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# INCORPORATING LOW-DOSE EPIDEMIOLOGY DATA IN A CHLORPYRIFOS RISK ASSESSMENT

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□ USEPA assessed whether epidemiology data suggest that fetal or early-life chlorpyrifos exposure causes neurodevelopmental effects and, if so, whether they occur at exposures below those causing the current most sensitive endpoint, 10% inhibition of blood acetylcholinesterase (AChE). We previously conducted a hypothesis-based weight-of-evidence analysis and found that a proposed causal association between chlorpyrifos exposure and neurodevelopmental effects in the absence of AChE inhibition does not have a substantial basis in existing animal or *in vitro* studies, and there is no plausible basis for invoking such effects in humans at their far lower exposure levels. The epidemiology studies fail to show consistent patterns; the few associations are likely attributable to alternative explanations. Human data are inappropriate for a dose-response assessment because biomarkers were only measured at one time point, may reflect exposure to other pesticides, and many values are at or below limits of quantification. When considered with pharmacokinetic data, however, these biomarkers provide information on exposure levels relative to those in experimental studies and indicate a margin of exposure of at least 1,000. Because animal data take into account the most sensitive lifestages, the use of AChE inhibition as a regulatory endpoint is protective of adverse effects in sensitive populations.

*Keywords: chlorpyrifos, neurodevelopment, risk assessment, epidemiology, acetylcholinesterase*

## INTRODUCTION

Chlorpyrifos is an organophosphorus (OP) insecticide that was widely used for agricultural and residential pest control until 2001, when its non-agricultural use was restricted in the United States (USEPA 2002). Non-occupational human exposures occur mainly *via* ingestion of residues in the diet, while dermal and inhalation pathways likely predominate for occupational exposure (Eaton *et al.* 2008).

Chlorpyrifos is highly absorbed after oral and inhalation exposures (Smith *et al.* 1967; Bakke *et al.* 1976; Ahdaya and Guthrie 1982; Nolan *et al.* 1984), but dermal absorption is low unless skin integrity is compromised (Shah *et al.* 1987; Aprea *et al.* 1994). Once absorbed, it is distributed to all organs and undergoes rapid metabolism. Chlorpyrifos is desulfated *via* cytochrome P450 (CYP450) enzymes to chlorpyrifos-oxon predominantly in the liver but also in other organs, including the brain (Chambers and Chambers 1989).

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Chlorpyrifos-oxon binds to and irreversibly inhibits cholinesterases, such as acetylcholinesterase (AChE), which terminates the action of acetylcholine at cholinergic synapses in the central and peripheral nervous system and at neuromuscular junctions (Palmer *et al.* 1980). Inhibition of AChE leads to an accumulation of acetylcholine at cholinergic synapses and overstimulation of nicotinic and muscarinic receptors throughout the body (Richardson 1995; ATSDR 1997; Eaton *et al.* 2008). Acute cholinergic toxicity occurs when cholinesterase inhibition exceeds 70% (Clegg and van Gemert 1999), but other adverse effects can occur with much lower levels of inhibition.

Several cohort studies have examined the association between chlorpyrifos exposure and neurodevelopmental outcomes in newborns and young children (*e.g.*, Perera *et al.* 2003; Berkowitz *et al.* 2004; Eskenazi *et al.* 2004; Whyatt *et al.* 2004; Young *et al.* 2005; Rauh *et al.* 2006; Engel *et al.* 2007; Eskenazi *et al.* 2007; Wolff *et al.* 2007; Barr *et al.* 2010). These cohorts include the Mothers and Newborns Study of North Manhattan and South Bronx (Columbia University); the Inner-City Toxicants, Child Growth and Development Study (Mt. Sinai Hospital); the Center for Health Assessment of Mothers and Children of Salinas Valley (CHAMACOS) (University of California/Berkeley); and the University of Medicine and Dentistry of New Jersey (UMDNJ) cohort. Exposures in these studies are at least 1,000-fold less than those that inhibit AChE in animals (Eaton *et al.* 2008; Prueitt *et al.* 2011). Multiple studies have been published for each cohort that evaluate several endpoints, including head circumference, infant neurobehavioral capacity, cognitive and motor development, and behavioral outcomes. Some statistically significant associations were reported in these studies, so it has been hypothesized that chlorpyrifos exposure may be associated with neurodevelopmental effects at doses below the threshold for AChE inhibition *via* a non-enzymatic role of AChE in brain development or by other non-cholinergic mechanisms in the developing nervous system.

