereas PD is a disease that appears most often in middle-aged or elderly people. The current two studies used the same animal model but extended the testing parameters to make the studies more relevant to humans: Study 1, modelling human exposure to paraquat with a physiologically based pharmacokinetic (PBPK) model, so that human-relevant doses could be determined; and Study 2, conducting a mouse study that incorporated functional activity testing, neuropathology, and stereology at 8 months and again at 16 months to determine whether any motor symptoms or neurotoxicity seen at 8 months progressed over time, consistent with a neurodegenerative disease process.

Study 1, PBPK model: Commercial Gramoxone, a PQ formulation, was spiked with radiolabelled PQ at a dose specific to the route of exposure, and tested for good detection in a pilot study. MB was administered using the formulation Hi-Yield. Intraperitoneal (i.p.) doses used technical compounds, not formulations, and the MB was administered in the 1:3 PQ:MB proportion used in the Thiruchelvam (2002) study on which the current in-life study protocol was based. Adults were 5 months old, and pups were 5 days old at dosing. The parameters for the PBPK model were taken from existing literature that describes adult mouse, juvenile mouse, and human physiological parameters (i.e., typical body weight, fraction of the body represented by the blood or brain, cardiac output, etc.). The PQ-specific parameters were measured, estimated from the data, or described in scientific literature (i.e., oral uptake rate, proportion of PQ that sequesters in blood or brain tissues *in vitro*, dermal permeability rate, tissue binding and release rates, urinary clearance, etc.). Altogether, the physiological parameters that affect distribution of a compound in the body and brain, and the parameters that describe the distribution of PQ in a mammalian body, were compiled into an equation, and the output of the equation was the model prediction for PQ distribution over time in an intact animal. This PBPK model was constructed using PQ parameters, and a duplicate set of data was taken using PQ+MB to determine whether the presence of MB changed the PQ kinetic parameters.

At a designated time after dosing, the animals were sacrificed and the amount of PQ in blood and brain was measured. The results were then plotted on a graph that extended over 24 hours. Metrics that compared the time course for PQ alone, PQ+MB, and the PBPK model prediction, showed that MB did not affect the absorption, distribution, or excretion of PQ, and that the model generally predicted the PQ data measurements well. The model was therefore considered acceptable, and was used to calculate the brain dose that existing literature suggests was received by human field workers; the model was then used to back-extrapolate from that human dose to an equivalent mouse dose, and used in the following *in vivo* study.

Study 2, *in vivo* mouse study: This study was designed to examine long-lasting effects of combined PQ+MB exposure using a protocol of 10 mg/kg PQ + 30 mg/kg MB by i.p. injection in pups, adults, or both ages, sacrifice at 8 months of age, and examination of motor activity and TH+ neuron counts. This study also used a low dose calculated from the PBPK model to produce brain PQ concentrations similar to exposed human field workers wearing tropical clothing (T-shirt, shorts and sandals), and a middle dose calculated from the Pesticide Operator Exposure Model to be representative of professional formulations handled by workers without personal protective equipment. Additionally, one group of animals was scheduled for sacrifice at 16 months of age to determine if effects seen at 8 months progressed at 16 months, consistent with a disease model. Pups were given daily injections on postnatal days 5-19 (15 doses), and adults were dosed twice weekly between 6.5 and 7.5 months of age (7 doses). Animals were scored for their movement on an open platform, and tested for motor activity at 7.5 months of age, and three of four groups were sacrificed at 8 months of age. The old group was tested again at 15 months of age, then sacrificed at 16 months. Brains were examined for glial cell activation (GFAP staining), neuronal membrane degeneration (silver staining), and TH+ neuron counts in the SN using stereology.

The essential criterion for an animal model of PD is selective loss of dopaminergic neurons in the SN. The major findings of this study are related to the observed mortality in high-dose males during adult dosing, and a decrease of 17% and 20% in TH+ neurons of the SN in high- and mid-dose males, respectively, that had been treated both as pups and as adults, and sacrificed at 16 months of age (no effects were seen at 8 months). Although the main effect of treatment with PQ+MB in high-dose males was significant by ANOVA, the post-hoc dose group comparisons with the concurrent controls were not statistically significant. The main effect of dose was also not statistically significant when PND 4 body weight and litter size were used as co-variates in the ANOVA. In other words, when pre-dose pup body weights were included in the analysis as a covariate, the main effect of PQ+MB exposure on number of TH+ neurons for 16 month old males was no longer statistically significant. These study results are considered equivocal based on non-random pup allocation to the treatment groups, and high variability and coefficient of error in the stereology data. Males are clearly more sensitive to the effects of PQ+MB treatment than females in the indices examined, and the high dose was at or above the maximum tolerated dose based on mortality and lung pathology.

Possibilities for further work include following up on the observation of the speed and high proportion of dose that was detected in the brain after intranasal exposure during the PBPK model study, which was ten-fold higher than the model predicted based on systemic blood flow. The explanation for under prediction in a PBPK model is that there is a significant route of tissue exposure that is unknown to the model. In this case, we hypothesized an additional and unexpected route of exposure in the nose that delivered ten times as much PQ to the brain as the systemic blood circulation. This preliminary observation has implications for exposure of humans to any neuroactive or neurotoxic substances that deposit in the nasal cavity during inhalation, including but not limited to pesticides. Second, it will be important to repeat the *in vivo* study with 16 month old males to determine if the reduced number of TH+ neurons at the middle dose level is consistent. If so, there are implications at dose levels that are at the top range of human-relevant doses.

Reference: Thiruchelvam, M., E. K. Richfield, et al. (2002). "Developmental exposure to the pesticides paraquat and maneb and the Parkinson's disease phenotype." Neurotoxicology **23**(4-5): 621-633.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:

- the objectives as set out in the contract;
- the extent to which the objectives set out in the contract have been met;
- details of methods used and the results obtained, including statistical analysis (if appropriate);
- a discussion of the results and their reliability;
- the main implications of the findings;
- possible future work; and
- any action resulting from the research (e.g. IP, Knowledge Exchange).

Objectives

The aim of the project is to determine whether developmental and/or adult exposure to a combination of paraquat (PQ) and maneb (MB) results in movement abnormalities and fewer tyrosine hydroxylase staining (TH+) neurons within the substantia nigra, a brain region that shows loss of TH+ neurons in human Parkinson's disease (PD). A proposed mouse model reports motor effects and fewer TH+ neurons in the substantia nigra after combined early postnatal exposure plus adult exposure to PQ and MB (Thiruchelvam *et al.* 2002). However, the dose administered in these previous studies is close to the maximum tolerated dose, and much greater than human occupational exposures, so the relevance of that high dose to humans should be examined. Also, the animal model protocol suggests evaluation at age 8 months when the mice are still young adults, whereas PD is a disease that appears most often in middle-aged or elderly people. The current two studies used the same animal model but extended the testing parameters to make the studies more relevant to humans: Study 1, modelling human exposure to paraquat with a physiologically based pharmacokinetic (PBPK) study, so that human-relevant doses could be determined; and Study 2, designing a mouse study that incorporated functional activity testing, neuropathology, and stereology at 8 months and again at 16 months to determine whether any motor symptoms or neurotoxicity seen at 8 months progressed over time, consistent with a neurodegenerative disease process.

