Three open access computational tools were used on pesticides with well-characterized toxicological profiles to determine concordance between modeled predictions and measured acute in vivo toxicity (most–least: chlorpyrifos-oxon/chlorpyrifos (CPFO/CPF), carbaryl (CB), endosulfan (ENDO), propyzamide (PZ)). Tools included 1) Toxicity Forecaster (ToxCast) and Tox21 (ToxCast/Tox21) assays (AC50s µM) to identify biological targets associated with modes of action; 2) Toxicological Prioritization Index (ToxPi) incorporating AC50s to rank and score toxicity; 3) Integrated Chemical Environment (ICE) tool for in vitro to in vivo extrapolation one-, three-, and multi-compartment models to predict human equivalent administered dose (EADhuman). ToxPi toxicity ranking was predictive for CPFO, CB and PZ but not for CPF or ENDO. Qualitative graphical visualizations and quantitative fold differences between EADhuman and acute oral in vivo endpoints for each pesticide were most predictive in the three-compartment model. Qualitative modeled toxicity rank among AChE inhibitors (CPFO>CPF>CB) was 100% predictive. Brain AChE inhibition endpoints (EADhuman vs. ADJBMD10) had better predictions (lower fold difference) than those with RBC AChE inhibition endpoints. Overall, the computational tools in this study could be useful in not only prioritizing pesticides for risk assessment but also providing insights into mechanistic data often lacking in traditional testing.

Keywords: Computational Tools, ICE/IVIVE/PBPK, Pesticides, ToxCast/Tox21, ToxPi, risk assessment

ABBREVIATIONS: AOP: Adverse outcome pathway; BMD: Benchmark Dose; CB: Carbaryl; CPF: chlorpyrifos; CPFO: Chlorpyrifos-oxon; ENDO: endosulfan; ICE: Integrated Chemical Environment; MOA: Mode of Action; PBTK: physiologically-based-toxicokinetic; POD: Point of departure; PZ: propyzamide; ToxCast: Toxicology Forecaster Program, Tox21: Toxicology in the 21st Century Program; ToxPi: Toxicological Prioritization Index Graphical User Interface

INTRODUCTION

Currently, pesticide registration, use and sales in the United States are controlled by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Federal Food, Drug and Cosmetic Act, the Food Quality Protection Act and the Pesticide Registration Improvement Act and the Code of Federal Regulations Title 40 (CFR, 2020). Specifically, the pesticide registration involves a comprehensive risk assessment that encompasses health effects from exposures through food and drinking water as well by residential or occupational uses and to bystanders living near agricultural fields or application sites (US EPA, 2014b, 2020d). Health Effects Test Guidelines studies (US EPA 1998) are submitted to regulatory agencies, to evaluate acute, subchronic and chronic effects related to carcinogenicity, genotoxicity (generally in vitro), development, reproduction, endocrine systems, neurotoxicity, immunology and other outcomes (US EPA, 1997, 2020e). Data contributing to the understanding of mode of action (MOA) or adverse outcome pathway (AOP), however, are not usually required or submitted by pesticide registrants.
There are hundreds of thousand chemicals, including pesticides in the environment, many with unknown toxicity. The United States Environmental Protection Agency (US EPA) and international agencies are committed to developing rapid and cost-effective computational tools to support risk assessment efforts for risk management applications (dos Santos and Nardocci, 2019; TSCA, 2020). In order to accomplish these goals, the Computational Toxicology Chemistry Dashboard (CompTox) provides a database repository that includes the Toxicity Forecaster (ToxCast) and Tox21 (ToxCast/Tox21) in vitro bioassays (US EPA, 2020a). There are almost 1600 unique assays for use in chemical screening, prioritization, and toxicity predictions through identification of potential disruptions in key biological pathways (Pearce et al., 2017; Williams et al., 2017; Zurlinden et al., 2020). The CompTox Dashboard allows easy access to ToxCast/Tox21 assay results for over 882,000 compounds obtained from numerous databases. These tools may, in some cases, replace the traditional methods for assessment of toxicity that rely on expensive, lengthy, and ethically challenging animal studies (Sipes et al., 2017; Turley et al., 2019; Zurlinden et al., 2020). In light of this goal, the US EPA has directed the Office of Chemical Safety and Pollution Prevention and the Office of Research and Development to "reduce requests for, and funding of mammal studies by 30% by 2025, and eliminate all mammal study requests by 2035," (US EPA, 2020c).

With the motivation in mind of moving to in vitro models, we conducted a case study examining whether the well-established acute oral animal bioassay endpoints for five pesticides coincide with results from three open access computational toxicology tools. To accomplish this we selected pesticides from four different classifications: chlorpyrifos (CPF: organophosphate insecticide) and the primary CPF toxic metabolite CPF oxon (CPFO), endosulfan (ENDO: organochlorine), carbaryl (CB: N-methyl carbamate insecticide) and propyzamide (PZ: amid herbicide) (CPDR, 2008, 2014, 2018; US EPA, 2007a, 2011, 2015c). Each of these compounds, has been shown to be highly toxic to agricultural workers, household residents and bystanders living near application sites (CDPR, 2008, 2014, 2018; US EPA, 2007b, 2014a, 2015d, 2017a). Data from the open access human health risk assessments determined the no- and lowest-observed-effect levels that served as a basis for comparison to in vitro predictions.

The open access tools for this study included the ToxCast/Tox21 Dashboard (US EPA, 2020a), the Toxicological Prioritization Index Graphical User Interface Program (Marvel et al., 2018; Reif et al., 2013; ToxPi, 2020) and the Integrated Chemical Environment tool (Bell et al., 2020; NTP, 2020). The tools were used with each pesticide for their utility in predicting: 1) biological activities of ToxCast/Tox21 assays at known targets; 2) ToxPi-generated chemical ranking and Toxicity Score based on relative activities in ToxCast/Tox21 intended target families; 3) human equivalent administered dose (EAD<sub>human</sub>) determined from three in vitro to in vivo extrapolation (IVIVE) and physiologically based toxicokinetic (PBTK) models in the ICE program (Bell et al., 2020; ICE, 2020) to compare with the established in vivo acute oral lowest effect levels. Concordance between predicted results from computational tools and measured in vivo bioassay results were examined for use in chemical screening and prioritization and in support of open access data associated with the presumptive mechanistic pathways.

**METHODOLOGY**

**In Vivo Acute Oral No Effect and Lowest Effect Levels Used in Risk Assessment**

In vivo acute oral studies for pesticides performed under Health Effects Test Guidelines were designed to generate a dose-response, that included a no observed effect level (NOEL) and lowest observed effect level (LOEL) (US EPA, 1998, 2014b). These studies have traditionally been conducted using doses at wide intervals, which may not accurately estimate a no effect level. However, when data can be modeled, a Benchmark Dose analysis may better represent the lowest effect (BMD) or an estimated no effect level (BMDL) than LOEL/NOELs conventionally used in risk assessment for non-cancer endpoints (US EPA, 2012). A Benchmark Response (BMR) of 10% (BMD<sub>10</sub>/BMDL<sub>10</sub>) was included in the open access oral acute data for CPFO, CPF and CB, as an acceptable normal range of variability for red blood cell (RBC) and brain acetylcholinesterase inhibition (brain AChE) (CDPR, 2014; Moser et al., 2010; US EPA, 2011, 2017a). The open access ENDO risk assessment used an acute oral NOEL as an endpoint for neurotoxicity in pregnant rabbits (CDPR, 2008). In some cases where a NOEL is not obtained and/or BMD analysis cannot be modeled, an estimated no-effect-level (ENEL) can be obtained by use of a 10-fold uncertainty factor to extrapolate from the LOEL (LOEL ÷ 10 = ENEL) (US EPA, 2015c).

The open access acute oral no effect and lowest effect levels for each pesticide were the most sensitive for that exposure duration (Table 1). This timeframe was also important because the ToxCast/Tox21 assays are performed after a single (acute) administration of chemical. Each in vivo acute low and no effect points of departure (POD) was reported as a LOEL/NOEL, a BMD<sub>10</sub>/BMDL<sub>10</sub> or LOEL/ENEL. The toxicity rank of each pesticide in Table 1 was from most toxic (CPFO) to least toxic (PZ) based on the listed PODs. These values were used to compare Toxicity Score/Rank predicted in the ToxPi program through use of selected ToxCast/Tox21 active hit-calls as data inputs.

