**Intracerebroventricularly and Systemically Delivered Inhibitor of Brain CYP2B (C8-Xanthate), Even Following Chlorpyrifos Exposure, Reduces Chlorpyrifos Activation and Toxicity in Male Rats**

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Chlorpyrifos is a pesticide that is metabolically activated to chlorpyrifos oxon (acetylcholinesterase inhibitor) primarily by the cytochrome P450 2B (CYP2B) enzyme subfamily in the liver and brain. We have previously shown that intracerebroventricular pretreatment with a CYP2B inhibitor, C8-Xanthate, can block chlorpyrifos toxicity. Here, we assessed whether delayed introduction of C8-Xanthate would still reduce toxicity and whether peripheral administration of C8-Xanthate could also inhibit chlorpyrifos activation in the brain and block toxicity. Male rats (N = 4–5/group) were either pretreated with C8-Xanthate (40 μg intracerebroventricular or 5 mg/kg intraperitoneal), or vehicle (ACSF or saline, respectively), 24 h before chlorpyrifos treatment (125 mg/kg subcutaneous) and then treated daily with inhibitor or vehicle until 7 days post-chlorpyrifos treatment. Additional groups received vehicle pretreatment, switching to C8-Xanthate 1, 2, 3, or 4 days after chlorpyrifos and then continuing with daily C8-Xanthate treatment until 7 days post-chlorpyrifos treatment. Neurotoxicity was assessed at baseline (before chlorpyrifos) and then daily after chlorpyrifos, using behavioral assessments (e.g., gait score). Neurochemical assays (e.g., serum and brain chlorpyrifos) were performed at the end of study. Pretreatment with C8-Xanthate completely prevented chlorpyrifos toxicity, and delayed introduction of C8-Xanthate reduced toxicity, even when started up to 4 days after chlorpyrifos treatment. Discontinuation of C8-Xanthate treatment 7 days post-chlorpyrifos treatment did not result in the reappearance of toxicity, tested through 10 days after chlorpyrifos treatment. These findings suggest that CYP2B inhibitor treatment, even days after chlorpyrifos exposure, and using a peripheral delivery route, may be useful as a therapeutic approach to reduce chlorpyrifos toxicity.

Key words: cytochrome P450; pesticide poisoning; neurotoxicity; brain metabolism.

As recently outlined in science, organophosphorus pesticide poisonings are a major therapeutic and biological problem worldwide (Enserink et al., 2013). Intentional organophosphorus pesticide poisonings result in up to 300,000 deaths each year (Bertolote et al., 2006; Gunnell et al., 2007), and account for one-third of the world’s suicides (Konradsen et al., 2007). Organophosphorus pesticide-mediated acetylcholinesterase (AChE) inhibition results in an accumulation of acetylcholine in the synapse, which causes cholinergic overstimulation and toxicity (Eddleston et al., 2005). Severe organophosphorus pesticide poisoning is difficult to manage, requiring intensive care and the use of atropine and oxime cholinesterase reactivators, and is further complicated by the scarcity of clinical trial evidence available to guide treatment (Eyer et al., 2007). One such organophosphorus pesticide used commonly worldwide is chlorpyrifos (CP); it is used for both agricultural and household purposes (Eaton et al., 2008), and is also the most common organophosphorus agent used in human self-poisonings (Eddleston et al., 2005). Dietary and occupational exposure to CP is widespread and CP metabolites were detected in the urine of 96–100% of U.S. residents tested (Barr et al., 2005; Morgan et al., 2011).

Acute CP exposure at high doses/poisoning can result in death or cause long-term neurological and behavioral toxicity. This toxicity usually presents itself as an early cholinergic crisis, intermediate syndrome, and delayed neuropathy, causing symptoms such as prolonged unconsciousness, central nervous system (CNS)-mediated respiratory failure or comas (Soummer et al., 2011). In rat models of CP poisoning, even months after a single high-dose exposure to CP, animals displayed functional CNS alterations detected using a repeated acquisition spatial task in the water maze as well as an amphetamine-induced place preference paradigm (Sanchez-Santed et al., 2004).

Current pharmacological treatments include atropine and acetylcholinesterase oxime reactivators (Hmouda et al., 2008). Atropine is a muscarinic antagonist that prevents cholinergic overstimulation by blocking the muscarinic acetylcholine receptor, but does not affect the inhibition of AChE (Hmouda et al., 2008). Oxime reactivators, such as pralidoxime are commonly used, but have not proven to be efficacious when tested...
in clinical trials (Eddleston et al., 2009) and in vitro investigations (Kuca et al., 2010). Moreover, because chlorpyrifos is highly lipophilic and can accumulate in adipose tissue (Soummer et al., 2011), its toxic metabolite CP oxon (CPO) continues to be produced through activation of the bioavailable CP. Therefore, novel therapeutics that can reduce the harm from CP by reducing the oxon load could prove beneficial.