Notice: the protocol for this mouse model was published by Mona Thiruchelvam and co-authors in a series of studies beginning in 2000. Since then, other authors have used components of the Thiruchelvam protocol for independent studies. However, in 2012, after completion of this present study, the U.S. Office of Research and Development announced that stereologic cell count experiments carried out between 2004 and 2006 at the University of Medicine and Dentistry of New Jersey by Dr. Thiruchelvam were fabricated using falsified methods [Federal Register, 2012, 77 [125]:38632–3]. The retraction by Dr. Thiruchelvam of two papers published in 2005 and a grant submitted in 2007 casts doubt on earlier work (e.g. Thiruchelvam *et al.* 2002) that was not investigated. This report fulfils Exponent's original agreement with UK DEFRA to compare results of our study with that of Thiruchelvam *et al*. (2002). However, this present study should be considered primarily as a stand-alone study, and caution should be used in placing too much emphasis on comparisons with Thiruchelvam *et al*. (2002). Other laboratories using different protocols but the same high dose of PQ (10 mg/kg-day) or PQ+MB (10 and 30 mg/kg-day, respectively) have reported decreases in TH+ cells in mice or rats (McCormack and Di Monte 2003; Cicchetti *et al.* 2005; McCormack *et al.* 2005; Saint-Pierre *et al.* 2006; Kachroo *et al.* 2010). Thus, there is a good scientific basis to study the potential effects of PQ+MB independent of the papers published by Dr. Thiruchelvam.

Study 1: Modelling Exposure

Purpose

This study was designed to generate data on which to fit a PBPK model of human field exposures to PQ, using the brain as the target organ. Because children may also be exposed to pesticides brought into the home by adults, neonatal and adult mice were both used in the modelling. Adult mice were given single doses of PQ according to the three routes of exposure that are relevant to human field conditions for pesticides (oral, dermal, and intranasal [corresponding to inhalation of large drops]), and additionally to the intraperitoneal (i.p.) route of exposure commonly used in controlled laboratory studies with mice. Post-natal day 5 (PND 5) mice were dosed by i.p. and oral routes only. A second set of animals was dosed concurrently with PQ+MB to evaluate whether the presence of MB changed the PQ kinetics, but those data were not used to fit the PBPK model. The validated PBPK model was then used to make predictions about the amount of PQ reaching the brain under different exposure routes, and could be used to extrapolate between routes of exposure and species. In this case, the model was used to determine the brain PQ concentration from a worst-case human dermal field exposure, then to extrapolate to the i.p. mouse dose that would produce the equivalent brain PQ concentration. The use of this PBPK model therefore predicted an injection dose in a mouse model that would produce approximately the same level of pesticide in the brain as the dermal exposures in human field workers described in the published literature. This use of the PBPK model is one approach to examining the relevance of animal PQ studies to human exposures.

Introduction

The publishedstudies showing neurodegeneration after exposure to pesticides are usually high-dose studies designed to examine a selective mechanism of action and pattern of response, or to show whether exposure

creates an animal model of a disease. In their 2006 review of the studies examining a possible link between pesticides and PD (Brown *et al.* 2006), Brown and co-authors from the UK's Medical Research Council Institute for Environment and Health comment that they could not identify any study that administered pesticides at a level comparable to that experienced by pesticide users, or were relevant to routes of exposure experienced by pesticide users (oral, inhalation, or dermal exposure, as opposed to i.p. injection). Therefore, Brown *et al*. considered it difficult to interpret the relevance of high-dose animal studies for human levels of exposure.

Literature exists for human field exposure to PQ that helps our understanding of realistic human exposure levels. To compare human exposure to the results for PQ+MB i.p. exposure reported in the animal model literature, one available approach is to construct a PBPK model for PQ distribution in humans and in the laboratory species most often used for PD research, the C57Bl/6 mouse. PBPK models can be used to extrapolate doses across species and routes of exposure by creating a dosimeter for specific target tissue(s). The current study therefore evaluated the internal dose that reaches the brain using described routes of exposure that are relevant to humans or animal models, allowing estimation of equivalent doses. Male mice only were used to create the adult model, based on reported greater male susceptibility to PQ toxicity after adolescence (Cory-Slechta *et al.* 2005), but both male and female pups were used to create the model for PND 5 mice.

The physical and chemical properties of PQ, and fate and behaviour relevant to mammalian toxicology and the environment, are described in the European Union's "Review report for the active substance paraquat" (European Commission, 2003), in EPA's re-registration document for paraquat in 1997 (EPA 1997), and in the EPA's more recent risk assessment in 2006 (EPA 2006). PQ is not registered for residential use in Europe or the US, but mixers, loaders, and applicators have been exposed and monitored during agricultural use, and are therefore models for the higher end of the range of likely human exposure, as described in the public literature.

In Europe, the review report for the active substance MB indicates that current practices are protective of operators, workers, bystanders, and consumers (adults and toddlers) (European Commission 2005). MB was considered for 40 commercial uses at its EPA re-registration in 2005 (EPA 2005), although the EPA did not register MB for any residential use.

PQ and MB generally are not used simultaneously in time because of their different indications for use, although they can be used in overlapping geographic areas. Commercially, PQ is a broad-spectrum herbicide used to clear weeds or non-crop plant structures; for example, the practice of desiccating above-ground potato leaves before harvesting the below-ground tubers. MB is used to prevent fungal damage to crops in the field, and to protect harvested crops from deterioration during storage. Simultaneous exposure to both PQ and MB could be hypothesized from working at different job sites during a single day, or living in an area where both pesticides are in use; however, the likelihood of this occurrence was not evaluated in this study. The population with the greatest potential for exposure is pesticide applicators. A full applicator exposure assessment for Gramoxone, the field-strength formulation of PQ often used by professional applicators, was calculated by the UK Pesticide Safety Directorate, and is described in Directive 91/414/EEC, finalized in 2003 (Pesticides Safety Directorate 1996). The primary route of PQ exposure is dermal, based on deposition of pesticide droplets on the skin during spraying, or during mixing of concentrate. The EPA's 1997 Reregistration Eligibility Decision used dermal exposure exclusively to assess risk to PQ handlers. Dermal exposure was therefore considered the basis for extrapolation between human field applicator exposures and mouse i.p. injection doses in this PBPK model.

To further increase the applicability of this study to human-specific exposure conditions, the sources of the PQ and MB used in the PBPK model doses in the adult mice were common formulations used in the field. Commercial formulations include surfactants, stabilizers, and other ingredients that might affect the absorbance of PQ and MB across body surfaces, or might change the internal kinetics. However, the PQ and MB used for i.p. injections was the purified compound, because the route of exposure ensured that all of the compound passed body barriers.