For the Integrated Chemical Environment tool (ICE) models (described below), the acute BMD<sub>10</sub>/LOELs in
Table 1 were further adjusted by a default factor of 10 (acute ADJBMD_{10}/ADJLOE) to account for animal to human interspecies extrapolation (US EPA, 2002b; WHO, 2017). That is, the acute in vivo BMD_{10}/LOELs were obtained from animal studies but the ToxCast/Tox21 data were performed with human tissues/proteins and the ICE models predict human EADs (Bell et al., 2020; ICE, 2020; NTP, 2020). Acute ADJBMD_{10}/ADJLOE are the relevant data to compare to acute EAD_{human} generated from IVIVE models, such as those used in ICE as has been shown previously (Sipes et al., 2017).

**Chlorpyrifos (CPF) and Chlorpyrifos oxon (CPFO):** In the United States and internationally, CPF is widely registered for use because it is highly effective as an insecticide, miteicide and acaricide for application on food crops (e.g., nuts, grains, fruit, vegetables, and grains) in addition to non-food use (e.g., golf course turf, nurseries, wood products) (CDPR, 2017; PMRA, 2019; US EPA, 2020b). Residues on food, in water and air presented human health safety concerns and there are increasing reports indicating that CPF exposure at low doses leads to irreversible neurodevelopmental effects in humans and rodents (APVMA, 2017; EFSA, 2014, 2017; Rauh et al., 2015; Rauh et al., 2012; Silva, 2020). The main acute CPF MOA is through activation by CYP2B6 (desulfitation) to the toxic metabolite CPFO to inhibit plasma, or butyryl cholinesterase (BuChE), red blood cell acetylcholinesterase (RBC AchE) and brain AchE resulting in neurotoxicity in humans and animals (Eaton et al., 2008a; Testai et al., 2010). CPFO is detoxified by paraaxonase 1 (PON-1/A esterase) to form the main urinary metabolite 3,5,6-trichloropyridinol (TCPy) (Eaton et al., 2008b). A hydrolysis path involving CYP2C19 (pregnane-X-receptor: PXR-associated) and CYP3A4/5 directly detoxifies CPF to TCPy, followed by conjugation with glucuronide, sulfate, or glutathione-s-transferase. CYP3A is activated through PXR + retinoid-X-receptor (RXR) heterodimerization and interaction with the PXR-Element (PXRE) promoter region in the nuclear DNA (Wang and Negishi, 2003). Reversal of AchE inhibition by CPFO in humans occurs over 30 days (US EPA, 2016) and takes 2-5 weeks in rats (Ellison et al., 2011). Other CYPs involved with CPF dearylation/dealkylation include CYP1A2, 2B6, 3A4, 3A5, 3A7, 1A1, 2C19, 2C9, and 2D6 (Buratti et al., 2003; Mutch et al., 2004; Tang et al., 2001). Data indicated that RBC AchE inhibition is more sensitive than in brain for CPF and CPFO (Table 1). However, effects on each endpoint may lead to adverse outcomes during development as has been proposed (Mattsson et al., 2000; Moser and Padilla, 1998).

**Carbaryl (CB):** CB is broadly applied on food (e.g., nuts, fruits, vegetables, grains, oyster beds) and non-food (e.g., turf, golf courses, ornamentals, cut flowers) crops in residential and agricultural settings in the United States and Internationally (APVMA, 2012; EFSA, 2006; PMRA, 2016; US EPA, 2017a). In addition to acute neurotoxicity effects to human health through residues in food, air and water, CB is considered by the US EPA to be a “likely human carcinogen,” (US EPA, 2007b). Further, the benefits from killing mosquitoes carrying malaria, conflicts with the toxic and deadly effects on honeybees (NPIC, 2003). The acute CB MOA is through inhibition of AChE in plasma, brain and red blood cells (RBC AchE) by carbamylation of the serine hydroxyl group at the active site on the enzyme in humans and animals (Moser et al., 2010; US EPA, 2017a). While CB itself can inhibit AchE, CYP-activated metabolic pathways produce carbaryl methylol (PXR-associated CYP2B6), 5-hydroxycarbaryl (CYP1A1, CYP1A2-PXR-associated) and 4-hydroxycarbaryl (CYP1A1, PXR-associated CYP3A4) which are also toxic (Nong et al., 2008; Tang et al., 2002; US EPA, 2007a). Arylhydrocarbon hydroxylase nuclear receptor (Ahr) regulates CYP1A1 expression (Fallone et al, 2005) and the CYP3A, CYP1A and CYP2B families are activated through CAR/RXR and PXR/RXR with PXRE in the nuclear DNA (Ihunnah et al., 2011; Wang and Negishi, 2003).

**Endosulfan (ENDO):** ENDO agricultural use as an insecticide and acaricide was completely phased out in the United States (USEPA, 2010) from 2009-2016. It was globally banned for all uses as of 2011 because it was listed as a persistent organic pollutant by the Stockholm Convention’s Persistent Organic Pollutants Review Committee (UNSC, 2010). However, ENDO persistence in the environment and accumulation in fatty tissues may pose a risk to human health as well as wildlife (Cone, 2010; EFSA, 2011). ENDO functions acutely as a neurotoxic non-competitive inhibitor in the central nervous system (CNS) by binding to and blocking Cl- channels associated with the γ -amino-butyric acid receptor (GABA\(_{\alpha}\)R) (Casida, 1993; French-Constant et al., 2000; Lawrence and Casida, 1994). GABA\(_{\alpha}\) receptors are the principal inhibitory neuroreceptors in the mammalian brain and antagonism of GABAergic neurons in the CNS causes generalized brain stimulation (Cone and Casida, 1986; Gant et al., 1987). Normally when GABA binds to its receptor, the Cl- ion channels are opened, leading to an influx of Cl- into neurons through an electrochemical gradient. The result is hyperpolarization of the cell membrane and inhibited neuron firing. However, ENDO prevents Cl- from entering neurons, thus blocking the effect of GABA binding to its GABA\(_{\alpha}\)R, resulting in uncontrolled excitation (Kamijima and Casida, 2000; Ratra et al., 2001). This primary effect is observed acutely in both humans and animals (where clinical signs were recorded). Metabolism occurs through activation by CYP2B6 and CYP3A4/5 to form ENDO-sulfate (Bebe and Panemangalore, 2003; Casabar et al., 2006). The main metabolite, ENDO-sulfate, has about the same toxicity as the parent technical mixture (ATSDR, 2000). Nuclear receptors (e.g., constitutive androstane receptor (CAR), peroxisome proliferation receptor alpha (PPAR\(_{\alpha}\)), PXR) heterodimerize with RXR and bind at the PXRE (Sugatani et al., 2004) or at the peroxisome proliferator hormone response element (PPRE) promoter regions on the gene.
(Viswakarma et al., 2010; Wang and Negishi, 2003) to initiate the expression of CYP2B6 and CYP3A4/5 and other proteins involved in ENDO metabolism (Michalik et al., 2006).

Effects on dopamine and dopamine active transporter (DA/DAT) resulted from ENDO treatment in rodents during gestation (in utero), neonatally and postnatally. Weanling rats had a decrease in DA in the hippocampus after treatment with ENDO and a lowered ability to learn and retain a required task (Lakshmanan et al., 2013). ENDO administered intraperitoneally to neonatal rats resulted in decreased DA and increased foot-shock fighting behavior (Seth et al., 1986). Male C57Bl/6j offspring (age 3 months) treated in utero with ENDO showed a decrease in DAT expression in the brain. (Wilson et al., 2014). DAT was further depleted in these pups after treatment with the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). C57Bl/6 mice treated with ENDO as juveniles and again in adulthood had decreased DA (Jia and Misra, 2007). ENDO is also an endocrine disruptor that affects the androgen and estrogen receptor but usually at doses equal or greater than those inducing neurotoxicity (Milesi et al., 2015; Silva and Gammon, 2009).