The xenobiotic metabolizing enzyme cytochrome P450 2B (CYP2B; human: CYP2B6, rat: CYP2B1) subfamily is prominent in the activation of CP to CPO (Buratti et al., 2003; Foxenberg et al., 2007) and our previous study demonstrated that inhibition of rat brain CYP2B1 could block CPO formation as well as the resulting CP behavioral and neurochemical toxicities (Khokhar and Tyndale, 2012). Although CP is activated to CPO in the liver, this CPO is quickly hydrolyzed by liver and plasma esterases to 3,5,6-trichloro-2-pyridinol (TCP). This is consistent with a lack of detectable CPO in plasma and liver perfusate, even though CP is known to be bioavailable for an extended period of time (Sultatos and Murphy, 1983). Together, this suggests that the majority of the neurotoxicity from CP may result from the local activation of CP to CPO in the brain by CYP2B enzymes and subsequent inhibition of brain AChE (Khokhar and Tyndale, 2012).

CYP2B1 and CYP2B6 proteins are ubiquitously expressed in rat and human brains, respectively (Khokhar et al., 2010; Miksys et al., 2003), and can alter the response to centrally acting drugs and toxicants. Pre-treatment with an intracerebroventricular (ICV) injection of a CYP2B mechanism based inhibitor (MBI) inhibits CP-mediated toxicity as observed via a decrease in AChE inhibition, hypothermia and through a reduction in score severity in neurobehavioral tests (Khokhar and Tyndale, 2012). A single ICV injection of the MBI reduced toxicity for 1–2 days, whereas daily treatment blocked toxicity for the duration of testing. To utilize CYP2B inhibitors therapeutically, it would be useful to know whether delayed introduction of the C8X would also reduce CP toxicity and if these inhibitors could be delivered systemically. Thus, we assessed the utility of central and peripheral pre- and post-CP administration of C8X on the activation of CP by CYP2B in the rat brain and ensuing neurotoxicity. Due to CP’s long-lasting bioavailability, we also tested whether toxicity returned upon discontinuation of C8X treatment as part of assessing CYP2B inhibition as a potential therapeutic for CP poisoning.

**MATERIALS AND METHODS**

**Animals.** Adult male Wistar rats ($N = 164$; 200–225 g; Charles River, St-Constant, PQ, Canada) were housed in triplets under a 12-h artificial light/dark cycle (lights off at 6:00 p.m.), and handled daily to habituate them to handling; upon cannula implantation the rats were singly housed for the duration of the studies. All experimental procedures were approved by the Animal Care Committee at the University of Toronto, and were conducted within the guidelines of the Canadian Council on Animal Care.

**ICV cannulations.** As performed previously (Khokhar and Tyndale, 2012), rats were anesthetized with 2% isoflurane and placed in a stereotaxic frame where unilateral cannulas were then implanted into their right lateral ventricle (bregma coordinates: anterior-posterior $= -0.9$, lateral $= -1.4$, dorso-ventral 3.6 mm (Paxinos and Watson, 1986)). The cannulas were held in place using dental cement and screws and the rats were allowed to recover for a week after the surgery.

**MBI treatments.** C8X is a selective CYP2B MBI and does not inhibit other human cDNA-expressed CYPs including 1A1, 3A2, 3A4, 2C9, and 2D6 in vitro (Yanev et al., 1999), suggesting that C8X does not alter CP metabolism through other pathways such as the 3A-mediated activation or 2C-mediated inactivation of CP (Tang et al., 2001). There is some evidence that suggests that C8X may inhibit human CYP2C19 (Turpeinen et al., 2004), but the effects of C8X on rat CYP2C11 or CYP2C6 are unknown. C8X (Toronto Research Chemicals, Toronto, ON, Canada) was given ICV at a dose of 40 µg in a 2 µl total volume of artificial cerebrospinal fluid (ACSF) or at a dose of up to 20 mg/kg in saline intraperitoneally (IP). ICV injections were made via an injector (0.6 mm protrusion beyond the cannula) affixed to a Hamilton syringe by polyethylene tubing over 2 min, and the injector was left in place for 1 min post-injection. This ICV dose of MBI has previously been shown to inhibit CYP2B activity in brain, and alter behavioral responses to drugs and toxicants metabolized by CYP2B, while not affecting CYP2B levels (Khokhar et al., 2010; Khokhar and Tyndale, 2011, 2012; Miksys and Tyndale, 2009). Moreover, a structurally distinct CYP2B MBI (i.e., 8-methoxypsoralen), given ICV, also produced identical effects in our previous studies of CYP2B drug and toxicant-mediated pharmacology (including CP toxicity), supporting the in vivo selectivity of C8X (Khokhar et al., 2010; Khokhar and Tyndale, 2011, 2012; Miksys and Tyndale, 2009).