This PBPK model focused on PQ parameters alone. A duplicate set of data was taken using PQ+MB to determine whether the presence of MB changed the PQ kinetic parameters. The MB was administered in the 1:3 PQ:MB proportion used by the Thiruchelvam (2002) study on which the current in-life study protocol was based.

Materials and Methods, PBPK Model

The commercial PQ Gramoxone formulation was spiked with radiolabelled PQ at a dose specific to the route of exposure (target dose for adults was 10 mg/kg for i.p. and oral routes, 3 mg/kg for intranasal, and 40 mg/kg for dermal, and the target dose for pups was 0.3 mg/kg for both oral and i.p. routes based on good detection at those levels in pilot studies). The formulation of MB was Hi-Yield. Both compounds were dissolved in sterile water for dermal, oral, and intranasal exposure routes. Both compounds were dissolved in isotonic saline for the i.p. exposure route.

Pups were born on site for the study of kinetics in early postnatal animals, and dosed at PND5. The adult males were dosed at 5 months of age, and were chosen because the growth curve has levelled off and the animals are

physiologically mature. Pups were dosed only by oral and i.p. routes to model the expected human exposure (oral) and compare to the experimental exposure typical of animal models (i.p.). Adults were dosed by the expected human exposure routes (dermal, oral, and intranasal) for comparison with i.p. exposure.

After dosing, the animals were sacrificed by an overdose of anaesthesia at a specified time to create a 24-hour time course. Three animals were used for each time point to determine the reliability and variability of data at that time point. After sacrifice, blood and brain samples were taken and measured for PQ radioactivity. Additionally, the lungs were removed and analyzed in animals treated by intranasal and i.p. exposure to determine whether intranasal exposure increased PQ in the lungs; lungs are a known target tissue for PQ toxicity.

The parameters for the PBPK model were taken from existing literature that describes adult mouse, juvenile mouse, and human physiologic parameters (i.e., typical body weight, fraction of the body represented by the blood or brain, cardiac output, etc.). The PQ-specific parameters were measured, estimated from the data, or described in scientific literature (i.e., oral uptake rate, proportion of PQ that sequesters in blood or brain tissues *in vitro*, dermal permeability rate, tissue binding and release rates, urinary clearance, etc.). Altogether, the physiologic parameters that affect distribution of a compound in the body and brain, and the parameters that describe the distribution of PQ in a mammalian body, were compiled into an equation, and the output of the equation was the model prediction for PQ distribution over time in an intact animal.

The strength of a model is judged by how well the prediction matches actual data. To determine how well the PBPK model predictions fit the data generated in the current study, the area under the curve (AUC) was used as the most biologically relevant metric. The AUC describes the rise and fall of PQ concentration in the blood or brain over time, and allows statistical analysis of individual time points using t-tests, or analysis of the entire AUC using analysis of variance. All calculations were done using *Mathematica* 8.0 and related *RiskQ* software.

Results, Data, and PBPK Model Fit

After verifying that the correct dose was administered, the amounts of PQ recovered from the blood, brain, and lung (when applicable) were plotted, and various kinetic parameters were calculated. Below is an example of the 24-hour time course of PQ and PQ+MB in the blood and brain of an adult male mouse after oral administration.

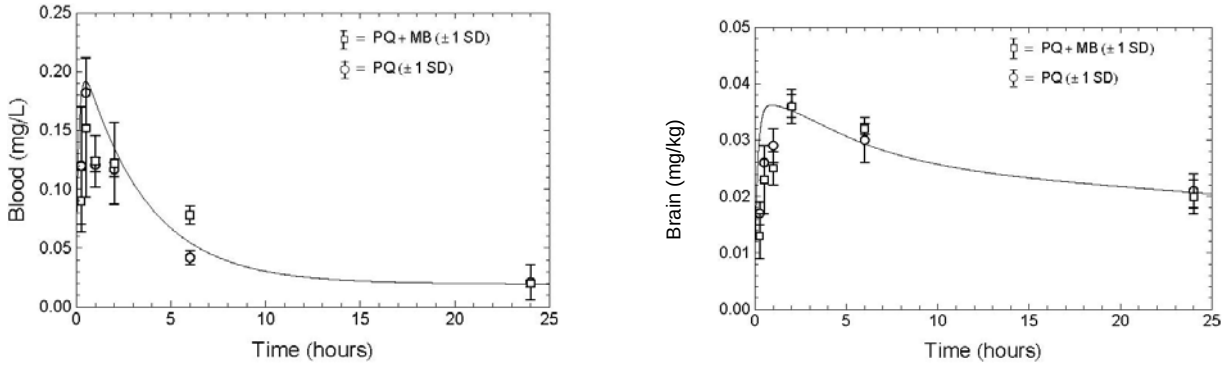


Figure 1. Adult pharmacokinetics of PQ in brain following an oral dose of 10 mg/kg. The model slightly overpredicts the initial peak of the data, but is generally consistent with both the shape and magnitude of the data. There is no significant effect of MB on PQ absorption; the 24-hour AUC for PQ-only is 0.645 μg-h/g, for PQ+MB is 0.653 μg-h/g, and for the model is 0.625 μg-h/g. N=3 for each time point.

In these figures, each circle or square represents the average of three measurements, and the vertical bar attached to the circle or square represents the amount of spread between the three measurements. The PBPK model prediction for PQ is drawn as a solid black line. In the left figure, the model predicts the rise of the blood PQ concentration well, and predicts a low level of PQ remaining at 24 hours, consistent with the PQ data. When the AUCs are calculated, the PQ-only AUC is 1.13 μg-hr/g tissue, the PQ+MB AUC is 1.52 μg-hr/g tissue, and the model predicts 1.14 μg-hr/g tissue for PQ only. These three AUCs are not different by statistical analysis, indicating that MB did not change the absorbance, distribution, and elimination of PQ in the blood over 24 hours, and that the PBPK model parameters correctly described the kinetics of PQ in the blood after oral exposure. In the right figure, the model again predicts the rise and fall of PQ concentration in the brain well, and agrees with the data that there is some residual amount of PQ in the tissue at 24 hours. The 24-hour AUC for the PQ-only data is 0.645 μg-hr/g, for PQ+MB is 0.653 μg-hr/g, and for the model is 0.625 μg-hr/g; there is no statistical difference between these numbers, showing no difference in PQ concentration with and without the presence of MB, and a good fit for the PBPK model.

Similar graphs and calculations were derived for blood and brain in adults after dermal, i.p., or intranasal exposure, for lung in adults after i.p. and intranasal dosing, and in PND5 pups after oral or i.p. dosing. In blood, there was no statistically significant difference between PQ-only and PQ+MB AUCs after i.p., oral, dermal, or

intranasal dosing in adults, or after i.p. or oral dosing in PND5 pups. This indicates that MB did not effectively increase PQ concentration in the blood or brain during concurrent exposure of the two pesticides, and that any MB effect would therefore be based on a different mechanism. Also in brain, there was no effect of MB on PQ concentration after i.p., oral, dermal, or intranasal administration, or after oral or i.p. dosing in PND5 pups.

