Abstract/Poster # 2884/P536

Chlorpyrifos: Neurodevelopment, AChE Inhibition &Weight of Evidence

Marilyn Silva, Ph.D., D.A.B.T. and Svetlana Koshlukova, Ph.D.

Department of Pesticide Regulation, California Environmental Protection Agency, Sacramento, CA

BACKGROUND

Chlorpyrifos (CPF) is an organophosphorus (OP) insecticide, acaricide and miticide. The toxicity is associated with binding and inhibition of acetylcholinesterase (AChE). Metabolic activation to CPF-oxon required for AChE Inhibition (AChEI). A risk characterization document (RCD) for CPF was produced by the Human Health Assessment Branch (HHAB) of the California Department of Pesticide Regulation (CDPR) due to concerns regarding (available at: http://www.cdpr.ca.gov/docs/risk/rcd.htm):

- Neurodevelopmental effects in fetuses, infants and children
- Human exposure (agricultural spray drift, food, drinking water)
- Infant exposure from hand-to-mouth activities

MODES OF ACTION (MOA) for CHLORPYRIFOS

1. Cholinergic MOA

AChE hydrolyzes ACh at synaptic clefts in the central nervous system (CNS), at the neuromuscular junctions in the peripheral nervous system (PNS) and erythrocytes (RBC). AChEI results in ACh accumulation and unremitting nerve impulses leading to continuous stimulation in the CNS or PNS. Butyrylcholinesterases (plasma ChE) are the major ACh-hydrolyzing enzymes in human plasma. CPF is metabolized to the ultimate toxin CPF-oxon which inhibits brain and RBC AChE, and plasma ChE (Figures 1 and 2). AChEI in RBCs is commonly used as a surrogate for inhibition in target tissues (Koshlukova and Reed 2014; Testai et al. 2010)

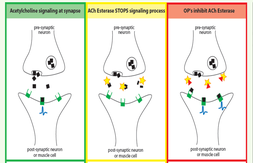

Figure 2. AChE is inhibited post-synaptically by CPF-oxon (2007 University of Washington opchild@u.washington.edu

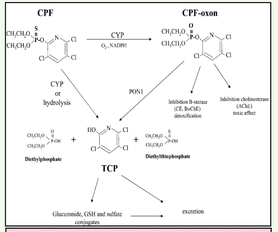

Figure 1. The major metabolic pathway for CPF (Testai et al. 2010)

2. Non-cholinergic MOA

CPF is implicated with toxicity via potentially more sensitive, non-cholinergic mechanisms. Potential targets include neurogenesis, macromolecule synthesis, neurotransmitter receptors, oxidative stress, cell signaling, nuclear transcription factors, and neuronal-glial cell interactions.

3. Toxicity Forcaster (ToxCast)Data and MOA

We reviewed the *in vitro* CPF ToxCast database for indications of noncholinergic effects at low CPF exposure. The in vitro ToxCast high-throughput screening assays (HTS) results were reported as activities at 50% concentration (AC_{50}; data available at: http://actor.epa.gov/dashboard2/). Results did not provide information that furthered knowledge of the MOA. This is likely because metabolic activation is needed to convert CPF to the toxic metabolite CPF-oxon. The Novascreen cell-free assay, however, was active with AChEI but other ToxCast assays gave no further insights to MOA for CPF-oxon.

ToxCast CPF and CPF-oxon active assays were used in the Toxicological Priority Index (ToxPi) to compare relative toxicities based on AC_{50} values (Reif et al. 2010; Reif et al. 2013). As shown in Figure 3, the active assays are categorized by components (e.g. DNA binding, receptor binding, etc.) which are designated by color. The size of the pie slice indicates relative activities between the two compounds. ToxPi results indicated that CPF-oxon (12.126) had a higher Toxicity Score than CPF (9.629). Although CPF needs metabolic activation to CPF-oxon to be toxic, the Toxicity Scores were very similar, indicating that in vivo effects are potentially related to numerous pathways. (Testai et al. 2010)