Rats were divided into six treatment groups ($N = 4–5$ / group); this treatment paradigm was tested with both ICV and IP C8X treatment in separate cohorts of rats (shown in Figs. 1 and 4 respectively). One group was pre-treated (24 h before CP), and then treated daily (including CP treatment day), with C8X; another group was pre-treated (24 h before CP) with the vehicle, ACSF or saline, followed by daily treatment; the remaining four groups were pre-treated (24 h before CP), and then treated daily, with vehicle until they were switched to C8X treatment 1, 2, 3, or 4 days post-CP (125 mg/kg SC) treatment. C8X (or vehicle) treatment was then continued daily for all six groups until 7 days post-CP treatment. In a separate cohort of rats, the dose range for IP C8X (to block all CYP2B enzyme activity) was tested as a single pretreatment injection, 24 h before CP, at doses of 0–20 mg/kg in 12 separate groups ($N = 3–4$ / group; 0 mg/kg refers to saline vehicle for C8X; Fig. 3). Lastly, another cohort of rats was used to assess the effects of discontinuation of both ICV...
and IP C8X treatment on behavioral toxicity (Fig. 6) and consisted of six groups (N = 4–5/group). For animals treated ICV (three groups), one group was pre-treated (24 h before CP) with C8X ICV, and then treated daily until 7 days post-CP treatment, one group was pretreated (24 h before CP) with vehicle (ASCF) ICV and then treated daily until 7 days post-CP treatment, and one group was pre-treated (24 h before CP) with vehicle ICV and then treated daily, until 3 days post-CP treatment, when they were switched to C8X ICV treatment until 7 days post-CP treatment. A similar treatment with IP (three groups) was performed, where one group was pretreated, and treated daily, with C8X IP, one pre-treated, and treated daily, with saline IP, and one that was pretreated, and treated daily, with saline until 3 days post-CP, when they were switched to C8X IP treatment until 7 days post-CP treatment. For both ICV and IP arms of this study, there were no further C8X or vehicle treatments after 7 days until 10 days post-CP treatment, but all behavioral measurements were still performed. Animal groups in the behavioral experiments are described by the route of treatment, followed by the treatment given (e.g., C8X or ACSF), followed by the days relative to CP treatment (e.g., (+1): pre-treatment; (+2): 2 days after CP treatment), such that the animals receiving ICV C8X pretreatment, followed by daily injections, would be referred to as ICV C8X (+1).

Chlorpyrifos dose and treatment. Based on previous studies (Bushnell et al., 1993; Khokhar and Tyndale, 2012), the rats subcutaneously (SC) received 125 mg CP/kg (maximum tolerated SC dose: 279 mg/kg (Chaudhuri et al., 1993); Fluka, Sigma-Aldrich, Oakville, ON, Canada) either 24 h after an ICV or IP injection of CYP2B MBI or vehicle, or without any prior MBI administration (2 ml/kg volumes were used for CP). This dose was chosen as the optimal dose in our previous study of the role of brain CYP2B in CP activation and toxicity and has also been previously used in mechanistic studies to model the neurotoxic behavioral and chemical changes following a single high-dose exposure to CP (Bushnell et al., 1993). This route of administration was used to model high-dose CP exposure and poisoning in humans (Bushnell et al., 1993). Even though most poisonings occur via an oral route, CP has also been used for self-poisoning via a subcutaneous injection (Soummer et al., 2011). Subcutaneous administration also resembles dermal absorption, which is one of the most common routes of accidental poisonings (Meggs, 2003); dermal and subcutaneous exposures to neurotoxicants produce similar levels of brain toxicity in mice (Chaudhary and Rao, 2010). Animals were sacrificed via decapitation for the measurement of ChE activity, and CP and CPO, either at 4 h (for IP C8X dose-ranging biochemical studies) or at 7 or 10 days (for behavioral experiments) after CP treatment (performed on day 0). No animal deaths were observed at this dose of CP or at higher doses in this rat strain (Bushnell et al., 1993; Khokhar and Tyndale, 2012).

Serum and brain chlorpyrifos and chlorpyrifos oxon levels. Serum and brain concentrations of CP and CPO were measured as before (Khokhar and Tyndale, 2012) upon animal euthanasia (4 h post-CP for IP dose range study; 7 or 10 days post-CP for behavioral experiments). Briefly, 0.5 ml of serum or brain homogenate supernatant (half brains homogenized: 1:2 wt/vol in cold 0.1M phosphate buffer, vortexed and centrifuged at 16,000 g for 10 min at 4°C) was added to 25 μl of 10 μg/ml 2-benzoxazolonine (internal standard) and 4 ml dichloromethane. The mixture was vortexed for 20 s, shaken mechanically for 10 min and centrifuged at 110 × g for 10 min. The organic layer was then dried under a nitrogen stream. The residue was dissolved in 110 μl of mobile phase and 90 μl of the solution was injected into the HPLC system (Agilent 1100 Series HPLC system (Palo Alto, CA) equipped with a degasser, quat pump, autosampler, and UV detector). Samples were separated using a ZORBAX Bonus-RP column (250 × 4.6 mm, 5 μm) with gradient elution conditions of acetonitrile and water starting at 40% acetonitrile, going up to 80% acetonitrile by 22 min and then returning to 40% acetonitrile at 24 min for 5 min to re-equilibrate. The acetonitrile gradient flow rate was 1 ml/min and UV detection was performed at a wavelength of 280 nm for chlorpyrifos and chlorpyrifos-oxon with retention times of 5.3, 13.6, and 22.8 min for 2-benzoxazolonine (internal standard), chlorpyrifos-oxon and chlorpyrifos, respectively, and a recovery of 93.1% for CP, and 85.2% for CPO. The limits of quantification were 10 ng/ml for CP in serum, and 10 ng/g for CP and CPO in the brain, and the assays were linear up to 1000 ng/ml and 1000 ng/g, respectively.