The route of exposure most relevant to humans is dermal, based on contact between exposed skin and pesticide droplets during mixing or field application. The dermal data showed the most variability, probably because the skin is both a barrier to entry and a reservoir for prolonged entry into the bloodstream. The data in the dermal adult group were variable both in determination of the curve describing blood concentration, and in the spread between individual animals at each time point. A pilot study showed that dermal exposure in adults was not well described by a 24-hour curve, so additional animals were included in the main study to allow samples to be taken for up to 72 hours. At 36 hours, the blood PQ concentration was less than at 24 hours, but clinical signs of hypoactivity were seen in the animals, and two died. At 48 hours, all animals were sacrificed because they showed signs of distress, and the blood PQ concentration was higher than had been seen at 24 hours. This demonstrates that the dermal exposure curve is complex, and suggests that PQ may be released from the skin into the bloodstream well past the time of exposure unless it is removed. The PBPK model described the 24-hour kinetics well, but did not model the later kinetics well. The assumption used by the model was that a worker exposed to pesticides would wash the skin thoroughly at the end of the day and would therefore remove a possible skin reservoir of pesticide.

Intranasal exposure is not commonly used for PBPK modelling, so the parameters used were not well established and should be validated further. The nasal mucosa is highly vascularised, so penetration of pesticide was expected to be fast; intranasal exposure was therefore modelled as a “slow” component that passed the nasal epithelial barrier following the parameters of dermal absorption, and a “fast” component that entered the circulation directly from the nasal cavity. Adding a “fast” parameter to the model was necessary, because the blood concentration peaked at 15 minutes post-dosing, similar to a direct systemic injection. The PBPK model predicted the AUC relatively well, but because a high peak dose can result in acute toxicity not described by a cumulative measure like AUC, maximum concentration (C_{max}) and time to reach maximum concentration (T_{max}) were also calculated for comparison across routes of exposure. The following table of calculations from the brain data shows that PQ that contacts the body surface at the nasal cavity, as could happen if pesticide droplets were inhaled, results in the greatest proportion of compound reaching the brain, and that proportionally, oral exposure results in the least amount of PQ reaching the brain.

Adult brain PK parameters*

	Dermal (40 mg/kg)		Intranasal (3 mg/kg)		Intraperitoneal (10 mg/kg)		Oral (10 mg/kg)	
Parameter	Minus maneb	Plus maneb	Minus maneb	Plus maneb	Minus maneb	Plus maneb	Minus maneb	Plus maneb
C_{max} (µg/g)	0.479	0.573	1.33	3.28	0.298	0.410	0.036	0.036
T_{max} (h)	4	4	1	2	0.25	0.25	2	2
AUC br	6.26	6.77	23.4	36.8	4.03	4.64	0.64	0.65
(24-hr **data**)	1.08	1.37	4.33	32.7	0.82	0.31	0.05	0.02
(mg.h/L)								
AUCBR	4.39		24.8		3.98		0.625	
(24-hr **model**)								
(mg.h/L)								

*Note that these numbers are based on mg/kg dosed for each route of exposure and are not standardized for direct comparison in this table.

In the studies with PND5 pups, the presence of MB again did not affect PQ concentration in blood or brain, and the PBPK model predictions were not statistically different from the data. However, the kinetic parameters differed between pups and adults (data not shown). To determine whether pups receive a greater proportional dose to the brain than adults, the percent of dose that reaches the brain was calculated for adults and pups for the routes tested at each age.

Percent dose reaching the brain across different routes and ages

	Dermal		Intranasal		Intraperitoneal		Oral	
Age	Minus maneb	Plus maneb	Minus maneb	Plus maneb	Minus maneb	Plus maneb	Minus maneb	Plus maneb

Adult	0.011%	0.011%	0.526%	0.828%	0.027%	0.031%	0.0044%	0.0044%
PND 5	-	-	-	-	0.982%	0.882%	0.403%	0.622%

The greater amount of the dose that reaches the brain in pups by both i.p. and oral routes indicates that the blood/brain barrier and the gut are different in PND5 mouse pups than in adults, and therefore, that developmental stage should be considered when estimating the brain dose of PQ.

PBPK Model Provides Human-Equivalent Dose for *In Vivo* Mouse Study

The PBPK model created in this study is predictive of the blood and brain concentration of PQ in adult mice after oral, dermal, intranasal, and i.p. injection (the model parameters are not included here due to limited space, but a full report has been submitted to the sponsoring DEFRA office). Human parameters derived from the literature can be substituted into the validated PBPK model and were assumed to be predictive in humans. The PBPK model was then used to calculate how much PQ would penetrate into the blood from a single dermal dose to a field worker based on three criteria: 1) a tropical climate scenario for exposed skin (short-sleeved shirt, short trousers, and open shoes; no hat, face mask, or gloves for protection), 2) a 6-hour dermal exposure time after which the worker washes pesticide residue from the skin, and 3) exposure to field-strength Gramoxone (2 g PQ/L). From the human dermal dose, the PBPK model was used to calculate an AUC for human brain exposure. Then, using the PBPK model with mouse parameters, the human brain AUC was back-calculated to find the i.p. dose that would produce the same brain AUC in mice. The PBPK model calculated that 8.6×10^{-5} mg/kg PQ injected into an adult mouse would produce the same brain AUC as a human field worker whose head, arms, and legs were fully exposed to field-strength Gramoxone for 6 hours. The PBPK model therefore provided a human-relevant dose that could be tested in a described mouse model of PD.

Study 2: *In Vivo* PQ+MB Mouse Study

Purpose

This study was designed to examine long-lasting effects of combined PQ and MB exposure in a previously described protocol for a proposed animal model of PD (Thiruchelvam 2002). In the original protocol, there was one treatment dose of 10 mg/kg PQ + 30 mg/kg MB, the combination was administered to three groups of mice (pups, adults, or both ages), and the animals were sacrificed at 8 months of age. The study reported decreased motor activity and a selective decrease in the number of tyrosine hydroxylase-staining neurons (TH+, a marker for dopamine) in the substantia nigra pars compacta (SNpc), the brain nucleus that shows a decrease in TH+ staining of neurons in PD. In the current study, the highest of three doses was the same as the Thiruchelvam *et al*. (2002) study, and two lower doses were derived from human-relevant exposure models. To provide additional human relevance in the current study, a fourth group of animals—including males and females, all dose groups, treated both as pups and adults—was maintained for an additional 8 months and sacrificed at 16 months of age, to provide information on appearance or progression of treatment effects with aging. Standard study observations such as body weight, food intake, clinical signs and mortality, and gross pathology of all animals were recorded. Neurobehavioral effects were evaluated with automated motor activity testing and a functional observational battery in an arena. Neurotoxicity was evaluated using serial brain sections stained for activated glial cells (GFAP) or degenerating neuronal membranes (silver stain), and by counting TH+ staining neurons in the substantia nigra (SN) brain region using stereology.

