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Behavioral Sequelae Following Acute Diisopropylfluorophosphate Intoxication in Rats: Comparative Effects of Atropine and Cannabinomimetics

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Abstract

The comparative effects of atropine and the indirect cannabinomimetics URB597 (a fatty acid amide hydrolase inhibitor) and URB602 (a monoacylglycerol lipase inhibitor) on functional and neurobehavioral endpoints following acute diisopropylfluorophosphate intoxication were studied. Male Sprague-Dawley rats were treated with vehicle or DFP (2.5 mg/kg, sc), immediately post-treated with either vehicle, atropine (16 mg/kg), URB597 (3 mg/kg), URB602 (10 mg/kg) or a combination of URB597 and URB602, and functional signs of toxicity as well as nocturnal motor activity were measured daily for seven consecutive days. Performance in the elevated plus maze (for anxiety-like behavior) and the forced swimming test (for depression-like behavior) was measured at days 6-8 and 27-29 after dosing. Twenty-four hours after dosing, DFP markedly reduced cholinesterase activity in selected brain regions and peripheral tissues (diaphragm and plasma). Substantial recovery of cholinesterase activity was noted at both 8 and 29 days after dosing but significant inhibition was still noted in some brain regions at the latest time-point. DFP elicited body weight reductions and typical signs of cholinergic toxicity, and reduced nocturnal ambulation and rearing. Atropine and the cannabinomimetics (alone and in combination) partially attenuated DFP-induced functional signs of toxicity. None of the post-treatments reversed the DFP-induced reduction in ambulation or rearing, however. No significant treatment-related effects on elevated plus maze performance were noted. DFP-treated rats exhibited decreased swimming and increased immobility in the forced swimming test at both time-points. None of the post-treatments had any effect on DFP-induced changes in immobility or swimming at day 8. At day 29, atropine and the combination of URB597/URB602 significantly blocked DFP-induced changes in immobility, while URB597 and the combination reversed DFP-induced changes in swimming. The results suggest that early blockade of muscarinic receptors and enhancement of eCB signaling can attenuate both acute and delayed effects elicited by DFP.

Keywords

atropine; diisopropylfluorophosphate (DFP); elevated plus maze; fatty acid amide hydrolase (FAAH); forced swimming test; monoacylglycerol lipase (MAGL); motor activity; URB597; URB602

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The authors declare that there are no conflicts of interest.

1. Introduction

Organophosphorus insecticides (OPs) are a major class of pesticides that elicit acute toxicity by inhibiting acetylcholinesterase (AChE), the enzyme responsible for the degradation of the neurotransmitter acetylcholine in the central and peripheral nervous systems. Acetylcholine accumulation following extensive AChE inhibition increases/prolongs the stimulation of cholinergic muscarinic and nicotinic receptors on postsynaptic neurons, myocytes and autonomic ganglia and end-organs, leading to an acute cholinergic syndrome characterized by autonomic dysfunction, involuntary movements, muscle fasciculations, respiratory distress, seizures and other signs.

The U.S. Environmental Protection Agency estimates that 65-90 million pounds of OPs are used each year in the United States, and they remain commonly used worldwide [24]. In addition to the well known acute toxicity, a number of epidemiological studies have also reported prolonged neurological changes following OP intoxication [48,52,58,62]. These studies indicate that individuals intoxicated by OPs can exhibit long-term deficits in cognitive and motor processes including abstraction, dexterity, memory, problem solving, visual attention and visuomotor speed. Interestingly, a prospective study of Nicaraguan patients showed that these neurological deficits tended to abate over a two year period and were replaced by an increase in neuropsychiatric symptoms including anxiety, depression, irritability and restlessness [10]. In addition, Sanchez-Amate et al. [49] reported that rats given the OP chlorpyrifos, at doses that failed to elicit overt signs of cholinergic toxicity, exhibited increased time in the open arms of an elevated plus maze, suggesting that OPs may affect anxiety-like behavior. Furthermore, higher rates of anxiety and depression have been reported in agricultural areas [46,51,57], and depression has consistently been identified as an important risk factor for suicide [5,20]. London et al. [27] and Jaga and Dharmani [21] proposed that exposure to OPs could have a causal relationship in the number of suicide attempts worldwide by inducing changes in affect.

