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Effect of *in vivo* nicotine exposure on chlorpyrifos pharmacokinetics and pharmacodynamics in rats

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ABSTRACT

Routine use of tobacco products may modify physiological and metabolic functions, including drug metabolizing enzymes, which may impact the pharmacokinetics of environmental contaminants. Chlorpyrifos is an organophosphorus (OP) insecticide that is bioactivated to chlorpyrifos-oxon, and manifests its neurotoxicity by inhibiting acetylcholinesterase (AChE). The objective of this study was to evaluate the impact of repeated nicotine exposure on the pharmacokinetics of chlorpyrifos (CPF) and its major metabolite, 3,5,6-trichloro-2-pyridinol (TCPy) in blood and urine and also to determine the impact on cholinesterase (ChE) activity in plasma and brain. Animals were exposed to 7-daily doses of either 1 mg nicotine/kg or saline, and to either a single oral dose of 35 mg CPF/kg or a repeated dose of 5 mg CPF/kg/day for 7 days. Groups of rats were then sacrificed at multiple time-points after receiving the last dose of CPF. Repeated nicotine and CPF exposures resulted in enhanced metabolism of CPF to TCPy, as evidenced by increases in the measured TCPy peak concentration and AUC in blood. However, there was no significant difference in the amount of TCPy (free or total) excreted in the urine within the first 24-h post last dose. The extent of brain acetylcholinesterase (AChE) inhibition was reduced due to nicotine co-exposure consistent with an increase in CYP450-mediated dearylation (detoxification) versus desulfuration. It was of interest to note that the impact of nicotine co-exposure was experimentally observed only after repeated CPF doses. A physiologically based pharmacokinetic model for CPF was used to simulate the effect of increasing the dearylation V_{max} based upon previously conducted *in vitro* metabolism studies. Predicted CPF-oxon concentrations in blood and brain were lower following the expected V_{max} increase in nicotine treated groups. These model results were consistent with the experimental data. The current study demonstrated that repeated nicotine exposure could alter CPF metabolism *in vivo*, resulting in altered brain AChE inhibition.

1. Introduction

Chlorpyrifos (CPF) is a widely utilized organophosphorus pesticide (OP), which has been extensively studied due to its potential neurotoxicity [1–3]. Although CPF is no longer registered for residential use in the US [4], there is still a high potential for human exposure, since it is widely utilized in agricultural applications [5]. Workers who are directly involved in agricultural operations may constitute a population who could face different risk from exposure to mixtures of chemicals [6]. The long-term goal of this research is to better understand the implications of the use of tobacco products on agricultural workers who may also be exposed to a range of insecticides. The primary mode of action for the toxicity of OPs, including CPF, involves the inhibition of acetylcholinesterase (AChE) activity [3,7]. Cholinesterases (ChE) are a broad class of ubiquitous enzymes that are present in many tissues including brain and muscle. CPF itself is a weak inhibitor of AChE, but it is bioactivated by cytochromes P450 (CYP450s) to the more potent inhibitor, chlorpyrifos-oxon (CPF-oxon). The inhibition of AChE by CPF-oxon results in an excessive accumulation of acetylcholine within the cholinergic synapse and neuromuscular junctions, thereby leading to a wide range of neurotoxic responses [8].

Chlorpyrifos is metabolized by CYP450s to several major metabolites, including CPF-oxon and 3,5,6-trichloro-2-pyridinol (TCPy) [9]. Once CPF is metabolized to TCPy, this metabolite can be directly excreted in the urine or undergo further conjugation prior to urinary excretion [10]. In addition, the conversion of CPF-oxon to TCPy is mediated by several esterases. While A-esterases, such as paroxonase-1 (PON-1), convert oxon to TCPy and are not inhibited

Abbreviations: OP, organophosphorus; CPF, chlorpyrifos; CPF-oxon, chlorpyrifos-oxon; TCPy, 3,5,6-trichloro-2-pyridinol; AChE, acetylcholinesterase; ChE, cholinesterase; CYP450, cytochrome P450; AUC, area-under-the-curve; PON-1, paroxonase-1; sc, subcutaneous; PBPK/PD, physiologically based pharmacokinetic and pharmacodynamic.

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by the oxon, whereas B-esterases, including AChE, BuChE and carboxylesterase (CaE), have a serine residue at the active site and are inhibited in the process. Since CYP450 plays a central role in both the activation and detoxification of CPF, changes in enzyme activity may have a profound impact on CPF dosimetry and the extent of esterase inhibition.

A number of studies have demonstrated the inductive effects of nicotine (the major component of tobacco products) on CYP450 metabolism both *in vivo* and *in vitro* [11,12]. Additionally, the influence of nicotine on physiological and pharmacodynamic processes, such as increases in heart rate, blood pressure, and energy expenditure, are well documented [13,14]. It has also been shown that prolonged exposure to even low concentrations of nicotine can cause desensitization of nicotinic receptors in the brain [15]. These diverse effects of nicotine exposure could subsequently modulate key internal pharmacokinetic and pharmacodynamic processes and alter the adverse effect due to exposures to OPs. There have been a few reports showing the combined effects due to co-exposures to CPF and nicotine [16,17], including one by Qiao et al. which suggested the combination of nicotine and CPF may result in increased thiobarbituric-acid-reactive species (TBARS) in developing rats [17].

We recently reported that repeated *in vivo* nicotine pretreatments impacted *in vitro* CPF metabolism in rat liver microsomes [18]. The influence of repeated pre-exposure to nicotine on CPF metabolism did not affect bioactivation to the CPF-oxon, but did result in increased dearylation of CPF to TCPy (1.9-fold induction of TCPy V_{max}). The current study was conducted to quantitatively assess potential pharmacokinetic changes in CPF and TCPy in blood and the pharmacodynamic ChE responses to CPF exposures in key tissues following pre- and co-exposures to nicotine. Levels of ChE inhibition in plasma and brain were measured to characterize changes in pharmacodynamic dose–response and also provide some qualitative perspective on the target tissue dose of CPF-oxon, which is too labile to measure analytically.

2. Materials and methods

2.1. Chemicals

Chlorpyrifos (CAS: 2921-88-2, 99% pure) and 3,5,6-trichloro-2-pyridinol (CAS: 6515-38-4, 99% pure] were kindly provided by Dow AgroSciences (Indianapolis, IN). Nicotine [as (−)-1-methyl-2-(3-pyridyl)pyrrolidine-(+)-bitartrate salt], the derivatizing agent, N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), along with Ellman reagents, 5,5′-dithiobis(2-nitrobenzoic acid) [DTNB] and acetylthiocholine chloride (ATC) were purchased from Sigma Chemical Company (St Louis, MO). The remaining chemicals and other solvents were reagent grade or better and were purchased from Sigma Chemical Company.