Materials and Methods

All chemicals were technical grade, and were diluted into sterile saline.

These studies were carried out by laboratories that specialized in animal care and testing, histology, and stereology. Evaluations were carried out by trained personnel without knowledge of the treatment level.

There were three dose levels for animals treated with PQ+MB, and an equivalent group served as control and was dosed only with the saline vehicle. The high dose in adult mice was 10 mg/kg PQ, the dose most widely used in the literature, and reported by various groups to cause motor effects and changes in brain chemistry and pathology in rodents. In pups, the high dose was 0.3 mg/kg PQ, consistent with Thiruchelvam *et al*. (2002). The middle dose was calculated from the United Kingdom's Predictive Operator Exposure Model (UK POEM), now updated as the EUROPOEM, that uses representative professional formulations (not home-use formulations), lack of personal protective equipment, and measured exposure data, to estimate maximum exposure to a pesticide worker. The UK POEM estimated possible worker exposure to be 0.055 mg/kg-day. A ten-fold multiplier was added, and the middle dose was set in adults at 0.6 mg/kg, approximately an order of magnitude lower than the high dose. In pups, the middle dose was 0.06 mg/kg, ten-fold less than the adult dose. The low dose was set from the PBPK model, which calculated that 8.6×10^{-5} mg/kg PQ would produce brain exposure in a mouse approximately equivalent to brain exposure in a highly exposed human field pesticide applicator. Similar to the middle dose, a ten-fold magnifier was used to create the adult dose of 7×10^{-4} mg/kg in the study. Because the adult dose was quite low, the pup dose was the same as the adult dose. For context of these doses with current regulatory guidelines for allowable PQ exposure in the general population, the low dose is within an order

of magnitude of the FAO/WHO acceptable daily intake of 5×10^{-3} mg/kg (WHO 2003), the EU Acceptable Operator Exposure Level of 5×10^{-4} mg/kg (European Commission Health and Consumer European Commission 2003), and the EPA's RfD of 4.5×10^{-3} mg/kg/day (EPA 1997).

MB was combined with PQ in a 3:1 ratio to maintain the proportion used by the Thiruchelvam *et al* 2002 study.

Treatment schedules[a]

Treatment Group	Pre-Weaning Treatment		Adult Treatment		Termination
	Days	Injections	Weeks	Injections	(Week/month of age)
A	5–19	Daily (15 in total)	28–31	Twice weekly (7 in total)	70/16
B	5–19	Daily (15 in total)	28–31	Twice weekly (7 in total)	33/8
C	5–19	Daily (15 in total)	—	—	33/8
D	—	—	28-31	Twice weekly (5 in total)	33/8

[a] Changes in treatment schedules are described in the methods section

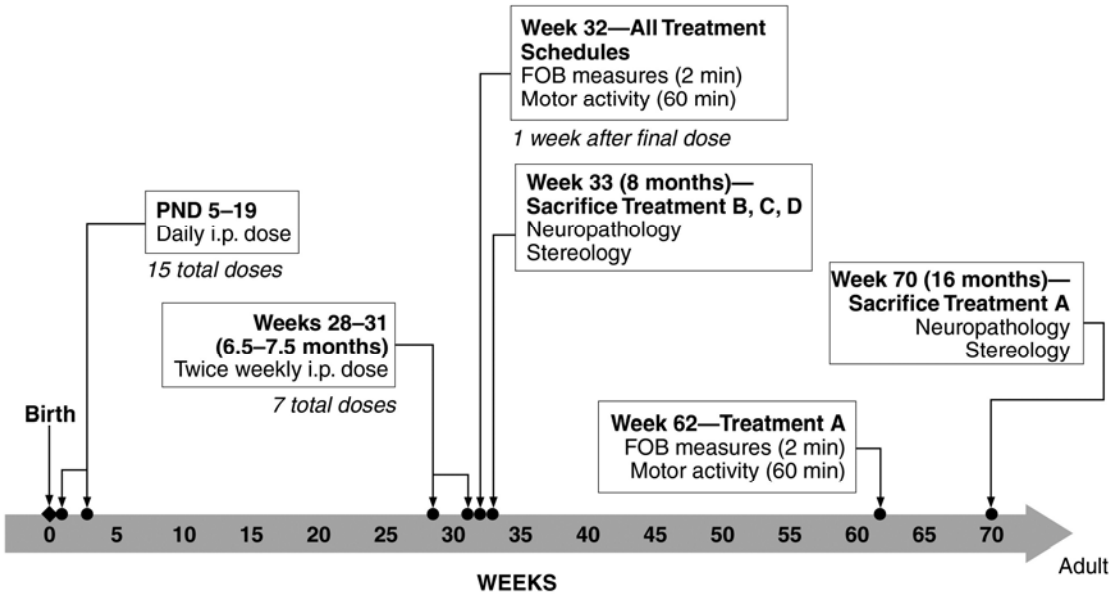
All study animals were maintained in a manner consistent with the United Kingdom's Scientific Procedures Act (1986). Ninety-nine mated female C57Bl/6J Crl mice of proven fertility were obtained, timed for receipt at 10–15 days' gestation. The animals arrived in four shipments spaced one week apart, to stagger dosing of the pups and testing of the pups as adults. The dams littered on site, and all pups within a litter received the same dose prior to weaning. At weaning, the pups were randomly assigned to one of three treatment schedules (A, B, or C) based on maintaining equal numbers of pups in all treatments as much as possible. Animals for treatment group D were brought on site at postnatal day 22 (PND 22). There were 12 pups per sex per dose per treatment group, derived from different litters, as much as possible. Overall, 9–12 litters were included in each group. The litter of origin was tracked for each animal, to ensure that litter could be included in the statistical analysis, as is standard for developmental neurotoxicity studies submitted for regulatory review.

Study Design

Animals from each treatment group were dosed as shown above. At week 32, all animals were evaluated for movement parameters using a standard functional observational battery (FOB) and an automated motor activity cage. Treatment groups B, C, and D were sacrificed at 33 weeks of age (8 months) similar to the protocol used by Thiruchelvam *et al*. (2002). Treatment group A was evaluated again at 62 weeks of age before sacrifice at week 70 (16 months).

One significant alteration of the study design was made. During the adult dosing period, 11 of the 12 high-dose males in treatment group D died or were sacrificed for humane reasons. Therefore, the dose level for all high-dose animals was halved after 2 or 3 doses. As a consequence of the deaths of the high-dose males from treatment D, there was concern about high-dose animals from treatment A surviving until the scheduled termination at 16 months. Therefore, some control animals originally assigned to treatment schedules B and C were allocated to treatment A, to have additional concurrent controls available in case high-dose animals became debilitated and had to be sacrificed earlier than the mid-dose and low-dose animals at the scheduled 16-month sacrifice time. This was allowable because, up to the point of sacrifice, controls from all treatment groups were treated similarly.