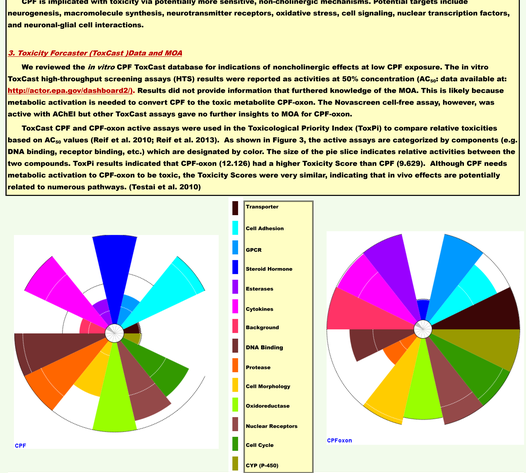

Figure 3. ToxPi figures comparing CPF & CPF-oxon activities in ToxCast assays. Toxicity Scores (CPF = 9.629; CPF-Oxon = 12.126)

POINTS OF DEPARTURE FOR RISK ASSESSMENT BASED ON 10% AChE INHIBITION

The critical Points of Departure (PoDs) used in the CDPR risk characterization document (RCD) are PBPK-PD-estimated human equivalent doses based on 10% inhibition of the RBC AChE activity after an acute (single day, 24 hr) or steady-state (21-day) exposure of CPF in humans (U.S. EPA, 2014). PoDs for CPF are typically based on RBC or brain AChEI, for which robust data in animals and humans are available (Table 1).

Table 1. PoDs for CPF and CPF-Oxon Used in the CDPR Risk Assessment (from U.S. EPA, 2014

Exposure Route[a]	PBPK-PD PoDs									
	Infants <1 yr		Children 1-2 yrs		Child 6-12 yrs		Youths 13-19 yrs		Females 13-49 yrs	
	Acute	SS[b,c]	Acute	SS[b,c]	Acute	SS[b,c]	Acute	SS[b,c]	Acute	SS[b,c]
Dietary (Drinking Water or Food-only) Exposure										
CPF-oxon mg/kg/d[b]	0.17		0.16		0.14		0.13		0.13	
Food CPF mg/kg/d	0.60	0.10	0.58	0.09	0.53	0.09	0.47	0.08	0.47	0.08
Spray Drift Exposure to Bystanders (http://www.cdpr.ca.gov/docs/risk/rcd.htm)										
Oral mg/kg	--	--	--	0.09	--	--	--	--	--	--
Dermal mg/kg/d	--	--	--	134	--	--	--	--	--	23.6
Inhalation mg/m³	--	--	--	2.37	--	--	--	--	--	6.15

a- Parent compound CPF for all estimates, except for drinking water where CPF-oxon exposure is estimated.
b- Acute PoDs for CPF-oxon in ppb (ug/L) converted to internal doses (mg/kg/d) using default drinking water consumption & body weight values.
c-SS = Steady State
"--" Not estimated

STUDIES SUPPORTING A WEIGHT OF EVIDENCE FOR NON-CHOLINERGIC CPF-RELATED NEUROTOXIC EFFECTS

Three main groups of studies (human, rat and zebrafish) have indicated that there are non-cholinergic adverse effects from CPF exposure that occur at exposures lower than those inducing brain AChEI (Tables 2-4).