In 2011, the United States Environmental Protection Agency (USEPA) conducted a risk assessment of chlorpyrifos using laboratory animal brain AChE inhibition data to derive a point of departure (POD) and for dose-response analysis (USEPA 2011). In a draft issue paper released this year, USEPA (2012) assessed whether the observational epidemiology data suggest that fetal or early-life chlorpyrifos exposure causes neurodevelopmental effects and, if so, whether these effects occur at exposures below those that inhibit AChE by 10%. If this were the case, then it might be more appropriate to use neurodevelopmental effects to determine the POD and dose-response.

To determine how to incorporate the epidemiology data in the chlorpyrifos risk assessment, it is critical that the data are first reviewed in a comprehensive, critical, and transparent manner. We recently conducted

a hypothesis-based weight-of-evidence (HBWoE) analysis of chlorpyrifos and neurodevelopmental effects to analyze the evidence regarding the hypothesis that chlorpyrifos can cause these effects below the threshold for inhibition of AChE activity in the nervous system (Prueitt *et al.* 2011). In this analysis, we systematically reviewed individual studies in tables and the text, and then integrated all of the relevant data and considered each line of evidence (*e.g.*, epidemiology, toxicology, and mechanistic) to inform the integration of other kinds of data and compare alternative hypotheses. Based on this, we concluded that a causal association between chlorpyrifos exposure and neurodevelopmental effects in the absence of AChE inhibition in the brain is not plausible in humans, and the few positive associations observed in epidemiology studies are most likely attributable to alternative explanations. We briefly discuss this analysis and our conclusions here, but the full evaluation can be found in the Prueitt *et al.* (2011) paper. Here, we use the results of that analysis to address the specific question of how the chlorpyrifos toxicology and mechanistic data can influence the interpretation of epidemiology data, and how this in turn can be used in a risk assessment.

## HYPOTHESIS-BASED WEIGHT-OF-EVIDENCE EVALUATION

Approaches for incorporating epidemiology data in a risk assessment are no different than those for incorporating other kinds of data. Because a risk assessment is quantitative, only one study generally can be used to calculate the POD. To determine which study is most appropriate and most consistent with the weight of evidence (WoE), a WoE analysis should be conducted. There are many WoE frameworks available (*e.g.*, Krimsky 2005; ECETOC 2009; Linkov *et al.* 2009; Rhomberg *et al.* 2010; USEPA 2010; Rhomberg *et al.* 2011). The HBWoE framework we used in the Prueitt *et al.* (2011) analysis incorporates several aspects of many of the others and can be summarized in seven steps:

1. Systematically review individual studies relevant to the causal question at hand, focusing on an evaluation of study quality.
2. Within a given line of evidence [*e.g.*, epidemiology, experimental animal, or mode of action (MoA) studies], systematically examine the data for particular endpoints across studies, evaluating the consistency, specificity, and reproducibility of outcomes.
3. Identify and articulate lines of argument ("hypotheses") that bear on the available data and on establishing potential human risk.
4. Evaluate the logic of the proposed hypotheses with respect to each line of evidence, considering plausibility and consistency across studies.
5. Evaluate the logic of the proposed hypotheses with respect to all lines of evidence holistically so that all of the data are integrated and inform the interpretation of one another.

6. Describe and compare the various alternative accounts of the observations at hand, with a discussion of how well each hypothesis is supported by all of the available data, the uncertainties and inconsistencies in the dataset, and any *ad hoc* assumptions required to support each hypothesis.
7. Formulate conclusions and any proposed next steps (*e.g.*, sharpening of proposed hypothesis, additional testing to clarify data gaps).