Study Timeline of Events



At the time of sacrifice, animals were given an overdose of barbiturate and perfused, and tissues were examined for abnormalities. All brains were cut into 50-μm serial sections, and every sixth section was stained with amino cupric silver to assess degeneration of cellular membranes, and another series of sections was stained using a glial fibrillary acidic protein (GFAP) antibody to assess astroglial reactivity known to occur in response to CNS cell damage. A third series was counterstained first with thionine/Nissl and then with tyrosine hydroxylase (TH) antibody. TH is the rate-limiting enzyme in the formation of both dopamine and norepinephrine, and is a marker for DA neurons in the SNpc. The GFAP and silver stained sections were examined by a certified veterinary pathologist. The total number of TH+ neurons in the SNpc was determined using stereological techniques.

Statistics

Statistical analysis was performed for the in-life data, such as body weight, food consumption, FOB parameters, and brain weights, according to standard protocols. These protocols used each animal as the individual unit of analysis, and only compared animals within the same treatment group (i.e. comparing four dose groups of males within treatment group A). Exponent provided statistical analysis for motor activity and stereology using the SAS Mixed procedure for analysis of variance (ANOVA), using the litter as the experimental unit of analysis, consistent with regulatory guidelines. Animals were compared across treatments (i.e. treatments A, B, C, or D), across sex, and across doses.

Results

The focus of this summary is on the mortality, FOB tremor, motor activity, and stereology data.

Mortality

No clinical signs or mortality were seen in preweaning pups at any dose level. Treatment-related mortality was seen in the high-dose males, in which 11 of the 12 males within treatment group D were found dead or were sacrificed for humane reasons. Dosing was stopped after five doses in all the high-dose animals of treatment group D; one male and all females survived. Two high-dose males in treatment group B also died during the adult dosing period, after exhibiting clinical signs similar to the high-dose adult-only treatment males, so dosing was stopped for that group, and the dose for all animals receiving treatment as adults was halved. No further treatment-related deaths occurred. However, there were clinical signs present during the dosing period in some surviving high-dose males and females.

Tremor

When each treatment group was evaluated separately for tremor incidence during the FOB, no effects were detected. However, when treatment A and B animal data were combined at 32 weeks (based on identical treatment up to that time), the incidence of tremors in males was 0 of 23, 1 of 23, 2 of 21, and 4 of 17 for control, low dose, middle dose, and high dose, respectively, which was statistically significant to $p<0.05$ by Cochrane-Armitage trend test. However, the trend was not exacerbated by age as would be expected in a progressive disease state. Of the three males within treatment A with detected tremor at week 32, only one had detected

tremor at week 62. The one animal with tremor at both 32 and 62 weeks was a high-dose male. Also, at age 62 weeks, two of the 22 controls had detected tremor, comparable to four of the 44 high-dose animals at week 32.

Motor Activity

In the 32-week global ANOVA of the total session activity for schedules A, B, C, and D combined, there was a significant sex effect for both the low ($F [1,323]=12.36$, $p>0.001$) and high beam ($F [1,326]=16.98$, $p<0.001$). Thus, analyses proceeded with each sex separately, and subsequent analyses indicated no statistically significant dose $\times$ treatment schedule or main effect of dose, indicating a lack of effect of PQ+MB injections when considering all treatment schedules together.

At 62 weeks (treatment A mice), there were statistically significant dose $\times$ sex interactions for both low- and high-beam activity (respectively, $F [3,113]=8.11$, $p<0.01$; $F [3,192]=4.73$, $p<0.001$). For males, no statistically significant effects were observed for either low- or high-beam activity. However, in females, there were statistically significant main effects of dose for low and high beam (respectively, $F [3,33.5]=4.83$, $p=0.007$; $F [3,32.5]=4.73$, $p=0.003$). Post-hoc comparisons with controls indicate that only the highest dose level of PQ+MB produced statistically significant increases in low- and high-beam activity (respectively, $p=0.002$, 0.004, using Dunnett's post hoc test). This increase in activity is not consistent with the reduced motor skills and activity seen in Parkinson's disease in humans.

In the repeated measures ANOVA for treatment A mice to compare week 32 and week 62 motor activity scores, there was a significant dose $\times$ sex interaction for the low-beam activity. Subsequent analysis proceeded separately for each sex. There were no statistically significant findings, except for the age-related increase in activity in 62 week old females described in the previous paragraph.

Neuropathology and Stereology

There were no findings related to treatment for GFAP or silver stained tissue sections.

Global analysis of the stereology data for treatment schedules A, B, and C resulted in a statistically significant effect of treatment schedule ($p=0.013$), so each treatment was evaluated separately. The ANOVA for each treatment schedule resulted in no statistically significant effects within treatment B or C. For treatment A, there was a significant dose $\times$ sex interaction ($F [3,68]=3.27$, $p=0.026$), so analysis was performed for each sex separately. The TH+ neuron count in high- and mid-dose males was 17% and 20% lower than control males, and there was a significant main effect of dose in males ($F [3,23]=3.26$, $p=0.040$), but not females. (See the table in Appendix A for the average number of TH+ neurons in each treatment group.) However, the planned post-hoc comparisons between treated and controls were not significant.

In the second *a posteriori* statistical analysis, PND 4 body weight and/or litter size were included as covariates in the analysis of treatment A males, because there was a tendency for larger litters to be assigned to the higher dose groups. The main effect of PQ+MB dose was no longer statistically significant when PND 4 weight or PND 4 weight and litter size were included as covariates in the analysis ($F [3,52]=1.94$, $p=0.1342$; and $F [3, 29]=1.76$, $p=0.1761$, respectively). An additional related analysis was conducted to determine whether a correlation existed between litter size and PND 4 body weights for male or female pups in treatment A using the Pearson correlation function in an Excel spreadsheet (Microsoft Office 2007). The Pearson product moment correlation coefficient for treatment schedule A males was –0.3854, and for females was –0.2617. These correlation coefficients are not high enough to conclude that litter size strongly influenced PND 4 body weight in treatment A pups.

Discussion

The major findings of this study are related to the observed mortality in high-dose males, and the decrease in TH+ neurons of the SNpc in high- and mid-dose males that had been treated both as pups and as adults, and sacrificed at 16 months of age. Males are clearly more sensitive to the effects of PQ+MB treatment than females in the indices examined.

The high mortality in high-dose males treated as adults indicates that changes in behaviour or cell counts in all high-dose males could be confounded by systemic effects, as shown by the abnormal pathology findings in the kidneys and lungs of the males that died or were sacrificed during treatment. Therefore, findings at the high dose in males should be interpreted cautiously. Adult females appeared to be resistant to overt toxicity at the 10/30 mg/kg PQ+MB adult high dose.