Non-Cholinergic Neurodevelopmental Effects

1. Human Studies:

The Columbia Cohort (Columbia Center for Children's Environmental Health, Columbia University, New York) focused on CPF levels in umbilical cord and maternal blood plasma as a direct biomarker for CPF in utero fetal exposure. The CHAMACOS study (Center for the Health Assessment of Mothers and Children of Salinas, UC Berkeley, CA) and Mount Sinai (Mount Sinai Hospital Children's Environmental Health and Disease Prevention Research Center, New York) measured TCPy (a metabolite of CPF and CPF methyl) in maternal urine, PON1 and genetic phenotypes (Table 2). Collectively, the results from these studies showed that exposure to CPF during pregnancy lead to neurodevelopmental decrements in children from birth to 11 years; including changes in brain morphology, delays in cognitive and motor functions attention deficits, tremors and respiratory symptoms (asthma). (References in Table 2)

Table 2. Human Studies Showing Neurodevelopmental/behavioral Effects

Primary Study	Biomarkers	Exposure Route	PoD or Detected Exposure	Developmental-Behavioral Neurotoxicity	Genotype-Phenotype
Columbia Birth Cohort	Maternal blood & urine, neonatal umbilical cord blood for CPF, TCPy	Air/dermal/ diet through CPF home use	0.35 µg/kg/d inhalation 0.43 µg/kg/d Ingestion (Hattis 2015)	Children: birth – age 11 ↑Mental & motor delays ↑ADHD ↑Working memory deficits ↑Abnormal neonatal reflexes ↑Brain (white matter enlargement) ↓Brain cortical thickness	Not Tested
CHAMACOS	TCPy Maternal urine Blood Sampled for PON1 genotype	Bystanders near agricultural activities	Risk ↑: 3.54 µg/L TCPy in urine (not adjusted for creatinine)	Children: birth – age 7 ↑Childhood asthma ↓Birth weight ↓Head circumference	–PON1R$_{192}$ ↑catalysis of CPF-oxon compared to PON1Q$_{192}$. – PON1 Q/Q ↓hydrolysis (homo- & heterozygotes) compared to R/R homozygotes.
Mt. Sinai	TCPy Maternal urine Blood Sampled for PON1activity & Phenotype/ genotype	Air/dermal/ diet through CPF home use	Risk ↑ at 7.6 (1.6-32.5) µg/L TCPy (uncorrected for creatinine) or 11.5 (1.8-35.4) µg TCPy/g creatinine	Children: birth – age 9 ↑risk for delayed mental development (12 mos).	Children of mothers with *PON1* Q192R QQ genotype (slow CPF-oxon catalysis) at greater risk for developmental delays

These opinions and conclusions expressed in this paper are those of the authors and do not necessarily represent the views or opinions of the Department of Pesticide Regulation, or the California Environmental Protection Agency . References are available on request msilva@cdpr.ca.gov

2. Preweaning Rat Developmental Studies (Endocannabinoid Disruption)

Some CPF-related CNS effects are associated with disturbance of the endocannabinoid system in rat pups (References in Table 3). The cannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA) affect neurodevelopment and behavior (Anavi-Goffer &Mulder 2009; Harkany et al. 2008). They bind to CB1 receptors in the CNS (Figure 4) and affect neurotransmitter release, resulting in reduced incoming synaptic input (presynaptic inhibition). This occurs when a postsynaptic neuron releases 2-AG and AEA to bind to CB1 receptors on the presynaptic terminal. CB$_1$ receptors then reduce the amount of neurotransmitter released (as well as Ca++ influx or K+ efflux), so that the excitation in the presynaptic neuron results in diminished effects on the postsynaptic neuron.

Glutamatergic cannabinoid receptors are responsible for mediating aggression, resulting in inhibition of excessive arousal (excessive excitation produces anxiety that limited the mice from exploration of both animate and inanimate objects). In contrast, cannabinoids in the GABAergic neurons lead to increased anxiety by limiting inhibitory transmitter release. Taken together, these two sets of neurons appear to help regulate the organism's overall sense of arousal during novel situations (Haring et al. 2011).

CPF causes developmental neurotoxicity in rats treated with CPF (PND 10-16) at doses that elicit minimal or no brain AChEI. CPF inhibited MAGL and FAAH responsible for 2-AG and AEA degradation, respectively at <0.5 mg/kg/d; with FAAH inhibited more and for a longer duration than brain AChE. Disruption of MAGL and FAAH results in accumulation of cannabinoids, leading to continued effects on ion channels and neurotransmitter inhibition manifest as neurobehavioral toxicity in preweaning rat pups at a LOEL of 0.5 mg/kg/d (Table 3).