These steps are described in detail in the Prueitt *et al.* (2011) publication. Using this approach, we found that the available epidemiology studies have imprecise exposure estimates that were measured at only one time point, sometimes reflected exposures to other pesticides, and often covered a small range and had many values below the limit of quantification. These studies do not report consistent associations, in that statistically significant associations were not reported for the same or related endpoints either within or among studies. That is, in cases when chlorpyrifos exposure was estimated using different surrogates, even in the same study, results based on different surrogates were not consistent. For example, results would be null for some metrics and positive for others, or sometimes in opposite directions for different metrics. There was also a lack of clear exposure-response relationships. We concluded that the reported associations in these studies are less likely to be indicative of causation and more likely to be a result of alternative explanations, such as exposure measurement error, bias, or confounding (Prueitt *et al.* 2011).

We also found that the animal toxicity data are themselves inconsistent in demonstrating neurodevelopmental impacts, and doses suggesting such effects are either associated with AChE inhibition or are at levels expected to cause such inhibition based on other experiments (Prueitt *et al.* 2011). This is also true for the majority of *in vitro* studies. Consequently, there is no positive evidence that chlorpyrifos is associated with neurodevelopmental effects at doses below the threshold for inhibition of AChE in the brain. Measurements and pharmacokinetic modeling show that human exposures, including those in the existing epidemiology studies, are far below those capable of causing meaningful AChE inhibition, which supports the interpretation that any associations reported in epidemiology studies are not causally related to chlorpyrifos exposure. Overall, the weight of available evidence more strongly indicates that a causal association between chlorpyrifos exposure and neurodevelopmental effects in the absence of AChE inhibition in the brain is not plausible for humans, and the few positive associations observed in epidemiology studies are attributable to alternative explanations (Prueitt *et al.* 2011).

## HAZARD CHARACTERIZATION

The mechanistic experimental toxicity data do not yet support a coherent set of key events in a MoA/adverse outcome pathway for neurodevelopmental effects of chlorpyrifos. As discussed in detail in the Prueitt *et al.* (2011) evaluation, the specific mechanisms proposed to support the hypothesis that chlorpyrifos induces adverse neurodevelopmental effects at doses below those that inhibit the activity of AChE in the brain involve the action of chlorpyrifos itself, rather than chlorpyrifos-oxon, and have been suggested to be involved in a large spectrum of effects. These include neuronal cell damage, disruption of systems controlling neuronal differentiation and synaptic function, and serotonergic dysfunction.

Evidence for the action of the proposed mechanisms at doses not affecting AChE activity comes mainly from *in vitro* studies, so relevance to potential outcomes in children with very low exposures to chlorpyrifos depends on tying levels in these *in vitro* experiments to the levels that would occur in children. Several physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) models are available for correlation of *in vitro* chlorpyrifos concentrations to systemic levels *in vivo* (*e.g.*, Timchalk *et al.* 2002, 2007). Recently, estimates of *in vivo* exposure equivalents determined with a LifeStage PBPK/PD model indicate that most doses in the *in vitro* chlorpyrifos studies were at or above levels that would inhibit AChE (Bartels *et al.* 2012). Of those *in vitro* studies with doses below those that cause AChE inhibition, it must be considered that confounding experimental factors (such as the use of DMSO as a vehicle or non-specificity of the assay) could be causing the biological effects rather than chlorpyrifos.