2.2. Animals

Adult male Sprague–Dawley rats (200–225 g, ca. 7 weeks old) were purchased from Charles River Laboratories, Inc. (Raleigh, NC). Prior to use, animals were randomly assigned to naïve and treatment groups and all animals were housed in solid-bottom cages with hardwood chips, and acclimated for 1 week in a humidity- and temperature-controlled room with a 12-h light/dark cycle. Rodent feed (PMI Certified Rodent Diet # 5002) and water were provided ad libitum. All procedures described in the present study were conducted in accordance with the guidelines for the care and use of laboratory animals in the NIH/NRC Guide and Use of Laboratory Animals, and were approved by the Institutional Animal and Care Use Committee (IACUC) of Battelle, Pacific Northwest Division.

2.3. Dose selection

There were three goals in selection of CPF dose, first that major metabolites could be quantified in plasma, second that cholinesterase inhibition could be measured in blood and brain, and finally so that subtle differences in cholinesterase inhibition could be detected. The selection of CPF doses employed in this study were based upon predictions from CPF PBPK model simulations in addition to previous experience and examination of ample literature regarding CPF extensively reviewed by Eaton et al. [7]. The repeated doses of 5 mg CPF/kg/day were predicted to elicit ∼75% cholinesterase inhibition in plasma and ∼50% inhibition in brain after 7 days of treatment, while they were not expected to result in extreme systemic cholinergic symptoms, based upon previous studies with repeated CPF treatments and dietary exposure in rats [19,20]. The oral single dose of 35 mg CPF/kg, equivalent to the total repeated dose, was expected to produce mild cholinergic symptoms, resulting in the similar levels of ChE inhibition in blood and brain with those from repeated 5 mg CPF/kg/day for 7 days, according to CPF PBPK model simulations (data not shown).

The dose and route for nicotine exposures (1 mg/kg/day for 7 days, sc) were based on nicotine CYP450 induction studies [11,12,21], due to the rapid metabolism of nicotine in rodent, these relatively high daily doses of nicotine are necessary to achieve blood nicotine concentrations comparable to those seen in smokers [22]. Doses of 1 mg nicotine/kg/day in rats were shown to produce similar CNS effects of nicotine found in smokers [23], and the pharmacological impacts of these nicotine doses compared to cigarette smoking have been extensively reviewed by Benowitz (1996, 2009) and Tricker (2006).

2.4. Animal treatment

Animals were randomly assigned to either the naïve group ($n = 4$) or CPF-treatment groups ($n = 4$–5 per group) with different post-dosing time points (1, 4, 8, 12 or 24 h) for sacrifice (Table 1). These CPF-treated groups included I) [CPF Repeat Dose]: subcutaneous (sc) saline + 5 mg CPF/kg (in corn oil, po) daily for 7 days; II) [Nicotine + CPF Repeat Dose]: 1 mg nicotine/kg/day + 5 mg CPF/kg/day (in corn oil, po) for 7 days; III) [CPF Single Dose]: saline for 7 days, and a single 35 mg CPF/kg (in corn oil, po) on the 7th day; and IV) [Nicotine + CPF Single Dose]: 1 mg nicotine/kg/day, for 7 days and a single dose of 35 mg CPF/kg (in corn oil, po) on 7th day. Rats were dosed with saline or 1 mg nicotine/kg (in sterile saline) subcutaneously (sc) at the nape in a dose volume of 1 ml/kg body weight, which demonstrated metabolic induction of CYP450s [18,21]. Oral CPF was dosed in corn oil (5 ml/kg b.w.) within 5 min of nicotine or saline dosing. Animals in the 24-h groups (both saline and nicotine treatments) were individually housed in plastic metabolism cages (Thermo Fisher Scientific, Rochester, NY) for at least 48 hr prior to the last CPF dose for acclimation. Urine was collected continuously with 12-h intervals post CPF treatment. Rats were euthanized by CO_2 asphyxiation at the specified time-points post-last CPF dosing (Table 1), and blood was collected via cardiac puncture into heparinized syringes. Plasma from each animal was prepared by centrifugation of blood at 1100 × *g* for 10 min. Immediately after blood collection, the brain was removed, and dry blotted, homogenized with nine volumes of ice-cold 0.1 M phosphate buffer (pH 7.4), all samples were stored at −80 °C until analyzed. The same volume of blood/urine from each individual animal and blank matrices from the naive animals were treated with 250 μL of NaCl-saturated 3N HCl. For analysis of conjugated TCPy in urine sample, urine aliquots were hydrolyzed by adding concentrated HCl and heated at 80° C for 1 h prior to extraction. Blood/urine samples were extracted 3× with 0.75 ml ethyl acetate, vortexed in a shaking incubator for 10 min, centrifuged to separate layers at 1100 × *g*

Table 1
Different treatment groups.

	Groups	1 mg/kg nicotine	Chlorpyrifos (CPF)	Post-last CPF dosing time-points
I.	CPF Repeat Dose	Saline 7 days, sc	5 mg/kg, po, 7 days	1, 4, 8, 12 or 24 h
II.	Nicotine + CPF Repeat Dose	Nicotine 7 days, sc	5 mg/kg, po, 7 days	
III.	CPF Single Dose	Saline 7 days, sc	35 mg/kg, po, once	
IV.	Nicotine + CPF Single Dose	Nicotine 7 days, sc	35 mg/kg, po, once	

Male S–D rats were treated with I) saline (sc) and 5 mg CPF/kg/day (in corn oil, po) for 7 days, II) both 1 mg nicotine/kg/day (sc) and 5 mg CPF/kg/day (in corn oil, po) for 7 days, III) saline (sc) for 7 days, followed by 35 mg CPF/kg (in corn oil, po) administration, or, IV) 1 mg nicotine/kg/day, (sc) for 7 days and a single dose of 35 mg CPF/kg (in corn oil, po) on 7th day. Animals were sacrificed at 1, 4, 8, 12, or 24 h post-last dosing of CPF.

for 20 min. Three subsequent organic layers were combined, the solvent was evaporated by blowing down under a gentle stream of N_2, and residues were reconstituted in toluene. For TCPy analysis, MTBSTFA (10 μL) was added for the derivatization of TCPy to its silylated form then heated at 60 °C for 1 h [24].