The acute LOEL/NOEL for ENDO is 1.8/0.7 mg/kg/day from a developmental toxicity study in rabbits was reported by CDPR (2008) and, US EPA (2002a). Although a developmental study in pregnant rabbits involves 22 days of gavage dosing, neurotoxic effects (hyperactivity) were observed within the first few days of treatment. This study exhibited the most sensitive endpoint for ENDO (Table 1).

**Propyzamide (PZ):** PZ has non-food uses (e.g., landscape maintenance, ornamental turf, golf courses, etc.), as well as food uses (e.g., lettuce, endive, artichokes, and radicchio) and is registered in the United States (US EPA, 2015b) and internationally (APVMA, 2020; EFSA, 2016; PMRA, 2009). Although PZ has comparatively low toxicity, it is widely used on crops and the residues in food and drinking water are a concern. There are also concerns for PZ effects on the thyroid from exposure during development (Marty et al., 2012; US EPA, 2017b). The PZ mode of action (MOA) involves activation of nuclear receptors and induction of liver enzymes as seen with phenobarbital treatment in rodents (Braeuning et al., 2014; Elcombe et al., 2014; LeBaron et al., 2014). Acute exposure activates xenobiotic sensors (e.g., CAR, PPARα and PXR) (Angrish et al., 2016; LeBaron et al., 2014) that heterodimerize with RXR to bind at PXRE (Sugatani et al., 2004) or PPRE (Viswakarma et al., 2010; Wang and Negishi, 2003) to initiate expression of the CYPs and other proteins involved in lipophilic xenobiotic metabolism (Michalik et al., 2006). CYP4A10, CYP2B10 (CAR-associated), CYP1A1 (AhR-associated nuclear receptor) and CYP3A11 (PXR-associated) are the primary P450s involved in PZ metabolism (LeBaron et al., 2014). An acute oral gavage neurotoxicity study in adult rats showed an increase in landing foot splay (female) and decrease in motor activity (males and females) at the LOEL of 40 mg/kg/day (US EPA, 2015c). There is not currently an MOA for acute PZ neurotoxicity and there were no other studies in the database showing evidence of neurotoxicity regardless of animal strain or treatment duration. However, nuclear receptor and CYP induction would have occurred to metabolize and eliminate PZ, since the effects were reversed by observation day 2. An ENEL of 4.0 mg/kg/day, achieved by adding an uncertainty factor of 10 to the LOEL (40 mg/kg/day), was determined by the US EPA to be the acute oral value for PZ (US EPA, 2015c) (Table 1).

**US EPA ToxCast/Tox21 from the CompTox Chemical Dashboard**

ToxCast/Tox21 results on the CompTox Dashboard characterize chemical interactions with intended target families and their biological molecular targets (e.g., cell cycle, CYP [P450s], receptor-ligand binding, steroidogenesis) were reported as concentration at 50% maximum activity (AC50) (Judson et al., 2011). These chemical interactions can provide support for presumptive MOAs, AOPs and/or downstream toxicity (NAS, 2017). ToxCast/Tox21 data are from in vitro and zebrafish (in vivo) HTS assays from numerous vendors and platforms (US EPA, 2020a). Complete descriptions of assays are available in the Dashboard download for individual chemicals (Supplemental Tables 1-5; Summarized in Supplemental Table 6). Data for each assay are filtered from level 0 (raw file processing; vendor-specific) through 6 (cautionary flags attached to detect potential false negative/positive) in the ToxCast Pipeline (Filer et al., 2017; Sipes et al., 2017; Williams et al., 2017). The CompTox Dashboard also presents graphical depictions of each active ToxCast/Tox21 concentration-response as well as the selection of a winning dose-response model and AC50 (Browne et al., 2015). Data characterizing the winning model include notations related to cautionary flags and the cytotoxicity limit (examples in Supplemental Figure1) (Judson et al., 2016; Williams et al., 2017). The cytotoxicity limit occurs in a narrow range of concentrations where assay results may indicate cell stress and cytotoxicity rather than compound-specific activity (“cytotoxicity-associated burst”) (Judson et al., 2016).

Biomolecular interactions in the ToxCast/Tox21 assays were measured for several intended target families (e.g., DNA binding, nuclear receptor, steroid hormone assay, etc.) and target enzymes or proteins (e.g., esterase; acetylcholinesterase inhibition AChE; CYP: cytochrome P450 CYP1A1; transferase: UDP-glucuronosyltransferase UGT1A1). All active hit-calls for CPF/CPFO, CB, ENDO and PZ were downloaded from the CompTox Dashboard for selection prior to use in the ToxPi ranking analysis. AC50s close to or greater than the cytotoxicity limit were less reliable but may be relevant to secondary effects from treatment (Judson et al., 2016).
Table 1: Acute In Vivo Endpoints for Chlorpyrifos-oxon, Chlorpyrifos, Endosulfan, Carbaryl, and Propyzamide

<table>
<thead>
<tr>
<th>Animal Strain</th>
<th>Treatment</th>
<th>Doses (mg/kg/d)</th>
<th>Acute Effect</th>
<th>Acute Endpoints mg/kg/day</th>
<th>Adjusted BMD&lt;sub&gt;10&lt;/sub&gt;/LOEL&lt;sup&gt;a&lt;/sup&gt; mg/kg/day</th>
<th>Ref&lt;sup&gt;b&lt;/sup&gt;</th>
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<td><strong>Chlorpyrifos-oxon</strong></td>
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<td>Pup: Sprague-Dawley Rat</td>
<td>Gavage PND</td>
<td>11</td>
<td>0 (corn oil), 0.005, 0.01, 0.05</td>
<td>↓ RBC AChE</td>
<td>BMD&lt;sub&gt;10&lt;/sub&gt; 0.093</td>
<td>ADJ BMD&lt;sub&gt;10&lt;/sub&gt; 0.0093</td>
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<tr>
<td>(Tested PND 11)</td>
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<td><strong>Chlorpyrifos</strong></td>
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<td>Pup: Sprague-Dawley Rat</td>
<td>Gavage PND</td>
<td>11</td>
<td>0 (corn oil), 0.005, 0.01, 0.05</td>
<td>↓ RBC AChE</td>
<td>BMD&lt;sub&gt;10&lt;/sub&gt; 0.5</td>
<td>ADJ BMD&lt;sub&gt;10&lt;/sub&gt; 0.05</td>
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<td>(Tested PND 11)</td>
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<td><strong>Carbaryl</strong></td>
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<td>Pup: Male Long-Evans Rat</td>
<td>Gavage, single PND 11 (Tested PND 11)</td>
<td>0 (corn oil), 3, 7.5, 15, 30</td>
<td>↓ RBC AChE</td>
<td>BMD&lt;sub&gt;10&lt;/sub&gt; 1.11</td>
<td>ADJ BMD&lt;sub&gt;10&lt;/sub&gt; 0.111</td>
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<td><strong>Endosulfan</strong></td>
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<tr>
<td>Pregnant New Zealand White Rabbit</td>
<td>Gavage GD 6-28</td>
<td>0 (corn oil), 0.3, 0.7, 1.8</td>
<td>Neurotoxicity signs from treatment day one</td>
<td>LOEL 1.8</td>
<td>NOEL 0.7</td>
<td>ADJ LOEL 0.18</td>
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<td><strong>Propyzamide</strong></td>
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<td>Adult: F344/DuCrI Rat</td>
<td>Gavage, single (corn oil)</td>
<td>40, 200, 600</td>
<td>↓ Motor activity (△)</td>
<td>LOEL 40</td>
<td>ENEL 4.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ADJ LOEL 4.0</td>
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| Abbreviations: ADJ: adjusted; BMD<sub>10</sub>/BMDL<sub>10</sub>: Benchmark Dose Lowest effect level/presumptive no effect level (10% benchmark response); ENEL: Estimated No Effect Level; GD: Gestation Day; LOEL: Lowest-observed-effect-level; NOEL: no-observed-effect-level; PND: Postnatal Day
<sup>a</sup> Lowest effect levels (BMD<sub>10</sub>/LOEL) were divided by a default interspecies factor of 10 to account for animal to human (interspecies) variability
<sup>c</sup> Oral ENEL = 40 (LOEL) × 10 (to extrapolate from a LOEL to an estimated no effect level) = 4.0 mg/kg NOEL was not achieved and the data were not modeled by BMD analysis (US EPA, 2015c).