Brain and serum cholinesterase activity. Cholinesterase (ChE) activity was measured as previously (Khokhar and Tyndale, 2012) using a Quantichrom AChE assay kit (BioAssay Systems, Hayward, CA). This kit uses a modified Ellman method, with acetylthiocholine as the substrate, and has been previously validated for the study of rat brain cholinesterase activity (Basselin et al., 2009). Serum samples were diluted 1:20 in the assay buffer, and supernatant from brain homogenates was diluted 1:10 to obtain optical density values within the assay’s linear range of detection. Sample (10 μl) was added to 190 μl of freshly prepared working reagent (per manufacturer’s instructions) and OD_{412 nm} was measured at 2 min and at 10 min in a plate reader. Cholinesterase activity values reported within this paper are a combination of acetyl- and butyryl-cholinesterase activities, as we did not use a butyryl-cholinesterase inhibitor. All ChE activities are reported as a percent of ChE activity observed in a separate group of animals treated with the vehicle for CP, peanut oil (food-grade 100% pure), which is the negative control for this experiment (brain: 782.43 ± 140 U/g; serum: 817.26 ± 291 U/l [mean ± SD]); peanut oil did not inhibit ChE activity or alter behavioral toxicity (Khokhar and Tyndale, 2012). The ChE activities were measured at 4 h post-CP in the IP C8X dose-ranging study, and upon sacrifice on days 7 or 10 in the behavioral experiments.
Body temperature measurement. Chlorpyrifos elicits an initial hypothermic state that lasts up to 24 h followed by hyperthermia lasting up to 72 h after CP exposure in rats; initial hypothermia is mediated by the cholinergic overstimulation of heat loss pathways in CNS thermoregulatory centers (Gordon and Grantham, 1999). Rectal body temperatures were measured using a digital thermometer at 4 h after CP administration in the IP dose-ranging study.

Behavioral assessments. All behavioral assessments were performed once at baseline (prior to CP exposure) and then every day post-CP exposure for 7 or 10 days, at which point the rats were euthanized. Two blinded observers scored the animals, and these scores are averaged and presented below. Animals will be compared with their own baselines (entire battery performed prior to C8X or CP treatments) as well as between treatment groups. These behavioral tests provide an indication of both central and peripheral (e.g., neuromuscular) neurotoxicity, and have been previously used to assess neurotoxicity associated with organophosphorus pesticides (Moser, 2011).

Incline plane. As previously described (Abou-Donia et al., 2003), rats were placed on a flat plane in the horizontal position, with their head facing the side of the board that is raised, and the angle at which the rat began slipping downward was recorded. The results of two trials were averaged.

Functional observational battery. The complete procedural details and scoring criteria for behaviors have been previously established, and are consistent with our previous study (Khokhar and Tyndale, 2012; McDaniel and Moser, 1993). Briefly, the rat was placed on a clean absorbent pad-covered laboratory cart (60 × 90 cm) with a perimeter barrier (6.5 cm high). The rat was undisturbed for 3 min and observed for locomotion and the frequency of rearing responses (ability of rat to place its weight on its haunches). At the same time, gait characteristics were noted and gait (1: least impairment; 4: most impairment), mobility (1: least impairment; 4: most impairment) and arousal scores (1: most impairment; 4: least impairment), were assigned. Aerial righting reflex (rat held supine and dropped from height of 30 cm, average of two measurements; 1: least impairment; 4: most impairment), body weight and rectal temperature, were then measured. The entire battery of tests took ~5 min per rat.

Statistical analyses. All data analyses were performed using SPSS 21 (IBM Inc., Chicago, IL). The biochemical measures (CP, CPO, ChE activity) were analyzed using a one-way ANOVA (OWA) and post hoc least significant difference (LSD) test. Linear trends were also assessed following the one-way ANOVA using polynomial contrasts to assess dose- or time of onset-dependency of the effects of CYP2B inhibition. Behavioral measurements were compared between baseline and between days +1 to +7 or +10, within- and between-treatment groups (C8X/vehicle pre-treatment or delayed C8X), across time using a repeated measures ANOVA (RMANOVA) followed by post hoc LSD test to compare between group differences. All statistical significances from the behavioral experiments are presented compared with the C8X pre- and daily-treatment group, whereas those from the IP dose ranging experiments are compared with the saline-treated (0 mg/kg C8X) group.

RESULTS

ICV C8X given after CP Reduces Toxicity, whereas Pre-treatment Prevents CP Toxicity

As previously shown (Khokhar and Tyndale, 2012), a single 125 mg/kg dose of CP resulted in behavioral toxicity when assessed via measures such as gait and incline plane angle as seen in the ICV ACSF pre-treated animals (Figs. 1A and 1B; N = 4–5/group). Consistent with previous findings (Bushnell et al., 1994), this dose of CP did not result in parasympatheticomimetic cholinergic signs of toxicity in these animals. Pre-treatment (24 h before CP) with ICV C8X (ICV C8X (−1)), followed by daily ICV C8X injections, prevented toxicity over the course of the full 7 days when compared with the ICV ACSF pre-treatment group. Introducing ICV C8X 1–4 days after CP treatment reduced toxicity and prevented worsening, with gait scores approaching those seen in the ICV C8X pre-treatment group (Fig. 1A). This occurred in a time-dependent manner: ICV C8X treatment 1 day after CP recovered before treatment 2 days after, etc. Even 4 days after CP exposure, ICV C8X results in a significant recovery compared with the ICV ACSF pretreated group. Within 1–2 days following the start of the delayed ICV C8X treatments (+1 to +4), CP toxicity was reduced to levels seen in the ICV C8X pre-treatment group, which was maintained throughout the study (Fig. 1A). Moreover, the group treated 1 day after CP with ICV C8X (ICV C8X (+1)) showed a lower maximal toxicity compared with the animals where the ICV C8X treatment was delayed for longer, consistent with an improved therapeutic outcome if brain CYP2B1 is inhibited as soon as possible after CP exposure. A significant within-group effect of time and treatment and a significant time × treatment interaction was seen, as well as a significant between-group effect of treatment (RMANOVA; p < 0.05). Similar findings were also seen for incline plane angle (Fig. 1B; p < 0.05) as well as other measures such as aerial righting reflex, arousal scores, and mobility (data not shown).