2.5. GC/MS analysis

Gas chromatography/mass spectrometry (GC/MS) analyses were performed using an Agilent 5975B Inert XL EI/CI mass selective detector (MSD), interfaced with an 7683B injector, G2614A autosampler and an Agilent 6890N GC equipped with ChemStation software for programming and data analysis (Agilent Technologies, Inc. Santa Clara, CA). Separation was achieved in splitless mode using a Restek RTX 1701 column (30 m × 0.25 mm i.d. × 1 μm df, Restek Co., Bellefonte, PA). The GC/MS conditions were utilized by following methods described in Brzak et al. [24]. The level of quantitation for TCPy analysis with the present method was 0.012 and 0.051 μg/ml in urine and blood, respectively. The background level of TCPy in urine from naïve animals was below the level of quantitation with the analytical method employed, although the background level of TCPy has been measured in other studies using different analytical methods [25].

2.6. Determination of ChE activities in plasma and brain

Cholinesterase activities in plasma and brain were determined spectrophotometrically using DTNB as the chromagen, and acetylthiocholine as a substrate for both AChE and BuChE [26]. Plasma and brain homogenates were diluted 1/200 and 1/1750, respectively to achieve optimal experimental conditions to place the optical density (OD)/min in the linear range [27]. Slopes from each tissue and group of animals were compared with those from naïve ($n = 4$) and nicotine-exposed groups ($n = 4$, 1 mg nicotine/kg only for 7 days), and expressed as percent of naïve group ChE activities.

2.7. PBPK/PD modeling

A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF, based upon the one described by Timchalk et al. [28,29], was used to simulate blood concentrations of CPF-oxon and TCPy, CPF-oxon concentration in brain, and urinary excretion of TCPy as well as ChE inhibition in plasma and brain, following oral exposure to CPF, with the simulation software acslX: 2.5.0.6 (AEgis Technologies Group, Inc., Huntsville, AL).

2.8. Data analysis

Means and standard deviations of TCPy concentrations and ChE inhibition rates were calculated from individual animals in each group. The statistical differences across the treatment groups were tested by one-way analysis of variance (ANOVA), followed by post hoc Tukey–Kramer's test for comparisons among different treatment groups using Prism 5 (Graphpad Prism Software Inc, La Jolla, CA), p values less than 0.05 were considered to be significant.

3. Results

For the first 3 days of exposure, nicotine administration resulted in cholinergic effects manifested by tremors. However, by day 4 of dosing, animals had adapted to nicotine's effects and tremors were minimal or not observed. Animals administered only a single 35 mg CPF/kg dose showed minimal tremors shortly after dosing (i.e. minutes); whereas, there were no observed cholinergic effects following daily repeated treatments with the low dose of CPF (5 mg/kg) without nicotine co-exposure.

3.1. Pharmacokinetics

Refer to Table 1 for an outline of study treatment groups. Previous studies have shown that CPF and CPF-oxon are difficult to detect in blood at lower doses due to rapid metabolism to a number of metabolites including the major metabolite, TCPy [30,31]. Thus, the current pharmacokinetic analysis primarily focused on TCPy. The time-course profiles of TCPy concentrations in blood after administration with either repeated 5 mg CPF/kg or a single dose of 35 mg CPF/kg are presented in Fig. 1.

The TCPy blood levels measured 4 h after a single 35 mg/kg dose of CPF were higher in nicotine pre-treated animals. However, the Area-Under-the-Curve through 24 h post-last dosing ($AUC_{0-24\,h}$) of blood TCPy was not affected by the nicotine pretreatment in the single high CPF dosing group (Fig. 1A). Following repeated CPF dosing, peak TCPy concentrations in blood measured at 4 h post-last CPF dosing were slightly higher (but not statistically significant) in the nicotine pretreatment group (~1.8-fold; 7.08 ± 4.4 μmol/L vs. 3.87 ± 0.9 μmol/L, respectively; Fig. 1B). Following the repeated CPF treatments, however, $AUC_{0-24\,h}$ of TCPy in blood was increased by ~2.5× in nicotine treatment groups relative to saline groups (59 and 23 μmol*h/L, respectively). These results suggest that repeated nicotine treatment enhances the metabolism of CPF to TCPy following a repeated low dose exposure to CPF. These also suggest that the repeated administration of nicotine had a greater impact on the TCPy pharmacokinetics after the repeated dosing with 5 mg CPF/kg/day than following a single larger bolus dose of CPF.

The amounts of TCPy excreted in the urine as free or conjugated TCPy for the various treatment groups are presented in Fig. 2. The total amount of TCPy in urine, which was considered as the sum of both free TCPy and conjugated TCPy, was measured after acid hydrolysis of the urine samples. Nicotine pretreatment had no impact on the ratio of free to total TCPy excreted in the urine after a single CPF dose (11.6 ± 4.3 and 13.5 ± 3.0% for saline and nicotine groups, respectively) [Fig. 2A]. However, the ratio of free to total TCPy was ~2-fold higher in repeated CPF dosing groups (33.3 ± 9.0 and 21.6 ± 10.9% for saline and nicotine pretreatment, respectively) relative to the single CPF groups. While these differences are not statistical, it is possible that repeated CPF dosing results in a depletion of glutathione levels over 7 days. Similarly,

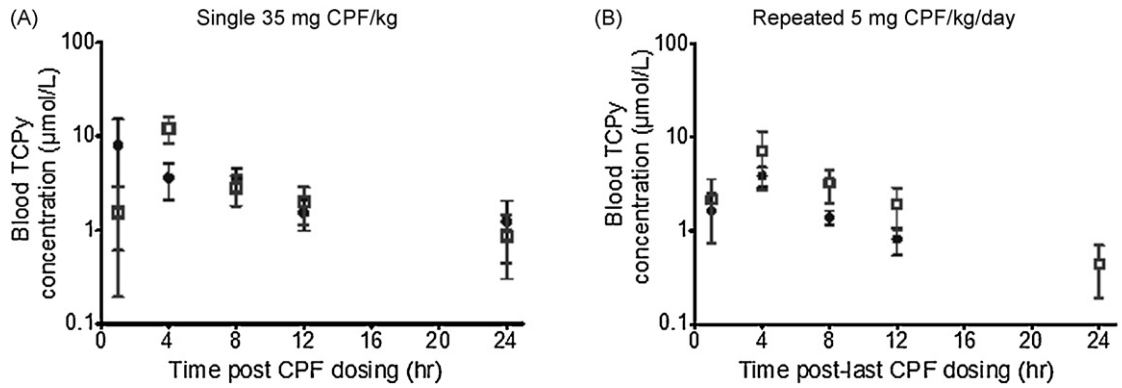

Fig. 1. Time–course TCPy concentration profiles in blood (μmol/L) after the administration of (A) a single dose of 35 mg CPF/kg (in corn oil, po), or (B) repeated 5 mg CPF/kg/day for 7 days combined with saline (sc) or 1 mg nicotine/kg/day (sc) treatments for 7 days. The animals were euthanized at 1, 4, 8, 12 or 24 h last-post dosing of CPF. Values represent mean TCPy concentration ± SD. Filled circles and open squares represent saline- and nicotine-treated groups, respectively.