Toxicological Prioritization Index Graphical User Interface (ToxPi GUI)

The ToxPi program (https://toxpi.org/ version 2.3, accessed 8-2020) (Marvel et al., 2018; Reif et al., 2010; Reif et al., 2013) was used to rank and prioritize chemicals for application in risk assessment processes. In this study, selected AC<sub>50</sub>s were used in the ToxPi program to visualize relative toxicity and ranking among the 5 pesticides. Since not all chemicals had active hit-calls for the same assays, in ToxPi version 2.3, data that are missing or invalid are disregarded (slice score = 0), and valid data would have a score between 0 and 1 (after slice scale modeling) (Marvel et al., 2018). The length of each ToxPi slice from the center of the circle is proportional to the normalized potency of the assay value (-log<sub>10</sub> (AC<sub>50</sub>) + 6) for the representative intended target family. This study used equally weighted slices of the same width irrespective of the number of measured AC<sub>50</sub>s. Predicted toxicity ranking was based on intended target family category and AC<sub>50</sub> potency. ToxPi ranks were compared to rank of in vivo endpoint values (Table 1) to determine concordance between predicted and measured toxicity. Assays included in the ToxPi analysis are described in Supplemental Tables 2b-6b.

High Throughput Toxicokinetics

Selected AC<sub>50</sub>s (µM) with active hit-calls below the cytotoxicity limit and relevant to the MOA were used in the open access ICE program (Bell et al., 2020) to predict an in vivo EAD<sub>Human</sub> (mg/kg/day). Figures 4-8 show the assay selections for the ICE program input (supplemental Table 7). Human pharmacokinetic parameters (PK) were used in the 1 (1C) and 3 (3C) compartment and physiologically based toxicokinetic multicompartment (PBTK) models to convert the AC<sub>50</sub> values to EAD<sub>Human</sub> (Bell et al., 2020; ICE, 2020). The data inputs (selected AC<sub>50</sub>s, chemical CASRN), along with reverse dosimetry (Tan et al., 2007) generated the estimated EAD<sub>Human</sub> (mg/kg/day) (Supplemental Table 7).

Qualitative ranking of the fold difference calculations (Thomas et al., 2013) between the 1C, 3C or PBTK EAD<sub>Human</sub> and in vivo endpoints were represented graphically for each of the 5 chemicals by plotting in R package “ggplot2” (Wickham, 2016). They were further examined for RBC versus brain AChE inhibition adjusted endpoints for CPFO, CPF and CB in the following manner: 1) Fold differences were determined between EAD<sub>Human</sub> and ADJBMD<sub>10</sub>s based on the RBC AChE inhibition 2) The same was done for fold differences between EAD<sub>Human</sub>.
and ADJBMD<sub>10</sub>s based on the brain AChE inhibition; 3) Final determinations indicated which endpoint, RBC or brain AChE inhibition had the most predictive EAD<sub>human</sub> and which model best represented those results. AChE inhibition is a biomarker for human exposure to organophosphates and carbamates and is the key event in the MOA for anticholinesterase compounds (US EPA, 2000). Inhibition initially occurs in blood and US EPA Guidance Document on use of cholinesterase inhibition in risk assessment indicated that RBC measurements serve as a surrogate for brain AChE inhibition (i.e., inhibition of RBC AChE presumes occurrence of brain AChE inhibition) (Chen et al., 1999; Coban et al., 2016; US EPA, 2000). For each anticholinesterase pesticide in this study the brain BMD<sub>10</sub> was higher than the RBC BMD<sub>10</sub> for AChE inhibition, which would be expected since the pesticides initially enter the blood where RBC AChE is the first line of defense (Table 1) (Coban et al., 2016; US EPA, 2000).

Quantitative fold differences were calculated for each EAD<sub>human</sub> in each model and were reported as 3 separate categories: 1) Fold differences within one order of magnitude of the in vivo ADJBMD<sub>10</sub>/ADJLOEIs (<10-fold difference) were predictive; 2) those with a 1–2 order of magnitude (11–100-fold difference) were marginally predictive and 3) those with greater than 2 orders of magnitude (>100-fold) difference were not predictive.

**One Compartment IVIVE Model:** A single compartment in a population-based PK model assumed 100% chemical absorption (route not applicable; Bell et al. (2020)) and treatment at 1 mg/kg/day (Wetmore et al., 2012). Measured plasma concentration at steady state (Css µM) is simulated at the 50<sup>th</sup> (median) and upper 95<sup>th</sup> percentile by Monte Carlo distribution to incorporate interindividual variability. Hepatic metabolism and passive glomerular filtration of chemicals is the assumed form of elimination (ICE, 2020). The predicted oral 1C EAD<sub>human</sub> is related in a linear fashion to the in vitro AC<sub>50</sub> (µM) and inversely related to the Css (µM) (Wetmore et al., 2012), hence the upper 95<sup>th</sup> percentile Css was used as a conversion factor, along with reverse dosimetry (Tan et al., 2007) to provide a conservative 1C EAD<sub>human</sub> (mg/kg/day; 50<sup>th</sup> and 95<sup>th</sup> percentile Css data in Supplemental Table 9). The EAD<sub>human</sub> calculation for the steady state 1C model used in this study corresponded to the total chemical concentration administered (Casey et al., 2018; Wetmore et al., 2012) according to Equation (1):

\[
\text{Steady-state EAD}_{\text{human}} \space \text{mg/kg/day} = \frac{AC_{50}(\mu M)}{\text{50th percentile Css (µM)}} \times (1 \space \text{mg/kg/day} + \text{95th percentile Css (µM)})
\]

Equation (1)

**Three Compartment IVIVE Model:** The 3C model has perfusion rate-limited compartments (i.e., equilibrium is achieved rapidly for tissue, RBC and plasma compared to flow of blood) comprised of gut, liver, and rest-of-body (e.g., fat, brain, bones). The “Solve_3comp” model is from the open access high-throughput TK-R package that calculates plasma concentration over time (httk v 1.9.2) (Pearce et al., 2017). An oral dose of 1 mg/kg/day in an acute interval (24-hr) was used to calculate maximum human plasma concentration (C<sub>max</sub> µM) at the 50<sup>th</sup> percentile using the average values for the PKTK parameters. Selected inputs (AC<sub>50s</sub> and chemical CASRN), C<sub>max</sub> and reverse dosimetry produced acute oral EADs (mg/kg/day). Hepatic metabolism and passive glomerular filtration of chemicals is the assumed form of elimination (Bell et al., 2020; ICE, 2020).

**PBTK Model:** The “Solve_pbtk” multicompartiment (gut, artery, vein, lung, liver, kidney, rest-of-body) function calculates C<sub>max</sub> at the 50<sup>th</sup> percentile using the average values for the PKTK parameters over time (httk v 1.9.2; Bell et al. (2020); Pearce et al. (2017)). Each compartment is perfusion rate-limited and has mass balance differential equations describing rate of change for quantity of chemical. The model used median PBTK parameters to calculate C<sub>max</sub> (µM) at an acute oral dose of 1 mg/kg/day for 24 hours. Selected inputs (AC<sub>50s</sub> and chemical CASRN), C<sub>max</sub> and reverse dosimetry produced acute PBTK EADs (mg/kg/day).

Both the “Solve_3comp” and Solve_pbtk” models, used the C<sub>max</sub> in the reverse dosimetry calculations as follows in Equation (2) (ICE, 2020):

\[
\text{Acute Oral EAD}_{\text{human}} \space (mg/kg/day) = \frac{AC_{50}(\mu M)}{\text{24hr exposure}} \times (1 \space \text{mg/kg/day} + C_{\text{max}} (\mu M))
\]

Equation (2)

Visual depictions of and calculations for each model are in Pearce et al. (2017).