In addition, there is very little *in vivo* evidence for the proposed mechanisms acting through chlorpyrifos at doses below those that inhibit AChE activity (as reviewed by Prueitt *et al.* 2011). Only one study reported effects on synaptic proliferation and activity observed in rats exposed to 1 mg/kg-day chlorpyrifos during gestational day (GD) 17-20 (Qiao *et al.* 2003), a treatment that was not associated with AChE inhibition in the brain in another study when assessed 24 hours after exposure cessation (Qiao *et al.* 2002). In all but this *in vivo* study, there is no evidence for an absence of AChE inhibition. Because the metabolite chlorpyrifos-oxon is markedly more potent than the parent chlorpyrifos in inhibiting AChE (Huff *et al.* 1994; Das and Barone 1999), it is presumed to be present in the nervous system during any exposures sufficient to inhibit AChE. Thus, chlorpyrifos-oxon could be driving the specific mechanisms through its propensity to bind to proteins, an effect that would be absent at doses below those causing AChE inhibition.

The large body of neurodevelopmental data in experimental animals is limited by the small numbers of doses used in each study. Other issues

with study quality are discussed in our recent evaluation of these data (Prueitt *et al.* 2011). For example, the majority of studies used subcutaneous injection as the exposure route, which, by avoiding the extensive first-pass metabolism of ingested chlorpyrifos, is not as relevant to humans (*e.g.*, see Smith *et al.* 2009). Studies using the more relevant oral exposure route do not appear to be more likely to report associations with neurodevelopmental effects. In fact, when we examined the studies with the most weight separately (*e.g.*, those that used dose groups with a relatively high number of animals of each sex), considering both oral and subcutaneous exposures and including the one study that complied with USEPA Pesticide Assessment Guidelines and Good Laboratory Practice (GLP) regulations, we found largely null effects were reported across various neurodevelopmental tests. Those that did report treatment-related effects often reported inhibition of AChE activity in the brain at the same doses, suggesting that effects occur *via* this pathway or at least at doses that can cause AChE inhibition. Overall, we found that the animal data available to date do not indicate that chlorpyrifos causes neurodevelopmental effects at exposure levels below those associated with AChE inhibition (Prueitt *et al.* 2011).

Chlorpyrifos exposures in epidemiology studies are much lower than those used in the animal and *in vitro* studies discussed above. For example, five-day-old rat pups exposed to 1 mg/kg chlorpyrifos had maximum blood concentrations at two hours post-dosing between 16 and 140 nM, resulting in estimated brain concentrations of 0.5-4.6 μM (Marty *et al.* 2007). AChE inhibition and mechanistic effects were also reported at micromolar concentrations in the *in vitro* studies, with three exceptions (Das and Barone 1999; Schuh *et al.* 2002; Howard *et al.* 2005). In contrast, chlorpyrifos concentrations in the brain of subjects in the Columbia cohort were estimated to be from 0.33-3.3 nM (Eaton *et al.* 2008). These data indicate that estimated exposures to chlorpyrifos in the cohort studies are at least 1,000-fold lower than those used in the animal studies or those at which effects, including AChE inhibition, were observed in most of the *in vitro* mechanistic studies.

Regarding the epidemiology literature, we found that it is just as likely that reported positive associations are a result of alternative factors (*e.g.*, bias, confounding, exposure misclassification, statistical artifact) as opposed to indicative of a causal relationship (Prueitt *et al.* 2011). The results are not consistent or coherent across studies, and no clear exposure-response association has been demonstrated. Issues with exposure estimates also likely biased results. The animal and mechanistic data described above also cast doubt on the plausibility of a causal association in humans at exposures below those that inhibit AChE. This informs the hazard characterization in that the lack of consistent effects supports AChE inhibition as the relevant MoA for risk assessment.

## DOSE-RESPONSE ANALYSIS

Although the conclusions of the hazard assessment above, which is based on our WoE evaluation (Prueitt *et al.* 2011), indicate that the epidemiology data are not sufficient to conclude effects at exposures below those that cause AChE inhibition, epidemiology data has been used in the past for deriving a POD and in dose-response analyses. In 2008, USEPA's Science Advisory Panel (SAP) specifically cautioned against using Columbia cohort data for deriving a POD for chlorpyrifos because biomarkers were only measured at one point in time and effects may have been due to chlorpyrifos in combination with other pesticides (USEPA 2008). The 2008 SAP also advised against using any of the chlorpyrifos cohort studies because of limitations of the exposure assessment, "*e.g.*, lack of repeated exposure estimates to ascertain more specifically the variability and periodicity of exposure over time." Other issues with exposure estimates, described below, affect the reliability of these measurements and confirm that they should not be used in a dose-response assessment.