nicotine treatment has been shown to deplete hepatic glutathione [32]. In addition, the total amount of TCPy excreted in urine during 24 h post-last dosing after the repeated 5 mg CPF/kg/day administration showed a marginal increase in nicotine-treated groups over saline groups (5.3 ± 1.5 and 4.2 ± 0.67 μmol, respectively) [Fig. 2C]. This is consistent with the observed increase in $AUC_{0-24\,h}$ of blood TCPy following nicotine pretreatments. Finally, a comparison of the total (free + conjugated) amount of TCPy excreted (0–24 h) across treatment groups showed that after a single 35 mg/kg CPF dose, there was a ~6.5-fold increase in total TCPy excretion relative to the repeated 5 mg/kg CPF dosing groups over the first 24 h. Since TCPy is the end-product from both bioactivation and detoxification pathways of CPF before conjugation, total urinary excretion would not be expected to be different due to nicotine treatment.

3.2. Pharmacodynamics

Changes in cholinesterase activity/inhibition, which is the important toxicological biomarker and endpoint of OP exposure, were evaluated in plasma and brain. ChE activities in CPF and/or nicotine treated animals were compared to those from naïve (neither nicotine nor CPF dosed) animals and with nicotine-exposed animals (1 mg nicotine/kg only for 7 days). When compared to naive rats, nicotine-exposed animals showed no significant differences in plasma and brain ChE activities (111.8 ± 16.8 and 101.3 ± 6.5%, respectively). Hence, the ChE activity for all treatment groups were compared to results from naïve animals.

The ChE activities in plasma and brain of animals exposed to nicotine and a single dose of 35 mg CPF/kg were not significantly different than in animals treated with saline. At this high CPF dose, ChE in plasma was maximally inhibited with or without nicotine exposure throughout the 24 h post-dosing time period (Fig. 3A). Levels of AChE inhibition measured in brain were also comparable with and without nicotine pretreatment [Fig. 3B].

Plasma ChE activities again showed near maximal inhibition after 7 daily repeated doses of CPF (5 mg/kg/day), with saline or nicotine co-administration (14 ± 4% and 23 ± 5% of those from naïve animals, respectively). However, for all time-points evaluated, the extent of plasma ChE inhibition was slightly less (but not statistically significant) for the nicotine versus saline treatment groups (Fig. 3C). The most significant changes due to repeated nicotine treatments were associated with the AChE activity in brain following repeated administration of 5 mg CPF/kg. Animals that had repeated co-exposures with both nicotine and CPF had substantially less brain ChE inhibition due to CPF than animals that were administered saline and CPF (Fig. 3D).

3.3. Model simulation of CPF pharmacodynamics and pharmacokinetics

To help understand observed differences in pharmacodynamic response resulting from repeated exposures to nicotine and CPF, the CPF PBPK/PD model [29] was modified as previously discussed,

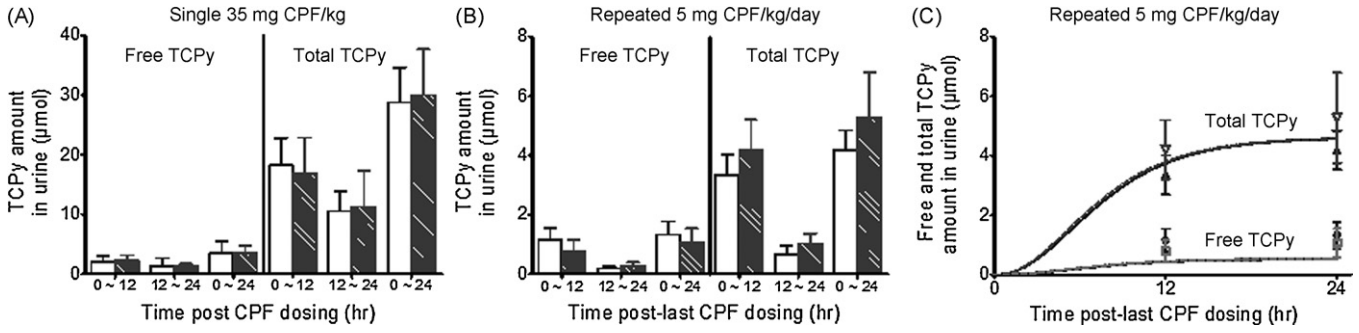

Fig. 2. Amounts (μmol) of free and total TCPy excreted in urine over 24 h after the last dosing of (A) a single 35 mg CPF/kg (in corn oil, po), or (B) repeated 5 mg CPF/kg/day for 7 days combined with saline or 1 mg nicotine/kg/day (sc) treatments for 7 days. Bar heights represent means ± SD (n = 5 per group per each time point). Blank and filled bars represent saline- and nicotine-treated groups, respectively. Note different y-axis scales. Urine was collected (1) before CPF administration, (2) from 0 to 12 h, and (3) 12–24 h after the last CPF dosing. (C) Comparison between experimental data and model predictions of amounts (μmol) of free and total TCPy excreted in urine during 24 h last-post dosing of seven repeated treatments of 5 mg CPF/kg/day. Closed circles and open squares represent free urinary TCPy of saline- and nicotine-treated groups, while closed and open triangles denote total urinary TCPy from saline- and nicotine-treated groups, respectively. Symbols represent means ± SD (n = 5 per group per each time point). The lines (solid: saline group and dotted line: nicotine groups with 1.9-fold increased TCPy V_{max}) represent the simulation after the repeated 5 mg CPF/kg/day dosing. Two lines representing free and cumulative TCPy amount in both saline and nicotine groups are nearly superimposed on top of each other.