Consistent with our previous investigation over 3 days, on day 7 ICV ACSF pre-treated animals did not have detectable levels of CP in the brain (significantly lower than the ICV C8X pretreated group (p_{OWA} < 0.05, Fig. 2A)). Moreover, brain CP levels were also significantly lower in the animals that started receiving ICV C8X 3 or 4 days after CP treatment (p_{OWA} < 0.05, Fig. 2A), compared with the C8X pre-treated group and displaying treatment-duration dependency (p_{trend} < 0.05, Fig.
FIG. 1.  ICV C8X pre-treatment prevents CP toxicity, whereas delayed onset of ICV C8X treatment reduces toxicity. ICV C8X (40 μg) pre-treatment (24 h before CP; ICV C8X (−1)), followed by daily treatment, reduced CP toxicity as measured by (A) gait score and (B) incline plane angle between 1 and 7 days after CP treatment (125 mg/kg SC). Delayed onset of C8X treatment, 1–4 days after CP treatment, reduced CP toxicity, reaching toxicity levels similar to C8X pretreatment (ICX C8X (−1)) in a stepwise manner with ICV C8X (+1) reaching ICV C8X (−1) levels first followed by (+2), (+3), and (+4). All rats received an ICV injection daily: C8X or ACSF in the pre-treated groups, or ACSF until the onset of C8X treatment for the other four groups. Significant within-group effects of time and treatment and significant time × treatment interactions on gait and incline plane angle were seen, as well as significant between-group effects of treatment (p < 0.05). Six separate groups of N = 4–5/group, *p < 0.05 compared with the C8X pretreated group, arrows indicate direction of toxicity and figure depicts group mean ± SD, BL indicates scores at baseline (prior to CP treatment; the entire behavioral battery was performed at baseline), and dashed lines indicate time of CP treatment.

FIG. 2.  ICV C8X pre-treatment results in higher brain CP levels and reduced impact on ChE activity, and delayed onset of C8X treatment reduces CP toxicity. (A) Brain and (B) serum CP levels in rats pretreated (followed by daily treatment) with either ICV C8X (40 μg) or ACSF or those treated with C8X 1–4 days after exposure to CP (ICV C8X (+1)−(+4) [followed by daily treatment thereafter]). (C) Brain and (D) serum ChE activities in rats sacrificed on day 7 post-CP treatment after the last behavioral measurement. Six separate groups of N = 4–5/group, *p < 0.05, #p < 0.07 post hoc differences (LSD) compared with the C8X pre-treated group, and figures depict group mean ± SD.
Peripheral administration of C8X inhibits both brain and peripheral CP metabolism and resulting toxicity in a dose-dependent manner. IP C8X treatment resulted in significantly higher (A) brain and (B) serum CP, (C) brain ChE activity, and (E) body temperature in rats sacrificed 4 h after CP (125 mg/kg SC) treatment (one-way ANOVA). (D) No differences were seen in serum ChE activity. Twelve separate groups of $N = 3-4$ / group, $^p < 0.05$, post hoc differences (LSD) compared with 0 mg/kg C8X (saline vehicle), figures depict group mean ± SD, dashed line represents average body temperatures in untreated rats (36.4°C).

Essentially no differences in serum CP levels on day 7 were observed, as expected from previous experiments, showing no effect of ICV C8X on peripheral metabolism of CP (Fig. 2A). Consistent with this effect of metabolic inhibition of brain activation of CP to the CPO, ICV ACSF pre-treated animals had significantly lower brain ChE activity than the ICV C8X pretreated group after sacrifice on day 7; brain ChE activities were also significantly lower in the animals receiving ICV C8X 2, 3, and 4 days after CP treatment compared with the ICV C8X pretreated group ($p_{\text{trend}} < 0.05$, Fig. 2B) and displayed treatment-duration dependency ($p_{\text{trend}} < 0.05$, Fig. 2B), but these groups were not significantly different from the ICV ACSF pretreated
FIG. 4. IP C8X blocks CP toxicity, and delayed onset of IP C8X treatment reduces toxicity. IP C8X (5 mg/kg) pre-treatment (24 h before CP), followed by daily treatment, reduced CP-associated toxicity seen through decreased (A) gait score and (B) incline plane angle between 1 and 7 days after CP (125 mg/kg SC) treatment. Delayed onset of C8X treatment reduced CP toxicity and prevented worsening of toxicity. All rats received an IP injection daily: C8X or ACSF in the pre-treated groups, or ACSF until the onset of C8X treatment for the other four groups. Significant within-group effects of time and treatment and significant time × treatment interactions on gait and incline plane angle were seen, as well as significant between-group effects of treatment (p < 0.05). Six separate groups of N = 4–5/group, *p < 0.05 compared with C8X pretreatment group, arrows indicate direction of toxicity and figures depict group mean ± SD, BL indicates scores at baseline (prior to CP treatment; the entire behavioral battery was performed at baseline), and dashed lines represent CP treatment.