FAAH inhibition is the more sensitive endpoint than MAGL and AChEI. Data support a potential endocannabinoid-affected pathway for neurobehavioral disruption after pre-weaning treatment in rats at doses lower than those inhibiting AChE in brain. Mohammed et al (2015) showed behavioral effects and inhibition of factors in the dopaminergic system (DOPAC; 3,4-Dihydroxyphenylacetic acid; HVA: homovanillic acid) which works in conjunction with the endocannabinoid system at a LOEL of 0.5 mg/kg/d (Table 3).

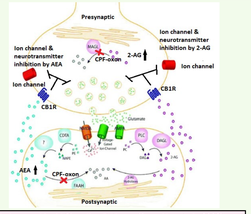

Figure 4 Endocannabinoid Signaling Pathway (from Ahn et al., 2008)

Table 3. Behavioral and AChEI Effects after CPF by Oral Gavage in Preweaning Rat Pups

ChE Inhibition	Effects	NOEL[a] mg/kg/d	LOEL[a] mg/kg/d	Ref[b]
↓ Brain AChE ↓Plasma ChE	↑Brain MAGL & FAAH inhibition; ↓2-AG & AEA hydrolysis (4 hr termination)	Brain AChE <1.0 Plasma ChE <1.0 Brain MAGL & FAAH <1.0	Brain AChE 1.0 Plasma ChE 1.0 Brain MAGL & FAAH 1.0	1
↓ Brain AChE ↓Plasma ChE	↑Brain MAGL & FAAH inhibition; ↓2-AG & AEA hydrolysis. At LDT peak inhibition of FAAH (52%) > plasma ChE (24%) leading to persistent pattern of elevated AEA	Brain AChE <1.0 Plasma ChE <1.0 Brain MAGL & FAAH <1.0	Brain AChE 1.0 Plasma ChE 1.0 Brain MAGL & FAAH 1.0	2
↓Plasma ChE & 12 h (No ↑Brain AChE)	No MAGL inhibition ↑FAAH inhibition at 4 & 12h ↑AEA	FAAH & AEA <0.5 Brain AChE <1.0 Plasma ChE <0.5	FAAH & AEA 0.5 Brain AChE 1.0 Plasma ChE 0.5	3
Not Tested	↑Open field effects, elevated plus maze, chasing crawling over/under, play fighting, playing behavior (↑anxiety, risk	Behavior <0.5	Behavior 0.5	4
↓ Brain AChE	↑ Brain MAGL & FAAH inhibition ↑2-AG & AEA ↓time to emergence from a dark container to a novel	Brain AChE 0.5 FAAH, AEA & Behavior<0.5	Brain AChE 0.75 FAAH, AEA & Behavior 0.5	5
Not Tested	↑time of emergence into illuminated area (↑anxiety) ↑DOPAC	Behavior <0.5	Behavior 0.5	6

a- NOEL = No-observed-effect-level and LOEL = lowest-observed-effect-level based on animal data
Abbreviations: AEA: anandamide; 2-AG: 2-arachidonoylglycerol; DOPAC: 3,4-Dihydroxyphenylacetic acid; FAAH: fatty acid amide hydrolase; LDT: lowest dose tested; MAGL: monoacylglycerol lipase; HVA: homovanillic acid
References: 1. Carr et al. (2011); 2. Carr et al. (2013); 3. Carr et al. (2014); 4.Carr et al. (2015a); 5. Carr et al. (2015b); 6. Mohammed et al. (2015);
"--" = No NOEL

3. Zebrafish Studies:

Zebrafish embryos (intact chorion) had developmental toxicity such as malformations and abnormal behavioral first observed with CPF treatment that persisted into adulthood (Table 4). These effects included anxiety-related behaviors (decreased swim speed and thigmotaxis [edge preference/anxiety]). Developmental and behavioral effects occurred at 0.01 µM CPF; 10-fold lower doses inhibiting AChEI (0.1 µM). (References in Table 4)