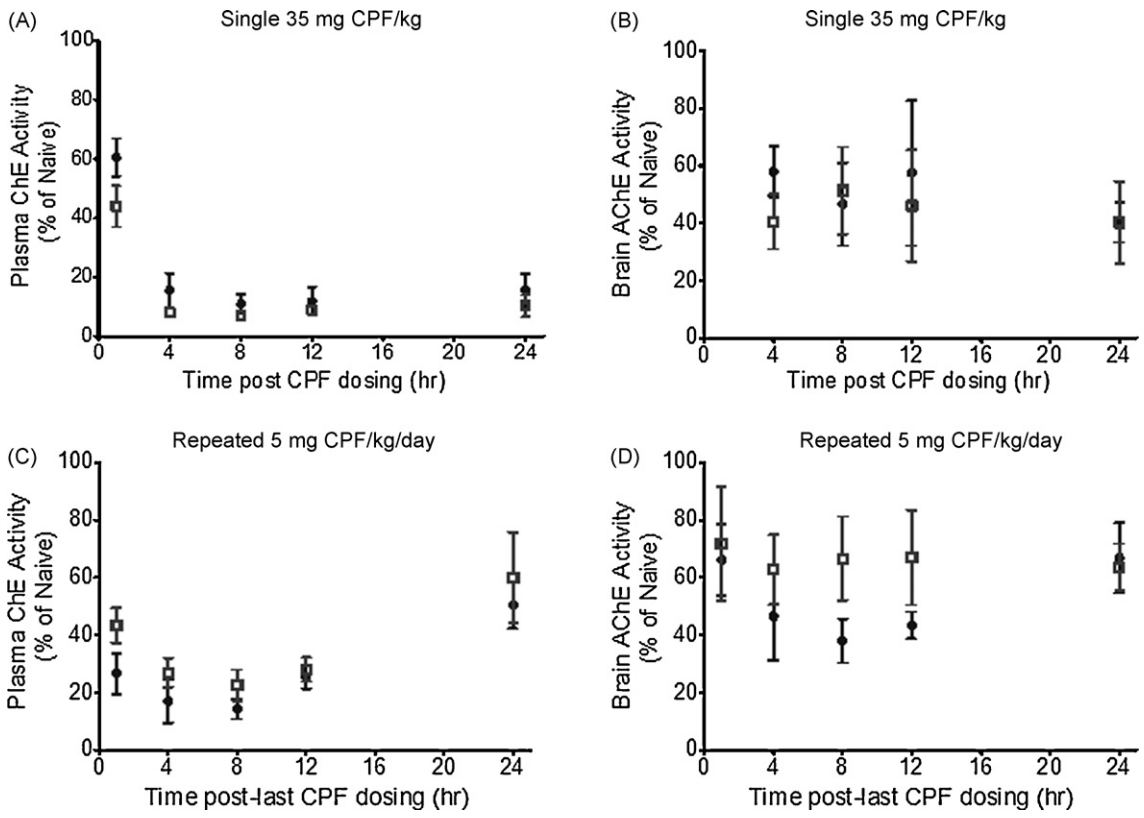

Fig. 3. ChE activities in plasma and brain during 24-h period after the last CPF dosing of a single 35 mg CPF/kg (A and B) or repeated 5 mg CPF/kg/day (C and D) administrations for 7 days, combined with nicotine or saline treatments. Symbols represent the means (±SD, $n = 5$) as percentage of those activities from naïve animals. Closed circles and open squares depict saline- and nicotine-treated groups, respectively.

to accommodate CYP450 metabolic induction. In previous in vitro metabolism studies with hepatic microsomes from animals which were repeatedly treated with 1 mg nicotine/kg/day for 7 days and killed 24 h after last-post nicotine dosing, it was shown that V_{max} for the metabolism from CPF to TCPy was increased 1.9-fold [18]. Therefore, to reflect influences of nicotine exposures on CPF metabolism in vivo, the V_{max} for the metabolism of CPF to TCPy was increased by this same factor in the model simulation.

When the increased TCPy V_{max} was incorporated, model simulations for the repeated oral administration of 5 mg CPF/kg/day predicted a decrease in C_{max} of CPF in blood by 38% in nicotine treated groups (Fig. 4A), while TCPy peak blood concentrations were only nominally different (~2%). Model simulations also showed substantial differences in blood CPF-oxon concentrations, with a decrease of 27% in CPF-oxon C_{max} (Fig. 4B). When CPF-oxon concentrations in brain were simulated for repeated CPF dosing groups, CPF-oxon C_{max} in brain were again predicted to decrease by 31% following an increase in TCPy V_{max}. With repeated CPF exposures the PBPK/PD simulations suggests a progressive increase in C_{max} of brain CPF-oxon such that the 7th dose is ~2-fold higher than the 1st; (Fig. 4C). In contrast, simulations of the blood CPF-oxon C_{max} rose only slightly over the week-long simulations (Fig. 4B).

The time-course profiles of ChE activities in plasma and brain for the repeated nicotine and CPF (5 mg/kg/day) treatments were likewise simulated using the PBPK/PD model, and matched well with experimental data (Fig. 5). The simulation for both CPF dosing groups predicted near maximal ChE inhibition in plasma within 3–5 dosing days, and plasma ChE activities were predicted to have maximum inhibition of 22 and 29% of naïve, after the 7th CPF dose in the saline and nicotine-treated groups, respectively (Fig. 5A). The simulation of brain AChE activities may not have reached steady-state by the 7th dose (Fig. 5B). Peak inhibition of brain AChE activities for repeated CPF dosing groups were predicted at 49.3 and 36.8% of naïve brain activity, with and without nicotine pretreatments after 7 days, respectively.