FIG. 5. IP C8X pre-treatment results in higher brain CP levels and ChE activity and lower brain CPO levels, and delayed onset of C8X treatment produces reduces CP toxicity by reducing CP activation. (A) Brain and (B) serum CP, and (C) brain CPO levels in rats pretreated, followed by daily treatment, with either IP C8X or ACSF or those treated with C8X 1–4 days after exposure to CP (IP C8X (+1)–(+4)). (D) Brain and (E) serum ChE activities in rats sacrificed on day 7 post-CP treatment after the last behavioral test. Six separate groups of N = 4–5/group, *p < 0.05 post hoc differences (LSD) compared with the C8X pre-treated group, and figures depict group mean ± SD.
FIG. 6. Discontinuation of both IP and ICV C8X treatment does not result in toxicity. ICV (40 μg) and IP (5 mg/kg) C8X pretreatment (−1; 24 h before CP), followed by daily treatment, prevented CP (125 mg/kg SC once) toxicity by decreasing (A) gait score and (B) incline plane angle, whereas delayed onset (3 days after CP treatment) of both ICV and IP C8X treatment reduced CP toxicity. Discontinuation of C8X treatment on day 7 did not alter behavioral score in any groups, suggesting no appearance of toxicity after 7 days of C8X treatment (behavior was measured daily until day 10). Significant within-group effects of time and treatment and significant time × treatment interactions on gait and incline plane angle were seen, as well as significant between-group effects of treatment (p < 0.05). Six separate groups of N = 4–5/group, *p < 0.05 compared with respective C8X (−1) groups, arrows indicate direction of toxicity and figures depict group mean ± SD, BL indicates scores at baseline (prior to CP treatment; the entire behavioral battery was performed at baseline), dashed lines represent CP treatment, and solid lines represent end of C8X treatment.

Peripheral Administration of C8X Inhibits both Peripheral and Brain CP Metabolism and Resulting Toxicity in a Dose-Dependent Manner

To optimize the dose of C8X for peripheral treatment (via an IP injection), we assessed doses of C8X between 0 and 20 mg/kg and measured the effects of C8X treatment on CP metabolism in the periphery and brain and the resulting impact on toxicity upon sacrifice at 4 h after CP treatment. IP C8X treatment dose-dependently increased brain CP (p_{OWA} < 0.05, p_{trend} < 0.001, Fig. 3A; N = 4–5/group), suggesting an inhibition of CP activation to CPO. This resulted in decreased inactivation of the ChE enzyme and higher brain ChE activity (p_{OWA} < 0.05, p_{trend} < 0.001, Fig. 3C; ~85% of ChE activity seen in animals not treated with CP (peanut oil alone)) and prevented CP-induced hypothermia (p_{OWA} < 0.05, p_{trend} < 0.001, Fig. 3E), both indicative of decreased CP toxicity in the presence of C8X. The 5 mg/kg IP dose resulted in significant increases in all of these measures (CP, ChE, and temperature) compared with IP vehicle (saline). IP C8X treatment also increased serum CP in a dose-dependent manner (p_{OWA} < 0.05, p_{trend} < 0.001, Fig. 3B) with doses between 2 and 20 mg/kg resulting in significantly higher serum CP (due to inhibited CYP2B1-mediated metabolism in the liver) compared with IP vehicle (saline), whereas no effects of peripheral C8X treatment were seen on serum ChE activity (Fig. 3D). Therefore, IP pre-treatment with C8X resulted in reduced activation of CP to CPO, blocked ChE inhibition (higher ChE activity in the brain), and attenuated the CNS-mediated hypothermia. The effects of peripheral CYP2B1 inhibition by IP C8X treatment on serum CP levels did not appear to have a significant effect on serum ChE suggesting that even low levels of CPO formed in the periphery reduce serum ChE activity, but do not contribute to central toxicity observed via the hypothermic response. We chose 5 mg/kg as a dose that significantly affected all measures, and resulted in higher brain CP and ChE activity (due to reduced brain CPO production and resultant ChE inhibition), as well as blocked the central hypothermic effects of CP administration for the behavioral experiments.

IP C8X Pretreatment Prevents CP Toxicity, and Delayed Onset of IP C8X Treatment Reduces Toxicity

IP C8X pre-treatment, followed by daily treatment, significantly reduced CP toxicity when compared with the IP vehicle (saline) pretreated group. The introduction of IP C8X treatment 1–4 days after CP treatment resulted in a stepwise reduction in...
CP toxicity with the animals treated with IP C8X 1 day after CP treatment, returning to the toxicity levels seen in the IP C8X pre-treated group first, followed by those that started IP C8X treatment on days 2, 3, and finally 4 (Fig. 4A; N = 4–5/group). This reversal of toxicity occurred 1–3 days after IP C8X treatment onset; by day 7 only the IP saline pretreated group differed significantly from IP C8X pre-treated animals (Fig. 4A). A significant within-group effect of time and treatment and a significant time × treatment interaction was seen, as well as a significant between-group effect of treatment (RMANOVA; p < 0.05). Similar results were also seen in other behavioral measures such as incline plane angle (Fig. 4B; p < 0.05) as well as mobility and aerial righting reflex scores (data not shown).