Table 4. AChEI and Neurobehavioral Effects after CPF Treatment in Zebrafish Embryos

Exposure	Age of Testing	Enzymatic Effects	Behavioral Domain Affected and AChE Effects	NOEL µM	LOEL µM	Ref[a]
5 days	Day 6 post hatching up to 28 wks	Not Tested	↑Mortality at 38 weeks ↑ effects on choice accuracy ↑ latency response ↓ spatial discrimination ↓ swimming activity	<0.028	0.028	1
0-1, 0-2, 0-3, 0-4, 0-5 dpf	3 months	Not Tested	↓brain dopamine & norepinephrine ↓habituation to startle ↓overall startle response ↓escape diving response ↓ learning rate ↓swimming activity Persistent effects from dpf 5 to adult included	<0.28	0.28	2
5 d dpf	48, 60, 96 hpf	↓AChE protein ↓AChE mRNA ↓GSH & GST	↑ Spinal deformities, pericardial edema ↑Decrements in swimming activity ↑Decrements in behaviors (stimulation of light/dark photoperiod transition 5 d) ↓Body length ↓ hatchability ↓Free swimming distance Neurobehavioral effects were not reversible.	AChE-related 0.084 Developmental 0.028 Behavior 0.084 GSH, GST <0.028	AChE 0.28 Developmental 0.084 Behavior 0.28 GSH, GST 0.028	3
7 dpf	7 dpf	Not Tested	↓Swim speed ↑ Thigmotaxis (edge preference) without changes in avoidance behavior.	Behavior 0.001	Behavior 0.01	4
1-5 dpf or late development 3-5 dpf	1-5 dpf or late development 3-5 dpf	↑AChEI	↑abnormal behavioral ("fish at rest") ↑ Thigmotaxis (edge preference) in swim lane (signifies ↓anxiety) ↓ Swim speed	AChE 0.01 Behavior 0.001	AChE 0.1 Behavior 0.01	5

Abbreviations: dpf = days post-fertilization
References: 1. Levin et al., 2003 and 2004; 2. Sledge et al., 2011; 3. Jin et al., 2015; 4. Richendrfer et al. 2012a; 5. Richendrfer and Creton, 2015

DATA SUMMARY

PoD/LOELs for human AChEI and behavior and NOEL/LOELs for those parameters in preweanling rats and zebrafish (Table 5).
PoDs for AChEI (0.1 mg/kg/d):Neurodevelopmental/behavioral effects (0.00043 mg/kg/d) is 232-fold higher AChEI. OED was Oral Equivalent Doses (OEDs Wetmore et al 2012, 2015) for humans (based on zebrafish data) showed 10-fold AChEI.

Table 5. Comparative NOEL/LOELs for Brain AChEI and Neurodevelopmental Effects (ND/B) in Human Children, Preweanling Rats and Zebrafish

Species/Subjects	PoD/NOEL/OEDs (mg/kg/d)		Fold Difference AChEI:ND/B	LOEL/OEDs (mg/kg/d)	
	Brain AChEI	Neurodevelopmental Effects		Brain AChEI	Neurodevelopmental Effects
Children 1-2 & 6-12 Years	0.09-0.1[a]	0.00035 inhalation 0.00043 Ingestion (Hattis 2015)	232 fold ↑	>0.09-0.1[a]	>0.00035 inhalation >0.00043 Ingestion (Hattis 2015)
Preweanling Rats	0.5	None Determined (<0.5)	NA	0.75	0.5
Zebrafish	0.03[b] (Rat) 0.015 (Human)	0.003[b] (Rat) 0.0015 (Human)	10 fold ↑	0.3 (Rat) 0.15 (Human)	0.03 (Rat) 0.0015 (Human)

a-Brain AChE PoD estimates in children were based on use of 10% RBC inhibition from the USEPA (2014) PBPK-PD model as a surrogate indicator (Table 7).
b-Doses converted from µM CPF exposure in zebrafish to oral equivalent doses (mg/kg/d) in rat (Wetmore et al. 2012) and mg/kg/d in human (Wetmore et al. 2015).