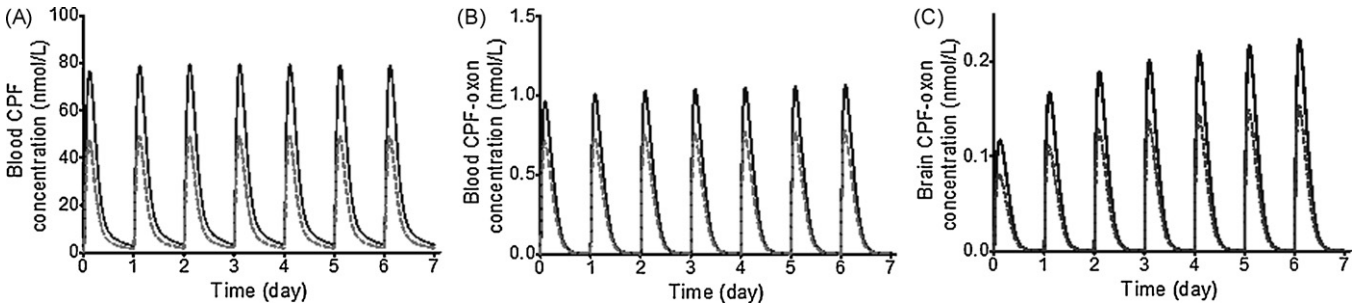

Fig. 4. PBPK model predictions for time–course profiles of (A) CPF concentration (nmol/L) in blood, (B) CPF-oxon concentration (nmol/L) in blood, and (C) CPF-oxon concentration (nmol/L) in brain after the repeated 5 mg CPF/kg/day dosing for 7 days. The solid and dashed lines represent saline-treated and nicotine (i.e. TCPy V_{max} increased) groups, respectively. Note different y-axis scales.

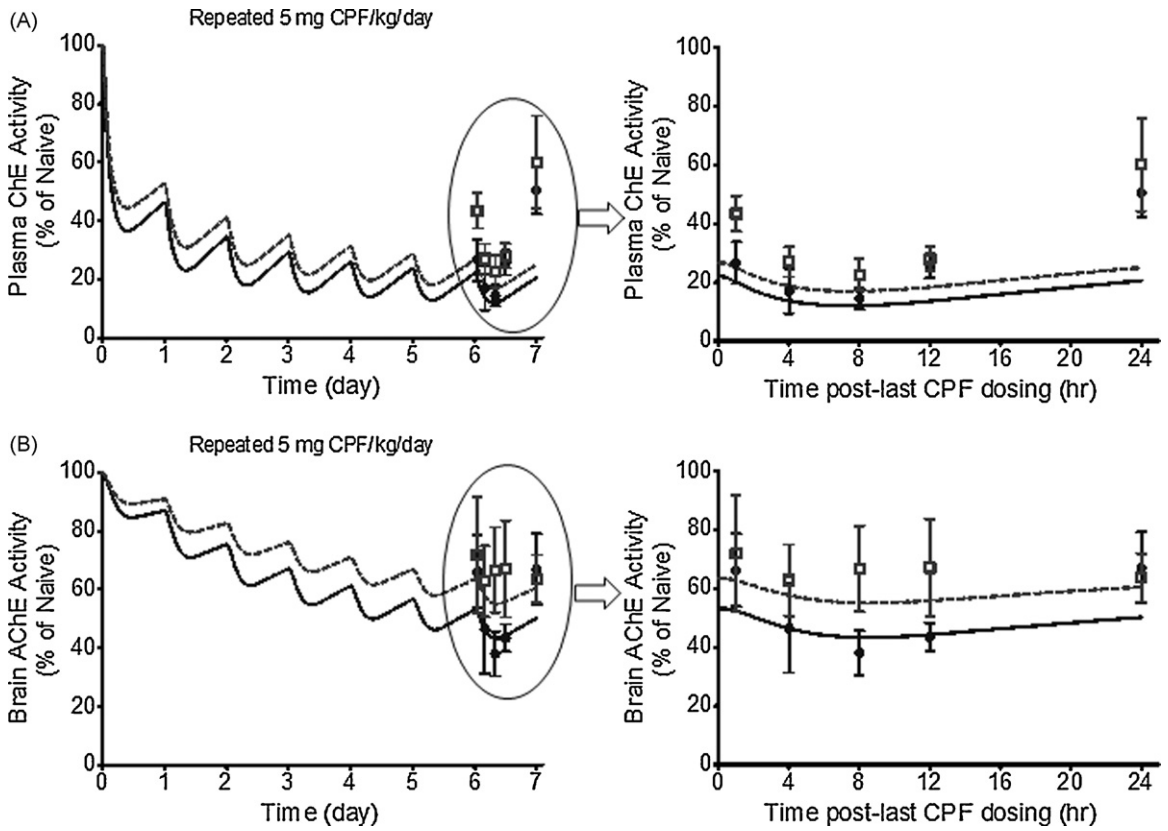

Fig. 5. Comparison between experimental data and model predictions of ChE activities in plasma (A) and in brain (B), after repeated 5 mg CPF/kg administration combined with nicotine or saline treatments. ChE activities during 24 h post-last dosing of CPF were expanded for the clarity. Closed circles and open squares represent the experimental values (means ± SD, $n = 5$) of saline- and nicotine-treated groups, respectively. The lines (solid: control, and dashed: TCPy V_{max} increased) represent the simulation of ChE activities of saline and nicotine treatment groups.

Interestingly, model simulations of both plasma and brain ChE activities after repeated nicotine and CPF dosing were higher than those from repeated saline and CPF administration, due to the increased V_{max} (1.9-fold) of CPF dearylation to TCPy. As noted, these predictions matched well with experimental data, where nicotine-pretreated groups demonstrated consistently higher ChE activities in plasma and brain. These simulations also predicted a larger relative difference (saline vs. nicotine) in CPF-oxon concentrations from brain than those from blood (Fig. 4B and C). This is consistent with the observed differences in experimental data and model simulations between saline and nicotine groups in ChE activities from plasma and brain (Fig. 5A and B). Overall, the influence of repeated nicotine treatments were more prominent following a repeated low dose of CPF (5 mg/kg/day) exposure than a single high dose (35 mg/kg) treatment.

4. Discussion

Humans are routinely exposed to complex chemical mixtures, yet for a majority of these chemical agents it is anticipated that exposures will be intermittent and quite low (i.e. at or below occupational and/or environmental exposure guidelines). Under these conditions, one might speculate that interactions would be minimal. However, drug/chemical mixture interactions are of great concern since drugs are routinely taken at high pharmacologically active doses. This suggests that pre- or co-exposure to drugs that modify metabolic processes (i.e. enzyme induction/inhibition) may potentially impact the pharmacokinetics of chemicals that are dependent upon those metabolism systems for activation and/or detoxification. In this context, the current study evaluated the impact of nicotine (i.e. pharmacologically active agent in tobacco), on the pharmacokinetics and pharmacodynamics of the OP insecticide CPF.