The basic principles of therapeutic intervention in cases of acute OP intoxication have not changed substantially over the last half century [31]. In fact, all countries worldwide use the same treatment strategy: an anticholinergic drug (e.g., atropine) to counteract the acute cholinergic syndrome, an oxime (e.g., pralidoxime) to reactivate AChE and an anticonvulsant (e.g., diazepam) to treat seizures and prevent neuropathology. Substantial clinical evidence questions the effectiveness of pralidoxime when given in combination with atropine [8]. In addition, the highest incidence of persistent neuropsychological symptoms in patients with prior OP intoxication is reported in cases requiring hospitalization, and thus almost certainly treated with the first line of defense, atropine [6]. While atropine effectively blocks postsynaptic muscarinic receptors to alleviate some clinical signs elicited by acetylcholinesterase inhibition, it can also block presynaptic muscarinic receptors mediating the adaptive inhibition of acetylcholine release, thus potentially contributing to more prolonged persistence of acetylcholine and potentially increased activation of nicotinic receptors. Thus, alternative therapeutic strategies may lead to more effective long-term recovery following acute intoxications.

Recent studies in our laboratory suggest that the enhancement of endocannabinoid (eCB) signaling can reduce the acute signs of toxicity following exposure to OP anticholinesterases [38,39]. Endocannabinoids (e.g., anandamide (AEA) and 2-arachidonylglycerol (2-AG)) are neuromodulators produced by neuronal depolarization that act in a retrograde fashion *via* presynaptic cannabinoid type 1 (CB1) receptors to inhibit the release of various neurotransmitters including acetylcholine [9,15,60], dopamine [4,59], glutamate [26,55], GABA [34], norepinephrine [53] and serotonin [37]. Endocannabinoid signaling is terminated

by enzymatic hydrolysis, a process that for AEA is mediated by fatty acid amide hydrolase (FAAH) and for 2-AG primarily by monoacylglycerol lipase (MAGL) (reviewed in [14,47]). Kathuria et al. [23] reported that URB597, a FAAH inhibitor [13], elicited anxiolytic behavior in rats. Moreover, URB597 both decreased immobility in rats in the forced swimming test and increased struggling in mice in the tail suspension test, two endpoints widely used for assessing antidepressant activity [17]. In both of these studies, the behavioral effects of URB597 were prevented by the pre-administration of SR141716A, a selective CB1 receptor antagonist/inverse agonist. Thus, we hypothesized that the enhancement of eCB signaling would block neurobehavioral changes following acute OP intoxication. We compared the effects of atropine with indirect cannabinomimetics (URB597; URB602, a MAGL inhibitor [25]; and a combination of these two drugs) on functional/neurobehavioral changes following acute diisopropylfluorophosphate (DFP) intoxication in rats.

2. Materials and methods

2.1. Treatments

DFP was purchased from Sigma-Aldrich Co. (St. Louis, MO), dissolved in peanut oil and administered (2.5 mg/kg, sc) in a volume of 1 ml/kg. Atropine was purchased from Sigma-Aldrich Co. (St. Louis, MO), dissolved in saline and administered (16 mg/kg, ip) in a volume of 1 ml/kg. DFURB597 (3′-carbamoyl-biphenyl-3-y-cyclohexylcarbamate) and URB602 (biphenyl-3-yl-carbamic acid cyclohexyl ester) were purchased from Cayman Chemical Company (Ann Arbor, MI), dissolved in vehicle (2% cremophor/2% dimethyl sulfoxide (DMSO) in saline) and intraperitoneally administered (3 and 10 mg/kg, respectively) in a volume of 3 ml/kg. Peanut oil (PNO) was used as a control for DFP and drug vehicles (either saline or cremophor/DMSO in saline) for atropine and the eCB drugs, respectively.