**RESULTS**

**ToxCast/Tox21 Active Hit-Calls**

The ToxCast/Tox21 data downloaded from the CompTox Chemicals Dashboard (US EPA, 2020a) are summarized in Table 2 (Data download: Supplemental Tables 2a-6a). The pesticides in this study were tested in 854-980 assays from 14 different vendors (Supplemental Table 1), but only 11-33% of the total assays had active hit-calls. Further, most of the active hit-calls had cautionary flags (Filer et al., 2017; Sipes et al., 2017; Williams et al., 2017). These potential false positives were reported as noisy data (low signal-to-noise ratio), low efficacy (i.e., capacity of a drug to activate or inactivate a receptor), borderline active (only at highest concentration above baseline), or confounding by overfitting the data (Browne et al., 2015; US EPA, 2020a). Of the total active hit-calls 32 to 68% were not selected for ToxPi because their activities were based on background measurements, cell viability/toxicity (Judson et al., 2016), were false positives or were otherwise unassociated with MOAs or other toxicity pathways.
Vendors with the greatest number of active hit-calls below the cytotoxicity limit were from Novascreen (NVS; cell-free) and CellzDirect (CLD; primary human liver cells) and were related to liver metabolic processes for all 5 chemicals (Supplemental Tables 2b-6b). Other intended target families with active hit-calls below the cytotoxicity limit, included estrogenic (CPFO, ENDO), androgenic (ENDO, PZ), neurotoxicity (CPFO, ENDO, CB) and steroid hormone (CPF, PZ). Active hit-calls with cautionary flags were selected for ranking in ToxPi given the following considerations: 1) Appearance of the dose-response curve; 2) relevance of the assay to the primary MOA as well as secondary effects; 3) if the flagged AC_{50} is below the cytotoxicity limit and there are more than one active hit-call for a similar target, then the data may be selected; 4) Selected assays had less than 3 flags, and AC_{50} value greater than the lowest concentration screened (Friedman et al., 2019). Assays with cautionary flags indicating the results were likely false positives were excluded from the ToxPi and ICE model analyses.

Chlorpyrifos-oxon: The intended target families associated with the CPFO metabolic pathway included nuclear receptors (PXR, PPAR, FXR, RXR and CAR) involved with CYP induction (Chang et al., 2003; Holick, 2005; Li et al., 2017; Michalik et al., 2006; Wang and Negishi, 2003), that may be specific to CPFO metabolism (Supplemental Figure 1; Supplemental Table 2a). Some were below the cytotoxicity limit of 11.72 µM, including many CYPs related to the MOA: CYP2C19, CYP2B6 (Foxenberg et al., 2007), CYP1A2 (Foxenberg et al., 2011) and AChE inhibition (Testai et al., 2010)). The cell-free Novascreen assay for BuChE (NVS_ENZ_hES) had a cautionary flag (AC_{50} below the lowest concentration tested) and was eliminated for use in ToxPi and ICE model analyses because the dose response curve indicated results were likely false positive.

There were active hit-calls for nuclear receptor estrogenic (8), androgenic (4), thyroid (2) and neurotoxicity pathways (4), some with AC_{50}s below the cytotoxicity limit (3 estrogenic, 1 neurotoxicity). One estrogen receptor assay (ATG_Era_TRANS_up) appeared to be a false positive based on the cautionary flag (hit-call potentially confounded by overfitting: Only highest conc above baseline, active). It also had an unacceptable curve appearance on the CompTox Dashboard; hence, it was not used in the ToxPi or ICE model analyses. The
neurotoxicity endpoints related to dopamine target (DRD1) and opiate receptors have been associated with CPF treatment (Aldridge et al., 2005; Slotkin and Seidler, 2010). Active hit-calls DRD1 and GHB (Tspan1 gene) are associated with autism spectrum disorder (Li et al., 2017) which is also linked to CPF exposure (De Felice et al., 2015; Lan et al., 2019). There were two mitochondria assays that, although the AC₅₀s were above the cytotoxicity limit and had only 50% efficacy, were selected for use in ToxPi because CFPO is a mitochondrial toxicant (Cole et al., 2011).

**Chlorpyrifos:** Of the active hit-calls for liver metabolism: nuclear receptors (i.e., PXRE, FXR, Ahr, RXR, CAR), CYPs (CYP1A1, 1A2D, 2B6, 3A4, 2C19) and transfer factor (UGT1A1), a few had AC₅₀s below the CPF cytotoxicity limit of 15.74 µM (Table 2, Supplementary Figure 1, Supplementary Table 3a) (Michalik et al., 2006; Wang and Negishi, 2003). The cell-free Novascreen assay for BuChE inhibition had an active hit-call, although it is primarily inhibited by CFPO (Testai et al., 2010). RBC AChE inhibition, as a sensitive endpoint, is preferable to BuChE inhibition because plasma has a mix of AChE and BuChE, where RBCs have only AChE (US EPA, 2000). As stated previously, RBC AChE inhibition also serves as a surrogate for brain AChE inhibition (US EPA, 2000).

There were active hit-calls with the estrogen (7), progesterone (1), androgen (5), thyroid (2) and steroid hormone (8) targets but none was below the cytotoxicity limit. CPF was tested in the Endocrine Disruptor Screening Program (EDSP: Tier 1) for potential effects on the estrogen, androgen and thyroid pathways, but based on the weight-of-evidence, it was not considered to be an endocrine disruptor (https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-tier-1-screening-determinations-and). An estrogen nuclear receptor assay by Odyssey Thera (OT: OT_ER_ERaERa_1440) had 3 cautionary flags (<50% efficacy: hit-call potentially confounded by overfitting: Borderline active) and graphically appeared to be a false positive, therefore it was not included in the ToxPi or ICE model analyses (Supplemental Table 3b). While endocrine ToxCast/Tox21 results may be unrelated to the primary CPF toxicity, depending on exposure, disruptions of these targets may have effects on endocrine parameters during development.

A single mitochondria assay had an active hit-call with CPF and although it was above the cytotoxicity limit, with 2 cautionary flags, it was retained for ToxPi because CPF is a mitochondrial toxicant (Park et al., 2017; Singh et al., 2018; Yamada et al., 2017).

**Carbaryl:** The intended target families in the CB metabolic pathway included DNA Binding (Ahr) and nuclear receptor (PXRE, PPRE, PPAR and CAR) assays; all of which are potentially involved with the CYPs related to CB metabolism (Chang et al., 2003; Faucette et al., 2007; Ihunnah et al., 2011; Lin et al., 2009; Michalik et al., 2006) (Table 2, Supplemental Figure 1 and Supplemental Table 4a). The CYP LTEA_HepaRG_CYP2C19_up assay had 4 cautionary flags (only highest conc above baseline, active: Borderline active: <50% efficacy: Hit-call potentially confounded by overfitting) and appeared graphically on the CompTox Dashboard as a false positive. It was not included in the ToxPi or ICE model analyses. The Ahr assays (induces CYP1A1; Ko et al. (1996)) related to liver metabolism, had AC₅₀s below the cytotoxicity limit (11.72 µM). Nuclear receptor assays for PPRE, PXRE, PPAR and CAR were above the cytotoxicity limit. Of the active hit-calls for the CYP intended target families, most had AC₅₀s below the cytotoxicity limit, including CYP1A2 (Tang et al., 2002), CYP1A1 (Tang et al., 2002) and Ahr (through induction of CYP1A1 metabolic activation; Ko et al. (1996)). These targets are associated with the CB MOA.

Esterase intended target family had active hit-calls for AChE inhibition which is focal to CB metabolism and toxicity (Blacker et al., 2010; CDPR, 2014). While AChE inhibition is rapidly reversed with CB, there remains concerns for non-cholinergic long-term effects on the developing brain, even after a single administration (Lee et al., 2015). For example, an assay for the serotonin-binding transporter (SERT) in the brain had an AC₅₀ below cytotoxicity limit. CB effects on this transporter is associated with increased stress sensitivity and stress-related disorders in rats (Brivio et al., 2019) as well as effects on motor activity and cognition (Piper et al., 2005) (Table 6). CB was also active with oxidoreductase monoxygenases (MAO) receptors that affect pathways in the liver and the brain (Beliaev et al., 2009; Gaweńska and Fitzpatrick, 2011).