A number of studies have demonstrated that pre-exposure to chemicals that induce CYP450s can alter OP metabolism and associated ChE responses [33,34]. For example, Carr et al. [33] reported that rats exposed to the PCB mixture Aroclor 1254 for 50 days then dosed with 60 mg CPF/kg showed less inhibition in brain AChE than in corresponding vehicle-treated rats. Sultatos et al. [35] reported that β-naphthoflavone, a CYP450s inducer [36], increased the toxicity of CPF, which was likely due to altered metabolism. Since the metabolism of CPF by CYP450s is a key event required for toxicity of this insecticide, enzymatic induction or inhibition will play a significant role in any manifestation of CPF toxicity due to shifts in production of the active metabolite, CPF-oxon.

Several CYP450s, notably CYP2B1 and CYP2E1, are inducible by nicotine *in vivo* [11,18]. Likewise CYP450s are responsible for CPF metabolism. Enzymes participating in the metabolism of CPF to CPF-oxon include CYP2B1/2 (rat), 2B6 (human), 2D6 (human) and 3A4/5, while dearylation of CPF to TCPy is likely mediated by CYP2C, 2D6, 3A4/5 and 1A2 [37–39]. Thus, there is some potential for nicotine to impact the pharmacokinetics and potentially the pharmacodynamics of CPF by modulating CYP450 activities.

We previously reported that repeated treatments with nicotine at relevant human exposure levels (i.e. approximate smoking dose) [14] increased the V_{max} for *in vitro* hepatic CYP450 metabolism of CPF to TCPy by approximately 2-fold, however CPF metabolism to CPF-oxon did not change [18]. Hence, the current study was designed to assess whether nicotine exposures likewise impacted the *in vivo* metabolism of CPF. Based upon our *in vitro* results, the working hypothesis was that nicotine co-exposure would

shift CPF metabolism towards dearylation (i.e. CPF → TCPy) versus desulfuration (i.e. CPF → CPF-oxon → TCPy). However, since both metabolic pathways result in TCPy formation, this metabolic shift would most-likely be observed as an initial increase in the amount and rate of TCPy formation (i.e. blood kinetics), but not in the overall amount formed and excreted since CPF is fully metabolized to TCPy via dearylation as well as via desulfuration followed by esterase metabolism of CPF-oxon to TCPy, then excreted in the urine [1–3,30]. In addition, it would be anticipated that with less CPF undergoing desulfuration, that levels of CPF-oxon would be decreased which would likewise result in less ChE inhibition following the nicotine co-exposures. Overall the results of the current study support this hypothesis. Specifically, repeated nicotine (1 mg/kg/day) and CPF (5 mg/kg/day) co-exposures clearly enhanced the metabolism of CPF to TCPy, as evidenced by increases in the measured TCPy concentration and $AUC_{0-24\,h}$ in blood. However, there were no significant differences in the amount of TCPy (free and total) that was eliminated in the urine, yet the amounts of excreted TCPy in urine were consistent with the time-course profile of TCPy in blood. The CPF PBPK model simulation with increased TCPy V_{max} predicted no significant differences in TCPy blood concentrations during 24 h post-last dosing of CPF after the repeated 5 mg CPF/kg/day (or a single 35 mg CPF/kg) dosing, and the same model simulation predicted minimal differences in the urinary profiles of total TCPy. In addition, the extent of ChE inhibition as reflected in the brain was reduced due to nicotine co-exposure, which is fully consistent with an increase in dearylation versus desulfuration.

Using the Ellman assay to quantify ChE responses has inherent variability, and is unlikely to discern subtle differences in pharmacodynamics [40]. Thus, to provide a quantitative estimate of the impact of nicotine-mediated differences in CPF metabolism to TCPy, an existing physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF was modified to accommodate changes in CYP450 metabolism based upon *in vitro* experiments [18] and exploited to simulate dosimetry and ChE inhibition dynamics in rats. The use of the model facilitated understanding of shifts in the overall metabolic products (i.e. CPF-oxon vs. TCPy), since it was only feasible to experimentally quantify TCPy in the current study. As described earlier, the only change incorporated into the model to reflect the influence of nicotine treatment was a 1.9-fold increase in V_{max} of the dearylation reaction (i.e. CPF → TCPy), from the previous *in vitro* CPF metabolism study [18]. When simulations of CPF-oxon concentrations in blood were compared, nicotine co-treatment resulted in a decreased CPF-oxon C_{max} by 26% (Fig. 4B). In brain, predicted CPF-oxon concentrations were 11–21% of those in blood (compare Fig. 4C and B), consistent with the anticipated tissue (i.e. blood vs. brain) dosimetry differences [28]. Also, consistent with the difference observed in the blood, the predicted CPF-oxon C_{max} in brain for the nicotine and CPF co-exposed group was ~30% lower than that of saline control. However, the CPF-oxon brain concentrations increase with repeated simulations of CPF exposures to a greater extent than in blood (Fig. 4C). These differences in simulated CPF-oxon concentrations were also consistent with measured and predicted ChE inhibition profiles in the blood and brain, which retained higher activity after nicotine pretreatments (Fig. 5A and B). These model simulations are very consistent with the observed experimental results, suggesting that a modest increase in the V_{max} for the dearylation pathway can substantially impact dosimetry resulting in less ChE inhibition.

It was of interest to note that following the single 35 mg CPF/kg dose, nicotine pretreatment resulted in no appreciable pharmacokinetic differences for TCPy (i.e. blood $AUC_{0-24\,h}$, urinary excretion profile). Likewise, there were no discernable differences in ChE activity (plasma or brain) as a result of nicotine pretreatment with 35 mg CPF/kg dose. These observed pharmacokinetic and pharmacodynamic differences relative to the repeated CPF exposure (5 mg/kg/day) is a consequence of repeated versus single exposure to CPF. This difference is a potential result of non-linear dose-dependent metabolism associated with the doses administered (5 mg/kg vs. 35 mg/kg), or as suggested in Fig. 6 an apparent magnification of small difference in both metabolism and ChE response that is manifested following repeated daily exposures to CPF. The *in vivo* metabolism of CPF following acute oral gavage administration has been previously evaluated over a range that encompasses the doses used in the current study [28,30]. Collectively these studies suggest that CPF pharmacokinetics is just beginning to lose linearity at the 35 mg/kg dose range. In addition, this conclusion is also supported by *in vitro* metabolism studies that reported relatively high K_m values for the hepatic metabolism of CPF to both TCPy and CPF-oxon, which is likewise consistent with linear metabolic rates over this dose range [41].