As seen with ICV ACSF pretreatment (Fig. 2) after euthanasia on day 7, IP saline pre-treated animals did not have detectable levels of CP remaining in the brain, which was significantly lower than the brain CP levels seen in the IP C8X pre-treated group (p < 0.05; Fig. 5A). Moreover, brain CP levels were also significantly lower in most of the delayed treatment onset groups compared with the IP C8X pre- and daily-treated groups (p < 0.05; Fig. 5A). All groups had significantly lower serum CP compared with the IP C8X pre-treated group (p < 0.05; Fig. 5B) and displayed treatment-duration dependency (p < 0.001, Fig. 5A). Brain CPO levels also displayed treatment-duration dependency (p < 0.05, Fig. 5C) and were significantly higher in the IP saline pretreated animals, and in those that started receiving IP C8X on day 3, compared with the IP C8X pre-treated group suggesting that in the absence of C8X inhibition there was greater activation of CP to the toxic CPO (p < 0.05, Fig. 5C). IP saline pretreated animals had significantly lower brain ChE activity than the IP C8X pre-treated group, suggesting greater CP activation to CPO and ChE inhibition in the brain. Brain ChE activities displayed treatment-duration dependency (p < 0.01, Fig. 5D) and were significantly lower in the animals that started receiving IP C8X on days 1 and 4 compared with the IP C8X pre-treated group (p < 0.05, Fig. 5D), whereas no differences in serum ChE activities were observed, as also seen with ICV C8X inhibition (Fig. 1) and over the wider dose range tested using IP C8X (Fig. 3).

Discontinuation of both ICV and IP C8X Treatment does not Result in Toxicity

Because daily C8X treatment resulted in higher CP concentrations in the brain 7 days after CP, it was possible this would result in toxicity once the C8X treatment was halted. Therefore, to test if the blockade of CPO production, and the resulting prolonged CP exposure, caused toxicity after the termination of CYP2B1 inhibition, we assessed the effects of C8X treatment termination on animals receiving either ICV or IP C8X pretreatment (or respective vehicles, followed by daily treatment), as well as rats that received either ICV or IP C8X from day 3 to day 7. The delayed C8X treatment (+3) groups, chosen because they were the group that resulted in complete disappearance of toxicity by day 7 across all measures and seemed a potentially likely treatment paradigm following accidental exposure, were used to assess whether C8X pretreatment would result in greater toxicity upon cessation. Furthermore, because this group had higher brain CPO levels (Fig. 5) than the pre-treatment group, we wanted to see whether that remaining CPO on day 7 could contribute to a return in toxicity upon discontinuation of C8X treatment. Pretreatment with C8X via both ICV and IP routes significantly blocked CP toxicity seen in the vehicle ICV and IP pre-treated rats; the pattern of toxicity and reversal was virtually identical for both routes again suggesting the main role of the inhibition of CP to CPO is within the brain. Following termination of ICV or IP C8X treatment 7 days after CP treatment (similar to Figs. 1 and 4), no increase in toxicity was seen, including in those groups where active CYP2B1 inhibition was started 3 days after CP (Fig. 6A; N = 4–5/group). A significant within-group effect of time and treatment and a significant time × treatment interaction was seen, as well as a significant between-group effect of treatment (p < 0.05). Only the vehicle treated groups were significantly different from the pre- and daily-treated C8X group on day 10 (Fig. 6A). A similar lack of rebound toxicity was seen for incline plane angle (Fig. 6B; p < 0.05) as well as other behavioral measures such as arousal and aerial righting reflex scores (not shown).

CP could not be detected in the brain or plasma on day 10, suggesting that all detectable CP had been cleared from the body by then. No significant differences were seen in brain or serum ChE (not shown), however, there was a slight trend toward lower brain ChE activity in the vehicle treated groups (p < 0.09), suggesting that in the absence of inhibition of CYP2B1-mediated CPO formation, there was a more prolonged inactivation of the ChE enzyme.

**DISCUSSION**

Using our rat model of a single high-dose CP exposure (similar to deliberate or accidental CP poisonings), we showed that pre-treatment and daily ICV or IP injections of a CYP2B MBI C8X inhibited local brain activation of CP to CPO (increasing brain CP, reducing inhibition of ChE, blocking the behavioral toxicity). Delayed introduction of CYP2B1 MBI (C8X) by either ICV or IP routes resulted in a reduction/reversal in toxicity, with the earliest treatments being most effective. The toxicity approached levels seen in the C8X pretreated rats within 1–2 days. This suggests that after commonly occurring CP poisoning incidents (Lein et al., 2012), via either self-poisoning, or accidental exposure, introducing a CYP2B inhibitor can effectively reduce toxicity and overall harm from CP exposure. Consistent with our previous findings (Khokhar and Tyndale, 2012), ICV administration of C8X did not affect serum CP levels, suggesting that C8X did not cross into the periphery in sufficient quantities to inhibit hepatic CYP2B-mediated CP metabolism.

Peripheral administration of C8X dose-dependently blocked CP activation in the rat brain and brain ChE inhibition as well
as the centrally mediated hypothermia, suggesting that C8X, the CYP2B1 MBI employed here, could effectively cross the blood brain barrier and inhibit brain CYP2B1, further confirming that the lack of toxicity seen in the IP C8X pre-treated group is due to continued inhibition of brain CYP2B1, and the resultant decrease in local activation of CP. IP administration was used here to model oral therapeutic drug (inhibitor) intake in humans due to the considerable first-pass effect with both oral and IP routes, and the relative ease of use of this route of administration in animals. Moreover, because peripheral administration of MBI produced very similar results to the ICV treatment experiments, this suggests the potential utility of peripheral administration of CYP2B MBI for the reduction of CP toxicity.