WEIGHT OF EVIDENCE (WoE) CONSIDERATIONS

Target Margin of Exposure and Uncertainty Factors for CPF

Toxicity risks were calculated as margins of exposure (MOE), quotients of the PoDs from the PBPK-PD model and estimated human exposure levels.

MOE = PoD ÷ Exposure by all known routes

In risk characterization, the calculated risks or MOEs are compared to a *target MOE* that takes into account uncertainty factors (UFs).

(1x interspecies UF) x (10x intraspecies UF based on database uncertainties) x (10x neurodevelopmental UF based on WoE) = 100 Target MOE for CPF

The default inter-species uncertainty factor of 10 was reduced to 1, because the toxicological PoDs for CPF were modeled from human data(Poet et al. 2014; Smith et al. 2014; Smith et al. 2011; Timchalk et al. 2002a; Timchalk et al. 2002b). The default UF of 10 was used to account for the sensitivity within the human population with respect to RBC AChE inhibition. An UF of 10 was used to protect against CPF neurodevelopmental effects in humans ß based on WoE) = 100 Target MOE for CPF

PBPK-PD Database Uncertainties

Intraspecies UF (10x): The use of 10% RBC AChEI as toxicological PoDs is associated with a lack of data for intraspecies variability.

(i)The current PBPK-PD model lacks critical data on physiological changes during pregnancy and genetic variability related to AChE metabolism (Poet et al. 2014).

(ii) Metabolism-related age- and ethnic-specific intraspecies parameters (variability of PON1 & CYP enzymes) were based on a very small sample size (30 human hepatic microsomal & 20 plasma samples). From few human tissue samples, the model generates intraspecies metabolism-related parameters that are applied to the general population (Smith et al. 2014; Smith et al. 2011).

(iii)There is a >10-100 fold difference between PoD/OEDs based on AChEI in humans or zebrafish and neurobehavioral effects in humans or zebrafish. Therefore RBC AChEI may not be the most sensitive indicator of toxicity in humans.

Weight of Evidence for Non-Cholinergic MOA for CPF

Neurodevelopmental toxicity associated with CPF Exposure (10x UF: Data and References in Tables 2-4):

(i)Results from the three major human prospective birth cohort studies, preweanling rats and zebrafish assays indicate that CPF may cause neurodevelopmental and neurobehavioral effects without brain AChEI.

(*ii*) Sufficient data are not available at this time to establish a human dose-response relationship for neurodevelopmental effects. Based on preliminary estimates of the oral *in utero* PoDs for working memory decrements in children 7 yrs old, the threshold for disruption of the endocannabinoid or dopaminergic systems in rats and the active concentration causing cognitive, anxiety and learning deficits in zebrafish, the neurodevelopmental effects could be predicted to occur at doses 3-10 fold lower than brain AChEI.

The HHAB RCD reported that children age 1-2 years old exposed to CPF by spray-drift inhalation alone as well as by multiple routes (e.g., diet, water, dermal, inhalation, mouthing behaviors, soil ingestion and others) have MOEs that are below the target of 100 (available at: http://www.cdpr.ca.gov/docs/risk/rcd.htm).

CONCLUSIONS

Selection of RBC AChE inhibition as the critical toxicity endpoint was intended to protect human populations from impacts on other endpoints that were not easily measured. More consideration needs to be given to non-cholinergic effects in the developing young due to mounting evidence supporting neurodevelopmental toxicity at exposures lower than those affecting brain AChE. Consequently, an extra 10-fold factor for sensitive populations should be considered to protect against CPF neurodevelopmental effects until sufficient data become available to derive PoD based on developmental neurotoxicity.