It has also been suggested that desulfuration (i.e. CPF → CPF-oxon) can result in a time-dependent reduction in CYP450, due to enzymatic reactions with the activated sulfur atom [2,42]; hence, repeated CPF exposures could alter the metabolism pathways as a result of suicide inhibition resulting from CYP450-mediated desulfuration [43]. In this regard, Cometa et al. recently evaluated the acute and repeated metabolism of CPF in mice where they also evaluated the impact of repeated oral CPF exposure on CYP450 metabolism [44]. They reported that repeated daily oral exposures (5–30 days) to CPF doses (1.6–25 mg/kg/day) had no impact on CYP450-mediated metabolism. Although it was not evaluated in the current study, these findings in mice do suggest that shifts in rat CPF metabolism are most likely not due to repeated exposure/time-dependent reduction in CYP450 activity.

To help understand the potential for repeated CPF dosing to impact pharmacokinetics, a PBPK/PD model was utilized to simulate the CYP450-mediated hepatic metabolism of CPF to TCPy. In Fig. 6, model simulations compared the amount of TCPy that was formed in the liver following daily oral administration of 5 or 35 mg CPF/kg/day in rats. Simulations included the induction of CYP450 metabolism associated with nicotine co-exposure that was previously described [18]. Comparing the impact of nicotine co-exposure, it appears that a high single dose (35 mg/kg) has minimal impact on TCPy formation from CPF; whereas, with repeated exposures the model suggests that the amount of TCPy formed from CYP450 metabolism will be greater for those animals that were co-exposed to nicotine. These model simulations are consistent with the results obtained in the current study where the impact of nicotine co-exposure was experimentally observed only for the repeated CPF doses.

Although the current study suggests that nicotine co-exposure enhances CYP450-mediated dearylation resulting in a reduction of brain AChE inhibition, the observed shifts in pharmacokinetics and pharmacodynamics was fairly modest. To help discern effects due to repeated nicotine exposure more clearly, administration of CPF with a lower dose (i.e., 1 mg/kg/day) would be helpful, since seven daily 5 mg CPF/kg doses from the current study seemed to approach near maximal inhibition in blood ChE activities, which may have obscure potential differences. It also would be desirable to increase the time for monitoring after the last dose to more fully characterize the pharmacodynamics profiles of ChE activity, including its recovery. The current approach can also be extended to simulate the potential implication of nicotine co-exposures in humans who are also exposed to CPF. In this regard, the existing CPF PBPK/PD model [28] has been validated in both rats and humans, and can easily be modified to accommodate nicotine modulation of human CYP450 activities. Human CYP450s in liver have greater inter-individual variability and susceptibility even within the same individual than those from animal models, averaging 5–10-fold differences in V_{max} of CYP450s [45].

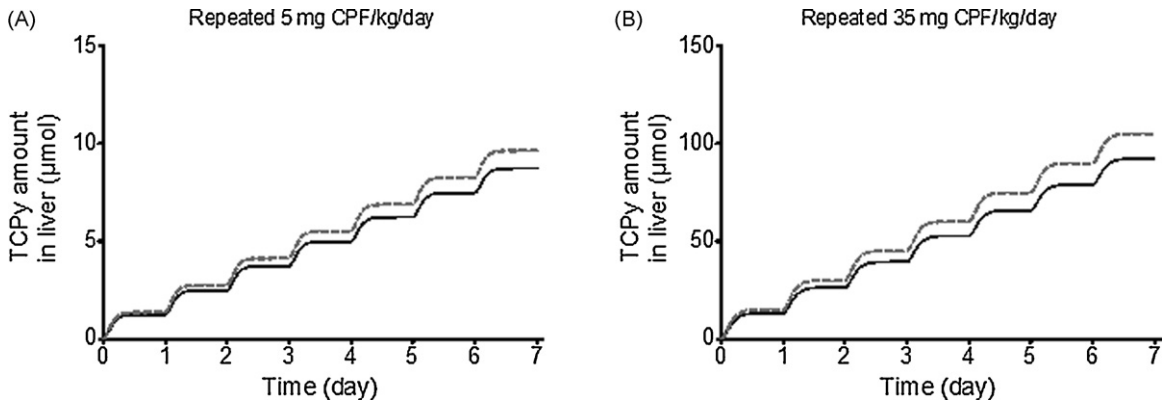

Fig. 6. Model predictions of TCPy amount (μmol) formed in liver compartment after the repeated dosing of 5 mg CPF/kg/day (A) or 35 mg CPF/kg/day (B), combined with saline (sc) or 1 mg nicotine/kg/day (sc) treatments for 7 days. The solid and dashed lines represent the simulations for saline and nicotine (1.9-fold increased TCPy V_{max}) groups, respectively. While the TCPy amount is greater for nicotine-treated groups than saline groups, simulations of repeated CPF dosing show that the difference between saline and nicotine groups at the end of the simulation is increased. Note different *y*-axis scales.

The induction and inhibition of other critical enzymes could also be considered in the current PBPK/PD model, including paraoxonase-1 (PON-1), which has more than 10-fold variations in human blood [46]. Gouedard et al. [47] also showed that PON-1 could be induced by 3-methylcholanthrene, which is a known CYP1A inducer [48]. The implication of PON-1 induction may be of significant importance, since PON-1 plays a bigger role in detoxification of corresponding oxons from OP pesticides than changes in CYP450 activity.

In summary, repeated nicotine treatments employed in this study resulted in higher blood concentrations of TCPy and less inhibition of brain AChE activities. Small differences in ChE activities, i.e., especially in plasma, due to repeated nicotine treatments, were difficult to ascertain accurately in experiments. However, simulations with the PBPK/PD model provided an adequate description, where overall profile of CYP450 induction due to repeated nicotine exposure was reduced to one metabolic parameter change, based on *in vitro* data. Thus, this study again emphasizes the importance of the balance between the bioactivation and detoxification of CPF in its toxicity manifestation. The current study demonstrates the utility of coupling focused *in vivo* studies with PBPK/PD modeling where these models can be exploited to help elucidate PKPD interactions between OPs and other environmental contaminants or drugs. In conclusion, we demonstrated an *in vivo* metabolic interaction between nicotine and CPF, which was explainable using PBPK/PD modeling.

Disclosures

Although Drs. Timchalk and Poet have received funding from the Dow Chemical Company, the manufacturer of chlorpyrifos, to conduct research, the Dow Chemical Company did not have any involvement in the study design, analysis and interpretation of data, in the writing of the report, or in the decision to submit this paper for publication.

Conflict of interest

The authors declare that they have no competing interests.

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