The only significant behavioural effect in the present study was an increase in both low-beam (cage floor) and high-beam (rearing) activity in high-dose females at age 62 weeks. The relevance of this observation to PQ+MB in mice as a PD model is unclear, because increased activity is not consistent with a movement and balance disorder. Furthermore, there were no motor-activity effects on male mice, even though males were more sensitive to the effects of PQ+MB, as shown by clinical signs, systemic toxicity, and TH+ neuron counts.

The essential criterion for an animal model of PD is selective loss of dopaminergic neurons in the SNpc. TH is a marker of dopaminergic neurons in the SNpc. Although decreased TH+ cells could be due to loss of dopaminergic neurons, it can also be due to suppression of neuronal TH+ expression (discussed in Prasad and Richfield 2008). This study did not differentiate between loss of neurons and loss of staining when the SNpc was counted using stereological techniques, similar to prior studies. Prior studies using the PQ+MB model have shown a statistically significant loss of TH+ neurons of up to 67% using 10/30-mg/kg PQ+MB i.p. doses in the C57Bl/6 male mouse at up to 8 months (Thiruchelvam *et al.* 2000; Thiruchelvam *et al.* 2002; Thiruchelvam *et al.* 2003; Kachroo *et al.* 2010) and in other studies that used PQ alone (McCormack *et al.* 2002; McCormack and Di Monte 2003; McCormack *et al.* 2005), although the effect of PQ alone was not consistent, and some authors found no effect (Thiruchelvam *et al.* 2002; Rojo *et al.* 2007).

The current study did not find an effect in treatment B and C animals for either motor activity or stereology, unlike prior studies, including Thiruchelvam *et al*. (2002), which was the basis for the protocol used in the current study. Different laboratories may use different tissue processing and stereological methods, so comparisons must be made cautiously, but the total TH+ neuron count in the current study falls within the range of numbers reported in the literature. The current study also used larger group sizes (9–12 per sex per dose per treatment) than prior studies, consistent with regulatory guidelines for developmental neurotoxicology studies.

This study is the first to test effects at 16 months of age (treatment A). There was a numerical decrease in TH+ neuron count in mid- and high-dose males of 20% to 17%, respectively, below the TH+ neuron count in control males. Although the main effect of treatment with PQ+MB in high-dose males was significant by ANOVA, the post-hoc dose group comparisons with the concurrent controls were not statistically significant. The main effect of dose was also not statistically significant when PND 4 body weight and litter size were used as co-variates in the ANOVA. In other words, when pre-dose pup body weights were included in the analysis as a covariate, the main effect of PQ+MB exposure on number of TH+ neurons for treatment A males was no longer statistically significant. The findings in the present study are strengthened by the relatively large treatment and control-group sizes in the 16-month-old males (n=11-12 for dose groups and 23 for control) and low standard error of the mean (SEM) (approximately 5%).

Another perspective of this analysis is to express the number of TH+ neurons as a ratio to PND 4 body weight of that animal. This resulted in an absence of any dose-related decrease in TH+ neurons. This *a posteriori* analysis should be viewed cautiously, because the litter sizes assigned to the higher dose levels were not abnormally large. Hence, there were no apparent maternal care issues that would be expected to cause nutritional deficits that would make these pups more susceptible. It also cannot be presumed that pups from the smaller litters assigned to controls were larger or healthier. Indeed, there was no correlation between litter size and PND 4 pup weight in treatment A males or females. Nevertheless, this *a posteriori* analysis with PND 4 body weight as a covariate raises uncertainty about whether the decrease in treatment A males at the mid- and high-dose level is due to PQ+MB exposure.

A second consideration is that the mean TH+ counts for the mid- and high-dose level (2395 and 2486, respectively) is within the range of control values for the six control groups, 2396 to 3440 for males and females in treatments A, B, C, respectively. Given the variability within the data, the mid- and high-dose groups in treatment A were within one standard deviation from B and C control males; the literature does not support a difference in TH+ neuron counts based on ages 8 and 16 months, so this comparison is informative.

A third consideration is that the magnitude of decrease in TH+ neurons in the high-dose and mid-dose males was comparable, despite the fact that the high dose was 1 order of magnitude higher than the mid dose. This lack of dose-response relationship would argue against a treatment-related effect, assuming no saturation of brain levels of PQ between the mid- (0.6 mg/kg) and high (10 mg/kg)-dose levels. Other authors have found rising brain levels from doses above 10 mg/kg of PQ (subcutaneous injections [Corasaniti 1991 and 1992], and single oral doses [Prasad *et al*. 2007]), indicating that the concentration in the brain is not saturated at the doses in this study, and that, therefore, the effect does not follow a dose-response. It is also possible that undefined technical issues limited the ability to detect decreases in TH+ neuron count greater than 20% (i.e., a “floor” effect).

Taken together, these considerations suggest that the decrease in TH+ neurons in treatment A males is an equivocal effect that requires replication before any firm conclusions can be reached.

Conclusion

The primary finding of this study is a numerical decrease in TH+ cell count in the SNpc of 16-month-old male mice treated with the high or mid dose of PQ+MB as pups and young adults, similar to the direction of effect observed by Thiruchelvam *et al*. (2002) in 8-month-old male mice. Although the main effect of PQ+MB treatment by ANOVA was statistically significant, the post-hoc treated-vs.-control group comparisons based on Dunnett’s and Tukey-Kramer tests were not statistically significant. There were no significant effects of PQ+MB on motor activity, except for an increase in activity in 16-month-old high-dose females (treatment A), which is inconsistent

with the direction of effect observed by Thiruchelvam *et al*. (2002), and inconsistent with a motor deficit disease like Parkinson's disease.

The current study does not replicate the results presented by the Thiruchelvam *et al*. (2002) study in 8-month-old mice—specifically, the motor activity decrement and lower TH+ neuronal counts in the SNpc (recognizing that dose adjustment to high-dose animals in the current study means that the current study group received approximately half the total dose of PQ+MB used by the prior study). The differences in our results and those of Thiruchelvam *et al*. (2002) could be due to methodological differences in frequency of motor activity testing, histopathology techniques, and stereological methods. However, the possibility for data manipulation also exists in the Thiruchelvam *et al*. (2002) paper, based on admitted data fabrication by this author in later publications (Federal Register 77(125): 38632-38633).