AChE inhibition data are the most robust source of dose-response data for deriving PODs for the chlorpyrifos risk assessment. These data take into account the most sensitive lifestages for exposure to chlorpyrifos (*i.e.*, pregnant women and their fetuses, newborns, and young children), and the use of AChE inhibition as a regulatory endpoint is protective of downstream cholinergic effects. Although some of the neurodevelopmental and mechanistic studies suggest there are non-cholinergic effects of chlorpyrifos on the developing brain, a quantitative method to relate the effect levels for AChE inhibition and potential non-cholinergic effects can help determine whether AChE inhibition continues to be the most sensitive endpoint – and therefore protective against other effects. To date, such analyses suggest that the levels would be similar (Bartels *et al.* 2012), which is consistent with the lack of evidence for non-cholinergic effects at doses that do not also inhibit AChE activity. That is, the models serve to help tie MoA data from different kinds of experiments into a coordinated set of bases for inferring possible human impacts and the exposures necessary to cause them. The dose-response for pharmacokinetic effects inform the plausible dose-response for mechanistic effects that have not been investigated directly at low doses.

## EXPOSURE CHARACTERIZATION

Chlorpyrifos exposures have been estimated using several different metrics across epidemiology studies. The best estimate, which was used in the Columbia cohort, is based on a chlorpyrifos measurement directly in blood. Plasma and cord blood were taken at delivery, however, so exposures during early central nervous system development are unknown.

The majority of samples were below the limit of detection and it is unclear how precise the measurements near the limit of detection were.

The chlorpyrifos metabolites 3,5,6-trichloro-2-pyridinol (TCPy), diethylphosphate (DEP), and diethylthiophosphate (DETP) have also been measured in urine (*e.g.*, Barr *et al.* 2005). Urinary TCPy originates not only from exposure to chlorpyrifos, but also TCPy itself (in the form of residues on foodstuffs from environmental degradation of chlorpyrifos) and other pesticides such as chlorpyrifos-methyl and triclopyr (MacIntosh *et al.* 1999; Morgan *et al.* 2005; Needham 2005). Urinary DEP and DETP can also originate from exposure to other pesticides, such as diazinon and disulfoton, and to pre-formed, environmental DEP and DETP (Needham 2005; Wessels *et al.* 2003). Activities of erythrocyte AChE or plasma butyrylcholinesterase (BuChE) also have been used as biomarkers of chlorpyrifos exposure, but these activities are not specific to chlorpyrifos; other chemicals, including other OP and N-methyl carbamate pesticides, can inhibit cholinesterases (ATSDR 1997; Barr and Angerer 2006).

The limitations in exposure estimates preclude one from determining precise exposures. When considered with pharmacokinetic data, however, they can provide information on exposure levels relative to those used in experimental studies. It appears that maternal exposures to chlorpyrifos are about three orders of magnitude lower than those that cause AChE inhibition and other effects.

## ESTIMATING THE RANGE OF POSSIBLE RESPONSES AT TYPICAL HUMAN EXPOSURE LEVELS

A WoE evaluation regarding the presence of risks at typical human exposure levels is an exercise in evaluating the uncertainty in the inferences about whether such risks exist from different available sources of data. Even when definitive quantitative modeling is difficult, using PBPK and dose-response models while varying assumptions in scientifically plausible ways can be an important tool in assessing whether proposed mechanisms are plausible causes at the low levels of human exposure.