CB was tested in EDSP because it was shown to have potential for effects on reproduction in females, and thyroid, testes, pituitary and adrenal glands (reviewed in CDPR (2014)) but the weight of evidence did not support CB as having endocrine disrupting activity (US EPA, 2015a). The nuclear receptor and oxidoreductase active hit-calls associated the estrogen (7), androgen (3), and steroid hormone (5) pathways exceeded the cytotoxicity limit and their activity was likely incidental.

**Endosulfan:** ENDO metabolic pathways in liver had active hit-calls for targets that were directly relevant to the MOA (e.g., CYP2B6, CYP3A4, FXR, PXRE, CAR, PBREM [phenobarbital response enhancer module], UGT1A). Many had AC₅₀s at or below the cytotoxicity limit (12.31 µM) (Table 2; Supplemental Figure 1 and Supplemental Table 5a-b) (Bebe and Panemangalore, 2003; Casabar et al., 2006; Ihunnah et al., 2011; Pavek and Dvorak, 2008). Two nuclear receptor assays and one CYP assay appeared to be false positives with unacceptable dose-response curves on the CompTox Dashboard (LTEA_HepaRG_CYP2C19_up, NVS_NR_hCAR_Antagonist, ATG_PBREM_CIS_up) and they were excluded from the ToxPi or ICE model analysis.
ENDO had active hit-calls for nuclear receptor target families related to estrogen (11), progesterone (1), and androgen metabolism (9), many of which had AC₅₀ below the cytotoxicity limit (Supplemental Table 6b). It is well-documented that in vivo ENDO treatment affects estrogenic and androgenic pathways (Silva and Gammon, 2009; Silva et al., 2015).

The thyroid target had active hit-calls, although ENDO is generally not known to interact with the thyroid receptor as a major pathway (CDPR, 2008; US EPA, 2002a). An acute high dose of ENDO in pubertal male rats (6.12 mg/kg/day) resulted in down regulation of thyroid stimulating hormone (TSH) along with decreased plasma concentrations (Caride et al., 2010). Caride et al. (2010) suggested that these effects were associated with pituitary toxicity, since prolactin, luteinizing hormone, growth hormone and TSH expressions were below the cytotoxicity limit. The DAT assays in human and rat were the only active hit-calls related to neurotoxicity but effects on this target are suggested to be associated with ENDO treatment (Jacob et al., 2008; Jia and Misra, 2007; Seth et al., 1986; Wilson et al., 2014).

Propyzamide: An intended target family in the PZ metabolic pathway included nuclear receptors (PXRE, PPRE, PPAR, PXR), with one AC₅₀ below the cytotoxicity limit (11.05 µM). These endpoints are potentially involved with induction of CYPs related to PZ metabolism in the liver (Chang et al., 2003; Ihunnah et al., 2011; LeBaron et al., 2014; Lin et al., 2009; Michalik et al., 2006) (Table 2; Supplemental Figure 1 and Supplemental Table 4). The CYP target family (CYP1A1, CYP1A2, CYP2B6 [PXR associated], CYP2A1, CYP3A4) had active hit-calls for CYPs that are known or likely to be involved with PZ metabolism (LeBaron et al., 2014). CYP1/2 families can be activated in liver by lipophilic xenobiotics and repeated dose studies with PZ in rodents have shown increased hepatocellular hypertrophy associated with CYP induction (LeBaron et al., 2014; Papineni et al., 2015; Rasoulpour et al., 2015).

The transferase intended target families had active hit-calls for UGT1A1, associated with elimination of thyroxine (T₄) in rodents, and disruption of the hypothalamus-pituitary-thyroid (HPT) axis (Hood et al., 2003; Papineni et al., 2015). This MOA involves PZ induction of UGT1A1, which then conjugates to T₄ leading to increased elimination (Papineni et al., 2015). Sustained thyroid stimulation by TSH will lead to thyroid follicular cell hypertrophy, hyperplasia, and tumorogenesis. UGT1A1 assays had AC₅₀ below the cytotoxicity limit. PZ also had an active hit-call below the cytotoxicity limit for sulfotransferase (SULT) targets. Sulfotransferases are associated with androgen and estrogen metabolism (Ihunnah et al., 2011; Khor et al., 2010).

PZ treatment leads to disruption of the hypothalamus-pituitary-gonad (HPG) axis leading to androgen imbalance (Rasoulpour et al., 2015). Further, estrogen (1), progesterone (1), androgen (3), and steroid hormone (8) assays had active hit-calls below the cytotoxicity limit. One of the three androgen assays (ACEAR agonist 80hr) had 4 cautionary flags (AC₅₀ < lowest concentration tested: < 50% efficacy: Hit-call potentially confounded by overfitting: Borderline active: Only one conc above baseline, active). The dose-response curve on the CompTox Dashboard indicated a false positive and it was not used in the ToxPi or ICE model analyses. The other endocrine assays were likely non-specific interactions due to the lipophilicity of PZ.

The ToxPi Program

ToxPi program inputs were AC₅₀ for the selected assays that could be associated with the known MOAs or other toxic endpoints (Supplemental Tables 2b-6b). The ToxPi model ranked the five chemicals from lowest to highest toxicity (rank 1 to 5) for the intended target family categories designated by the CompTox Dashboard (Figure 1 and Table 4). The dimensionless Toxicity Score is the sum of all the individual intended target family scores divided by the 11 categories (i.e., CYP, DNA Binding, Esterase, GCPR, Growth Factor Inhibitor, Mitochondria, Nuclear Receptor, Steroid Hormone, Transferase, Transporter, Oxidoreductase). For example, using CPFO data from Figure 1 in Equation (3) (Marvel et al., 2018):

**CPFO Toxicity Score** = ([Intended Target Family scores [5.6316]) ÷ (No. of categories [11])] × 0.5120 Equation (3)

The scores then determined the ranks, with the highest score having the highest rank and greatest toxicity (Reif et al., 2010). Output data with the rank from low (least toxic) to high (most toxic) and Toxicity Score (Figure 1: shown as color-coded slices) were as follows: PZ (Rank 1: 0.2633 < CPF (Rank 2: 0.3271) < CB (Rank 3: 0.3800) < ENDO (Rank 4: 0.4548) < CPFO (Rank 5: 0.5120).

The acute in vivo ranking as determined by the LOEL/BMD₁₀S was as follows: PZ (Rank 1: ENEL 4.0 mg/kg/day) < ENDO (Rank 2: LOEL 1.8 mg/kg/day) < CB (Rank 3: BMD₁₀ RBC AChE/Brain AChE: 1.11/1.46 mg/kg/day) < CPF (Rank 4: BMD₁₀ RBC AChE/Brain AChE: 0.5/1.42 mg/kg/day) < CPFO (Rank 5: BMD₁₀ RBC AChEB/Brain AChE: 0.093/1.06 mg/kg/day). Table 3 shows concordance between ToxPi ranking and in vivo endpoints for CPFO, CB and PZ, however ENDO and CPF were not. Since the ToxPi model was based on ToxCast/Tox21 AC₅₀, as well as selection of assays with active hit-calls a certain degree of variability was introduced. Further, three of the in vivo lower effect levels had endpoints that were almost equivocal (i.e., ENDO: 1.8 mg/kg/day; CB: 1.11/1.46 mg/kg/day; CPF: 0.5/1.42 mg/kg/day). Variability in the in vivo endpoints was dependent on expert
decisions by regulatory agencies (i.e., US EPA, CDPR), that must rely on protocols (e.g., treatment levels, dose spacing, duration, animal strain, chemical route of administration and other factors) determined by pesticide registrants or study authors. ToxPi, concordant in 3 of the 5 pesticide rankings, was predictive considering the numerous variables in both in vivo and in vitro models (Table 3).