The 20% and 17% reduced average TH+ neuron count in the mid- and high-dose males at 16 months of age, but not 8 months, indicates the possibility of delayed toxic response beyond two weeks post-dosing. These potential effects require replication before final conclusions can be made regarding the biological significance of these findings, because (a) the number of TH+ neurons in mid- and high-dose males was within one standard deviation of male and female control values for Treatment A and B+C, and there is no evidence of sex differences in TH+ neuron count based on limited data from Cory-Slechta *et al*. (2005); (b) there was no difference in magnitude of decrease in TH+ neurons between the mid- and high-dose groups, indicating lack of a dose-response relationship; and (c) the main dose effect by ANOVA was no longer statistically significant when PND 4 body weights were included as a covariate. However, the numerical decreases in TH+ neuron count in the SNpc are consistent with published reports by other investigators for PQ alone (10 mg/kg-day) or PQ+MB (8–10 and 24–30 mg/kg-day respectively) in rats or mice dosed as adults using different experimental protocols (McCormack and Di Monte 2003; Cicchetti *et al.* 2005; Li *et al.* 2005; McCormack *et al.* 2005; Saint-Pierre *et al.* 2006; Kachroo *et al.* 2010).

The high dose of PQ (10 mg/kg) was at or above the maximum tolerated dose based on mortality and lung pathology. The mid dose of PQ (0.7 mg/kg) was 10-fold higher than the UK POEM estimate for a knapsack sprayer without protective equipment wearing shorts and sandals (McIntosh *et al.* 2011). Thus, the mid- and high-dose levels for PQ are both relatively high-dose levels (McIntosh *et al*. 2011). The likelihood of simultaneous exposure to high levels of MB and PQ was not evaluated in this study.

Future Work

Intranasal Transport

In the competition details and project specifications for which this proposal was submitted, section (iii) raised the question of whether certain chemicals could be transported directly to the brain after intranasal contact. The conclusions drawn by the Dorman laboratory (Dorman *et al.* 2002; Dorman *et al.* 2004) that hypothesized a direct retrograde transport of manganese from the olfactory epithelium to the deep brain nuclei was reversed in a paper from the same laboratory a few years later (Leavens *et al.* 2007). However, of interest concerning this topic, intranasal dosing in the PBPK model portion of this study indicated a fast and extensive circulation of paraquat in the brain tissue—the amount of paraquat detected in the brain after intranasal exposure was ten-fold higher than the model predicted based on systemic flow. The explanation for under-prediction in a PBPK model is that there is a significant route of tissue exposure that is unknown to the model. In this case, we hypothesized an additional and unexpected route of exposure in the nose that delivered ten times as much PQ to the brain as the systemic blood circulation. The possibility is that a compound deposited in the nasal cavity can enter the arterial circulation through a plexus in back of the nasal cavity, and can be distributed directly to the brain before dilution in the systemic circulation. This hypothesis is supported by preliminary data indicating that PQ enters the brain in large amounts in less than an hour (unlike retrograde transport which would take days to weeks), the distribution in the brain is greater on the side of the head where a unilateral dose is administered (consistent with ipsilateral blood flow in the brain), and the data are variable (consistent with unpredictable contact with the arterial plexus in the back of the nasal cavity during dosing). This preliminary observation has implications for exposure of humans to any neuroactive or neurotoxic substances that deposit in the nasal cavity during inhalation, including but not limited to pesticides. To better understand the risk, a study would be needed to examine the intranasal arterial hypothesis, including the pattern of paraquat distribution in the brain, and confirmation of the amount in the brain regions after intranasal administration.

Repeat Relevant Portion of this In-Life Study

The conclusions of the in-life study presented here are equivocal because of non-random pup allocation that resulted in low body weight of high-dose-group males, and of high variability and coefficient of error in the stereology data. Nonetheless, an averaged reduction in SN neurons of 17%–20% was seen in high- and medium-dose males at 16 months using an adequate number of animals. A small follow-up study repeating the protocol for just 8- and 16-month males around the middle dose would be important for determining the relevance of effects at a dose at the top of the range of human-relevant doses, as determined by the UK POEM for paraquat.

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Acronyms and Abbreviations

ANOVA	analysis of variance
FOB	functional observational battery
GFAP	glial fibrillary acidic protein
i.p.	intraperitoneal(ly)
MB	maneb
PBPK	physiologically based pharmacokinetic (model)
PD	Parkinson’s disease
PND	postnatal day (e.g., PND5 = postnatal day 5)
PQ	paraquat
SEM	standard error of the mean
SNpc	substantia nigra pars compacta
TH+	tyrosine hydroxylase positive (stained neuron)
UK POEM	United Kingdom’s Predictive Operator Exposure Model

Appendix A.

Average number of TH+ neurons ± SEM in the SNpc of mice for each treatment schedule

	Treatment Schedule					
	A: (16 mo, pup+adult treatment)		B: (8 mo, pup+adult treatment)		C: (8 mo, pup only treatment)	
Dose	Female	Male [a]	Female	Male	Female	Male
Control	2396±163, n=19 Range 1094 to 3378	2998 [a] ±169, n=23 Range 1709 to 4895	2265±209, n=4 Range 1837 to 2817 2775±215[c] N=9 Range 1837 to 3790	3440±565, n=6 Range 2198 to 5283 3047±356[c] N=11 Range 1682 to 5283	3183±483, n=5 Range 2569 to 3790 Left: B and C controls combined [c]	2578±344, n=5 Range 1682 to 3337
Low	2514±161, n=12 Range 1563 to 3653	3187 [a] ±245, n=12 Range 1585 to 4153	3011±306, n=11 Range 1855 to 5050	2899±210, n=12 Range 1717 to 3793	2811±280, n=4 Range 2072 to 3428	2976±347, n=4 Range 2210 to 3675
Medium	2638±313, n=7 Range 858 to 3724	2395[a,b]±138, n=11 Range 1589 to 3091	3095±206, n=11 Range 2077 to 4526	3025±260, n=11 Range 1830 to 4782	2973±205, n=5 Range 2317 to 3406	2592±210, n=5 Range 1953 to 3183
High	2539±220, n=12 Range 1337 to 3471	2486[a,b]±122, n=11 Range 2051 to 3300	2985±242, n=10 Range 1954 to 4166	3041±230, n=10 Range 2051 to 3922	3220±229, n=5 Range 2444 to 3774	2961±311, n=6 Range 1705 to 3772

[a] Statistically significant main effect of dose based on overall ANOVA ($p<0.05$, see text)

[b] Numerical decreases compared to control were NOT statistically significant based on post-hoc Dunnett's and Tukey-Kramer ($p>0.05$).

[c] Treatment B and C controls combined. Treatment B and C controls were treated identically, with the exception that B controls received seven vehicle injections during the adult dosing period. This difference was judged not to be important to neuron counting. Additionally, there was no difference in neuron counts for B and C control groups ($p=0.941$, t-test).

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Actions Resulting from this Work

Three symposia were held at public scientific meetings, during which presentations were made by Abby Li or Laura McIntosh using data from this work:

- Society of Toxicology 2009
- International Neurotoxicology Association 2011
- NorCal Society of Toxicology 2011.

Nine posters were presented by Abby Li or Laura McIntosh at public scientific meetings related to this work:

- Society for Toxicology 2007, -8, -9, -10, -11
- Teratology 2008, -9
- Eurotox 2009
- International Neurotoxicology Association 2007.

Poster abstracts are available in the publications of these meetings.