Our previous research report showed that 2 days after a single ICV MBI treatment, considerable behavioral toxicity was seen in rats and was comparable to rats receiving ICV vehicle (Khokhar and Tyndale, 2012). Single injections of MBI resulted in de novo CYP2B enzyme synthesis 2 days later and as a result, remaining CP was activated to CPO by the uninhibited CYP2B, resulting in toxicity. We assessed whether discontinuation of daily MBI treatment 7 days after CP treatment would also result in toxicity, and found no evidence of toxicity between days 7 and 10 suggesting that the bioavailable levels of CP were low enough by day 7 that any activation to CPO was not sufficient to result in toxicity in the measures that we assessed; consistent with a lack of detectable CP and CPO in brain and plasma on day 10. These findings make the potential use of a CYP2B inhibitor as a therapeutic agent to reduce CP (and other organophosphorus pesticides activated by CYP2B, e.g., parathion, malathion, diazinon, azinphos-methyl, and methyl-parathion (Albores et al., 2001; Buratti et al., 2003, 2005)) activation even more plausible as they can be given systemically, even days after poisoning and rebound toxicity was not observed if inhibition was maintained.

Using CYP2B inhibitors, alone or as adjuncts to cholinesterase reactivators (e.g., pralidoxime), to prevent the further activation of CP may be useful in preventing subsequent inhibition of the reactivated AChE. Inhibiting CP activation also avoids the problem of “aging” usually associated with organophosphorus pesticides such as chlorpyrifos, where activated organophosphorus pesticides render the phosphorylated enzyme very stable so that recovery of AChE activity occurs only through the synthesis of new enzyme reducing the efficacy of AChE reactivators (Lotti and Moretto, 2005). Moreover, a recent study in humans suggested that CP-poisoned patients had the slowest rate of serum cholinesterase activity recovery after acute poisoning of all organophosphorus pesticides tested in the study including profenophos, dimethoate, malathion, and others, possibly due to its “aging” as well its fat-soluble nature and long-lasting bioavailability (Chaou et al., 2013). This emphasizes the need for therapeutics that could reduce the amount of CPO over some time, and as a result, the inhibition of AChE activity and toxicity.

Recent studies have also shown that genetic variants in human CYP2B6, namely CYP2B6*4, 5, 6, and 7, have higher V_{max} and Cl_{int} for CP activation to CPO compared with the wild-type, indicating a potentially heightened risk for CP toxicity in individuals with faster CYP2B-mediated metabolic activation of organophosphorus pesticides (Crane et al., 2012a,b). Moreover, CYP2B6 protein is found at higher levels in the human brain in individuals exposed to commonly used substances such as tobacco and alcohol (Khokhar et al., 2010; Miksys et al., 2003) which could put those that consume these drugs at greater risk for CP-induced toxicity due to greater CPO production. Furthermore, as female humans display higher CYP2B6 expression and activity (Lamba et al., 2003), females may be at a greater risk for CP activation and toxicity (Tang et al., 2001). This is consistent with the great interindividual variability in the formation of CPO in patients poisoned with CP, with a 100-fold difference in CPO to CP ratio between patients (Eyer et al., 2009). Moreover, the effectiveness of pralidoxime in reactivating the inhibited acetylcholinesterase was strongly dependent on the CPO concentration (Eyer et al., 2009). Inhibition of CYP2B-mediated CP activation would reduce the CPO burden, regardless of variability in brain CYP2B levels, and therefore promote a favorable response alone or with pralidoxime treatment.

This finding is the first in vivo evidence of both central and peripheral CYP enzyme inhibition being useful as a therapeutic agent to reduce neurotoxicity from a commonly used brain-activated neurotoxicant. This approach becomes especially interesting as a large variety of organophosphorus pesticide neurotoxicants are activated by CYP2B (e.g., parathion, malathion, methylparathion, diazinon (Albores et al., 2001; Buratti et al., 2003, 2005)). Of note, variation in other brain-expressed CYP subfamilies (e.g., CYP2D, 2E, and 3A) may alter neurotoxicity for separate classes of toxicants including MPTP, TIQ, paraquat, and harmaline. Depending on an individual’s ability to activate or inactivate toxicants, inhibitors or inducers of these enzymes could be used to reduce toxicity. Moreover, our approach of using both central and peripheral administration CYP enzyme inhibitors to alter CP activity in the brain might be useful in targeting the disposition of drugs to achieve preferred central therapeutic concentrations, as well as minimizing the side-effects associated with centrally acting drugs (Khokhar and Tyndale, 2011).

Based on the common exposure to chlorpyrifos, either intentionally or by accident, and the need for more efficacious therapeutics targeting organophosphorus pesticide-mediated toxicity, we present an effective potential therapeutic avenue that may help to reduce the harm from poisoning with organophosphorus pesticides such as chlorpyrifos. Blocking the CYP-mediated bioactivation of organophosphorus pesticide neurotoxicants within the brain using peripheral delivery of CYP2B inhibitors even days after poisoning may be a useful therapeutic approach alone, or in addition to AChE reactivators.
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