**Table 3: Concordance Between ToxPi Score/Ranking and Acute In Vivo Low Effect Level Rank**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Toxicity Score</th>
<th>ToxPi Toxicity Rank</th>
<th>In Vivo (mg/kg/day)</th>
<th>In Vivo Toxicity Rank</th>
<th>Concordance</th>
<th>ToxPi Predictions and Acute In Vivo POD Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZ</td>
<td>0.2633</td>
<td>1</td>
<td>40</td>
<td>1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>CPF</td>
<td>0.3271</td>
<td>2</td>
<td>0.5/1.42^a</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB</td>
<td>0.3800</td>
<td>3</td>
<td>1.11/1.46^a</td>
<td>3</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ENDO</td>
<td>0.4548</td>
<td>4</td>
<td>1.8</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPFO</td>
<td>0.5120</td>
<td>5</td>
<td>0.093/1.06^a</td>
<td>5</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Green Boxes indicate concordance and red boxes indicate no concordance

Toxicity ranking: 1 (least toxic); 5 (most toxic)

^a - BMD\textsubscript{10} for RBC/brain AChE inhibition, respectively

**Figure 1:** ToxCast/Tox21 AC\textsubscript{50} values for all ENDO, CPFO, CPF, CB and PZ active hit-calls are categorized and scored by intended target family. The top panel has the color codes for ToxPi Intended Target Families. The lower panel shows the calculated scores for each intended target family, the overall Toxicity Scores, and the rank, which goes from high (5=most toxic) to low (1=least toxic). Selected active hit-calls included assays associated with primary MOA or toxic endpoint determinations. Toxicity Score is calculated as:

**Toxicity Score = (ΣIntended Target Family scores) ÷ (number of Intended Target Family categories)**
High Throughput Toxicokinetics

Assays selected for the ICE 1C, 3C and PBTK modeling are shown in Table 4 (Supplemental Table 8). AC50 values were all below the cytotoxicity limit and there were 4, 14, 7, 10 and 6 AC50s for CPF, CPFO, CB, ENDO, and PZ, respectively as model inputs. Selection also involved elimination of identical assays performed at different durations for each chemical. For example, the AC50 for CYP2B6 at 24 hours (CLD_CYP2B6_24hr) with CPFO was 0.404 µM but at 48 hours it was 11.2 µM. The selection was made for the lower value, meaning that at 24 hours, this assay is a more sensitive endpoint.

Qualitative Comparisons Among Model Predictions

Qualitative data analysis demonstrated visually the performance among the 1C, 3C and PBTK models. Figures 2 and 3 show the "spread" of data as a means to compare the performance of the different models. The “ggplot2” calculated on R-studio provided an ideal graphical depiction of the overall fold differences per chemical as quartiles in a box-and-whiskers format with an overlay of individual assay points (Pearce et al., 2017; Wickham, 2016).

One Compartment Model: The 1C model had numerous outliers beyond the median value (Figures 2-3), indicating that it was not the best selection for modeling our data. Qualitatively, CPFO EADHuman fold differences had the most variability and the most outliers (least predictive), where PZ EADHuman fold differences had no outliers (highly predictive) (Figure 2).

3 Compartment and PBTK Models: Qualitative visual EADHuman predictions for both 3C and PBTK models showed the most fold difference variability around the median with CPFO and the least with both ENDO and PZ. It was also evident that the magnitude of qualitative differences was dependent on the number of assay inputs (Figure 2). However, ENDO and PZ do not need to be activated to a toxic metabolite; as such, their AC50s might be more representative of target activity. From a qualitative assessment, it is evident that the 3C and PBPK models provided equivocal fit-for-purpose in determining EADHuman predictions.

Figure 2: Quantitative fold difference comparison among models and pesticides. Box and whiskers plots show fold difference median, minimum and maximum values in quartiles. Colored circles indicate fold differences for individual assays in the three models.

Figure 3: Qualitative fold differences among the 3 models for EADHuman predictions and in vivo RBC or brain AChE ADJBMD10s are shown along with direct RBC vs. brain AChE inhibition fold difference comparisons. Box and whiskers plots show fold difference median, minimum and maximum values in quartiles. Colored circles indicate relative values for individual assays in the three models.
**Quantitative Comparisons Among Model Predictions**

Overall quantitative fold differences for each model and pesticide are shown in Figures 4-8. Quantitative fold differences are categorized as <10, 11 to 100 and greater than 101. Data are described below as number of assays in a category per total assays.

**One Compartment Model:** Quantitatively the 1C model was predictive of EAD\textsubscript{Human} (< 10-fold difference) in 6/6 assays for PZ and 4/14 assays for CPFO for the brain AChE ADJBM\textsubscript{D10} endpoint (Figures 4-8; Supplemental Table 9). 1C EAD\textsubscript{Human} with fold differences between 11 and 100 were 4/14 CPFO (RBC) and CPFO 10/14 (brain), 2/4 CPF (brain), 1/7 CB (brain) and 6/10 ENDO. 1C with EAD\textsubscript{Human} with fold differences greater than 101 were 10/14 CPFO (RBC), 4/4 CPF (RBC) and 2/4 CPFO (brain), 7/7 CB (RBC) and 6/7 CB (brain) and 4/10 ENDO.

**Three Compartment Model:** The 3C model was quantitatively predictive of EAD\textsubscript{Human} in 3/14 CPFO (RBC) and 14/14 CPFO (brain), 1/4 CPF and (RBC), 2/4 CPF (brain), 1/7 CB (brain), 10/10 ENDO and 3/6 PZ (Figures 4-8; Supplemental Table 9). 3C EADs with fold differences between 11-100 were 11/14 CPFO (RBC) and 14/14 CPFO (brain), 3/4 CPF (RBC) and 2/4 CPF (brain), 7/7 CB (RBC) and 6/7 CB (brain) and 3/6 for PZ.

**PBTK Model:** Quantitatively the PBTK model was predictive of EAD\textsubscript{Human} in 3/14 CPFO (RBC) and 12/14 CPFO (brain), 2/4 CPF (brain), 10/10 ENDO and 5/6 PZ (Figures 4-8; Supplemental Table 9). PBTK EADs with fold differences between 11 and 100 were 9/14 CPFO (RBC) and CPFO 2/14 (brain), 4/4 CPF (RBC) and 2/4 CPF (brain), 1/6 PZ. PBTK with EADs with fold differences greater than 101 were 2/14 CPFO (RBC).
**Figure 4:** Data identify fold difference between predicted EAD\textsubscript{Human} for RBC and Brain AChE and their ADJBMD\textsubscript{10} \textit{in vivo} endpoints using ToxCast/Tox21 AC\textsubscript{50} and ICE models.

**Figure 5:** Data identify fold difference between predicted EAD\textsubscript{Human} for RBC and Brain AChE and their ADJBMD\textsubscript{10} \textit{in vivo} endpoints using ToxCast/Tox21 AC\textsubscript{50} and ICE models.
**Figure 6:** Data identify fold difference between predicted EAD\textsubscript{Human} for RBC and Brain AChE and their ADJBMD\textsubscript{50} in vivo endpoints using ToxCast/Tox21 AC\textsubscript{50} and ICE models.

**Figure 7:** Data identify fold difference between predicted EAD\textsubscript{Human} for neurotoxicity and the ADJLOEL in vivo endpoint using ToxCast/Tox21 AC\textsubscript{50} and ICE models. The tables were divided based on the fold difference scale for 1C (24 to 309) compared to the 3C and PBTK models (0.03 to 11).
DISCUSSION

In pesticide risk assessment, the traditional in vivo Health Effects Test Guideline studies are designed to observe a low or no effect dose level and, if possible, identify a targeted system in a dose-related manner. This type of design can have large gaps between doses and events leading to a “tipping point,” where an organism may no longer be able to recover from chemical insult, can be masked (Frank et al., 2018; Saili et al., 2020; Shah et al., 2016). However, currently there are numerous open access computational tools available that can help reveal and prioritize chemical activities at their tipping points prior to irreversibly overwhelming metabolic systems. More sophisticated tools are in development that will replace animal studies for pesticide risk assessment by 2035 (Nyffeler et al., 2020; US EPA, 2020a). The user-friendly open access tools, such as ToxCast/Tox21 and ToxPi are used to identify MOAs, AOPs, and previously unknown molecular interactions that may impact pesticidal effects on human health (Browne et al., 2015; Kleinstreuer et al., 2017; Kleinstreuer et al., 2013; Kleinstreuer and Knudsen, 2011; Pham et al., 2016). Even if a molecular event is minimal, in combination with other disruptions it may prove critical to the understanding of chemical action that can support and facilitate the risk assessment process.