In particular, the chlorpyrifos animal and *in vitro* data that suggest non-cholinergic mechanisms of impact on the development of the nervous system are largely seen at rather high doses, and the extrapolation of the magnitude of these effects to humans at lower doses is hampered by lack of multiple-dose studies or because the relation of *in vitro* exposures to tissue levels in humans at sensitive stages is in question. Even if dose-response evaluation of such factors cannot be done definitively, it is possible to use PBPK and hypothetical dose-response patterns to identify the range of plausible measures of these factors that might appear in the human tissues at risk. For instance, as we argued in our HBWoE analysis on chlorpyrifos (Prueitt *et al.* 2011), the MoA of most proposed non-

cholinergic effects seems to involve the production and binding of chlorpyrifos or its metabolites, notably chlorpyrifos-oxon, to key proteins. Pharmacokinetic models and plausible dose-response patterns for such models, and for the relative degrees of protein binding implied, inform the evaluation of whether such processes would be likely to act with sufficient magnitude to result in adverse outcomes at expected human doses and at doses well below those that cause AChE inhibition. That is, even if these projections are in some degree hypothetical, it is possible to conclude that they would not plausibly operate with sufficient magnitude to cause dysfunction at human exposures – if even a wide range of projections does not include the values that would be of concern. Even such uncertain projections can be useful in ruling out the role of the factors discussed above in producing low-dose human hazard.

There is little evidence that proposed non-cholinergic mechanisms occur at doses lower than those that cause cholinesterase inhibition. The effects depend to a large degree on a common set of chlorpyrifos metabolites as the primary active agents. The diminution of the production of these metabolites and the consequent amount of protein binding with lower doses makes it implausible to consider that the effects are sufficient to cause dysfunction at human dose levels.

The evaluation of animal and mechanistic data provide evidence that calls into question the plausibility of chlorpyrifos to be able to affect neurodevelopment at low human doses. If there were any ability of chlorpyrifos to act at low doses, it would have to be by some novel hypothetical mechanism for which there is currently no strong evidence. The epidemiology data alone do not make a compelling case for the existence of a chlorpyrifos effect – a causative interpretation based solely on the observed patterns of association is not markedly different in plausibility from explanations that attribute these outcomes to chance or confounding. It is not merely that the animal data do not support a causal association in epidemiology studies, they actually cast doubt on the biological plausibility of such a conclusion, in that they provide evidence against a causal association at human-relevant exposures of chlorpyrifos.

Even though AChE inhibition is an acute endpoint from which one can recover, maintaining exposures low enough to avoid inhibition also protects against any chronic effect that requires ongoing exposures at levels above those limits. The issue arises when one exceeds the acute-effect dose restriction for a short time – one risks the acute effect but not the chronic one unless the exceedance goes on long enough. In other words, if an acute and a chronic effect had the same threshold, the issue would be whether protecting against the chronic effect (which needs superthreshold doses for some run of time) would protect against the acute effect (which needs only a single exceedance), not the other way around. If AChE inhibition is mechanistically required to bring about the high-

dose neurodevelopmental effects seen in animals, then exposures that avoid such inhibition clearly avoid the developmental consequences as well. But even if the neurodevelopmental impacts have a non-cholinergic MoA, the preponderance of evidence (as discussed by Prueitt *et al.* 2011) suggests that such mechanisms would require doses at or near those also causing AChE inhibition. Thus, the current approach of using AChE inhibition as a sensitive endpoint and the basis for limiting exposures should protect against any neurodevelopmental risk as well.

In conclusion, an HBWoE evaluation of the relevant epidemiology, toxicology, and mechanistic data indicates that statistically significant associations in epidemiology studies are not likely indicative of causation (Prueitt *et al.* 2011). There appears no substantial basis to abandon USEPA's stance that exposure restrictions that protect against AChE inhibition will be protective not only of the general population, but also those at the most sensitive lifestages for exposure to chlorpyrifos (*i.e.*, pregnant women and their fetuses, newborns, and young children). Since that basis is well established and well documented, it should be retained. No additional uncertainty factors need be applied to allow for the possibility of effects in sensitive populations at exposures already limited to avoid AChE inhibition because the MoA information indicates the implausibility of such effects, and the epidemiology data alone provide no compelling basis to conclude otherwise.

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