The acute in vivo endpoints used in this study were obtained from open access documents, including Health Effects Test Guideline studies as reported in risk assessments by the California Department of Pesticide Regulation (CDPR, 2008, 2014, 2018) or the US EPA (US EPA, 2011, 2015c, 2017a). BMD10 analyses for CPF, CPFO and CB were performed by the US EPA and had the advantage of a modeled endpoint, where ENDO and PZ data relied on registrant-determined dose levels for endpoint determination (i.e., NOEL/ENEL/LOEL). An ENEL could be considered an artifact of dose selection and the uncertainty factor used to derive it. On the other hand, comparative data across several laboratories indicate that motor activity is quite variable, such that an effect of more than 30% difference from control must occur to distinguish signal from noise (Crofton et al., 1991; Wolansky et al., 2006).

Open access ToxCast/Tox21 assays produced active hit-calls that supported, for the most part, the molecular mechanistic functions related to each pesticide. For example, many active hit-calls were for targets associated with reported liver metabolic pathways, as well as pathways for neurotoxicity and endocrine disruption for ENDO, CPFO, CB and PZ. Several, albeit a small percentage of overall active hit-calls, were below the cytotoxicity limit and supported the use of the ToxCast/Tox21 assays to accurately predict potential pathways where disruptions could lead to adverse effects.

CPF had the fewest active hit-calls and none was specific to the MOA, which strengthened previous findings requiring activation of CPF to CPFO for adverse effects to occur (i.e., AChE inhibition). PXRE/PXR are xenosensors that are activated as adaptive responses leading to chemical activation or detoxification, but they are not specific to the CPF MOA (Kliwer, 2003; Knudsen et al., 2013; Knudsen et al., 2011). CPF results also indicated that human primary cells or human-derived cell lines and cell-free biochemical assays may not adequately allow for chemical interaction at the target at a concentration that would be observed in vivo. CPFO and CB inhibited AChE and esterase activities for both compounds were below the cytotoxicity limit, indicating a likely specificity. The two cell-free Novascreen assays are the only AChE assays available in the ToxCast/Tox21 battery. The results for CPF show that this compound is not likely to inhibit AChE without metabolic activation.

The primary metabolic pathways for ENDO in liver (e.g., CYP2B6 and CYP3A4/5) had active hit-calls but the GABAAR, critical to the MOA, did not (Kamijima and Casida, 2000; Ratra et al., 2001). The GABAARβ3 subunit is the major site of binding for this pesticide (Jacob et al., 2008; Kamijima and Casida, 2000; Ratra et al., 2001) but there was not an assay for this endpoint on the CompTox Dashboard. Only one GABAAR assay (NVS_LGIC_bGABARa5; inactive) was performed with ENDO but it was not active. Although neurotoxicity assays were for DA and DAT, both of which are associated with ENDO toxicity in vivo and in vitro (Jacob et al., 2008; Jia and Misra, 2007; Lakshmanan et al.; Seth et al., 1986; Wilson et al., 2014).

The primary MOA for PZ is initiated in the liver and while there were numerous active hit-calls involving liver metabolism, ToxCast/Tox21 assay results also indicated effects on endocrine endpoints that also occur in vivo (LeBaron et al., 2014; Papineni et al., 2015; Rasoulpour et al., 2015). However, the lack of activity with thyroid and
testosterone assays supported the in vivo data indicating that effects on these hormones occur through disruption of the HPG/HPT axes and are secondary to effects in the liver (Papineni et al., 2015; Rasoulpour et al., 2015).

The open access ToxPi model used in this study was dependent on individual ToxCast/Tox21 AC50s for selected assays within relevant intended target families. Therefore, the ranking was only as predictive as the potency (AC50 value) of the active hit-calls for each chemical/target. While the selected assays in the intended target families may be associated with the MOAs, their relative AC50 values may or may not represent what would occur in vivo. For example: 1) cell-free assays involve chemical to target protein activity that is not physiological; 2) many of the AC50s were above the cytotoxicity limit, thereby rendering the relevance of those assay results as undetermined; 3) while selected active hit-calls identified many of the enzymes and proteins in the metabolic pathways supporting the mechanistic weight-of-evidence for each pesticide, these targets would also be active for a variety of lipophilic compounds; 4) active hit-calls may be false positives based on cautionary flags due to automated processes that are prone to error (Filer, 2019; Filer et al., 2017; Ryan, 2017). However, the use of ToxCast/Tox21 AC50s is supported by the knowledge that ToxPi is a model for generalized ranking (prioritizing) chemical toxicity.

ToxPi predicted Toxicity Scores and ranks were least to most potent: PZ < CPF < CB < ENDO < CPFO, while the in vivo toxicity lowest effect levels were as follows: PZ < ENDO < CB < CPF < CPFO. The comparison showed concordance for 3 of 5 compounds, which is fairly predictive considering the following potential confounders: 1) number of active hit-calls relevant to the MOA or associated toxic effects, which is dependent on metabolic capability of the cells, assay design and other limitations of cell or cell-free systems (NAS, 2017); 2) selection of relevant, MOA-related ToxCast/Tox21 assays for ToxPi analysis; 3) Variability of the in vivo endpoints are dependent on study protocols determined by Health Effects Test Guidelines (US EPA, 1998), pesticide registrants or study authors. Due to the degree of variability among in vitro and in vivo parameters, ToxPi ranking may not be exact.

The open access ICE program provided the 3 IVIVE/PBTK models for this study (Bell et al., 2020; Bell et al., 2018). The 1C linear model was comparable to a constant infusion exposure (Css 95th percentile) and was only predictive for PZ at less than 10-fold difference from in vivo endpoints (Figures 2-8). The oral acute PBTK model for exposure at Cmax was less predictive than the 3C model. One explanation could be that the PBTK model, with multiple compartments, incorporating a wide range of mechanistic information and complexity may also introduce increased variability with each added PBTK parameter. The 3C model may have a more appropriate fit-for-purpose, considering the data available in this study (Sipes et al., 2017). That is, the PBTK model, with higher data requirements may require more precision than we needed.

Qualitatively fold differences between EADhuman predictions and acute oral in vivo ADJ8MD10/ADJLOE values for each compound and model were graphed in quartiles and indicated that rank among models and chemicals was: 1C < PBTK < 3C. Quantitative fold differences for each pesticide provided a more in-depth evaluation of the EADhuman predictions for each model. Divided into three categories of fold difference (<10; >11<100; >101), three of four of the direct acting pesticides (CPFO [brain], ENDO, PZ) had the most predictive EADhuman, primarily in the 3C model, but also in the 1C model where PZ EADhuman were 100% predictive. The exception was CB which had almost 100% non-predictive EADhuman in the 1C and only one predictive EAD (brain AChE inhibition endpoint) in the 3C model. EADhuman predictions for AChE inhibitors (CPFO, CPF, CB), especially CB was not as well captured in the ToxCast/Tox21 assays since liver metabolism would likely be the initial pathway but not the toxic pathway for these compounds. The most striking overall observation was the difference among the three ICE models indicating that model refinement/complexity did not necessarily provide more accurate results.

In pesticide risk assessment, where open access in vivo Health Effects Test Guideline results were available, ToxCast/Tox21 data, ToxPi ranking and ICE modeled EADhuman predictions provided support for the presumptive MOAs and mechanistic pathways in most cases. These tools, as applied in this case study, were easily accessible, user friendly and provided a variety of methods for assessing chemical toxicity. They will prove to be even more useful as technology progresses in not only prioritizing pesticides for risk assessment but also providing insights into mechanistic data often lacking in traditional testing.

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