2.2. Animals

Fifty-seven experimentally naïve, male Sprague-Dawley rats weighing 175-199 g were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) over a 6-month period as part of four separate cohorts. Rats were individually housed in polycarbonate cages with unlimited access to food (Laboratory Rodent Diet 5001, PMI Feeds, Richmond, IN) and tap water in a temperature controlled room (22 ± 2°C) under a 12-h light/dark cycle (lights on at 07:00). After being allowed to acclimate to the environmental conditions for a period of at least five days, rats were treated with DFP and immediately given vehicle (DFP/VEH; n = 17), atropine (DFP/ATR; n = 13), URB597 (DFP/URB597; n = 8), URB602 (DFP/URB602; n = 5) or a combination of URB597 and URB602 (DFP/COMBO; n = 5). Control rats (PNO/VEH; n = 9) were injected with peanut oil and immediately treated with vehicle (four with saline and five controls with cremophor/DMSO in saline). Body weight was recorded daily. All animal care procedures were in accordance with guidelines set forth in the NIH/NRC *Guide for the Care and Use of Laboratory Animals* and were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

2.3. Functional signs of toxicity

Signs of cholinergic toxicity were recorded at 1, 2, 4 and 8 h post-injection and then daily for seven consecutive days based on the method of Moser et al. [36] by a trained observer “blinded” to the treatment groups. Involuntary movements were scored as: 2 = normal; 3 = mild tremors localized to head and neck regions; 4 = whole body tremors; 5 = myoclonic jerks. SLUD (an acronym for salivation, lacrimation, urination and defecation) signs were scored as: 1 = normal (no signs); 2 = mild (one or multiple, slight signs); 3 = moderate (multiple, obvious signs); 4 = severe (multiple, overt signs).

2.4. Motor activity

Nocturnal motor activity was measured daily for seven consecutive days post-injection using a home cage-based photobeam activity system (PAS-HC) from San Diego Instruments (San Diego, CA). A 4 × 8 photobeam configuration on the frame of the apparatus measured ambulation while another set of photobeams, fixed at a height of 12 cm from the cage floor, detected rearing. The PAS-HC application software was used to record the number of photobeam interruptions during each test session (20:00-02:00).

2.5. Elevated plus maze

The elevated plus maze consisted of four equal-sized arms (50 cm long × 10 cm wide) extending from a central area (10 cm × 10 cm) in the shape of a plus sign, physically elevated 72.5 cm from the floor surface. Two of the arms were enclosed by solid walls 20 cm high (closed arms), whereas the other two arms had no walls (open arms). At 6 and 27 days after treatment, rats were placed in the central area facing an open arm and allowed to explore the maze for 5 min. Each test session (conducted during 13:00-17:00 h) was recorded with a video camera positioned above the maze such that the entire maze was in the recording frame, and the videotape was subsequently scored by a trained observer. The frequency and duration of two behaviors were recorded: open arm occupancy (all four paws were completely in an open arm) and closed arm occupancy (all four paws were completely in a closed arm). From these behaviors, the total number of arm entries and the percentage of time spent in the open arms [total amount of time spent in the open arms / (total amount of time spent in the open arms + total amount of time spent in the closed arms) × 100] were calculated. The percentage of time spent in the open arms is widely accepted as a valid measure of anxiety-like behavior [42].

2.6. Forced swimming test

The forced swimming test was conducted in a plastic cylinder (40 cm high × 48 cm diameter) filled to a level of 28 cm with tap water (25 ± 2°C). At 7 and 28 days post-injection, rats were placed into the center of the cylinder on two consecutive days for 10 minutes (day 1, pretest session) or 5 minutes (day 2, test session), after which they were removed, dried with a towel and returned to their home cages. The pretest session facilitates the development of immobility, which is widely accepted as a valid endpoint for depression-like behavior, and increases the sensitivity for detecting behavioral effects during the subsequent test session [29]. Each test session (conducted during 13:00-17:00 h) was recorded with a video camera positioned such that the entire cylinder was in full sight, and the videotape was subsequently scored by a trained observer using the sampling technique described by Detke et al. [11]. Briefly, each test session was broken down into sixty 5-s periods, and the observer recorded the rat's behavior at the end of each time period as one of three behaviors: climbing (upward-directed movement of the forepaws usually against the wall of the cylinder), swimming (horizontal movement throughout the cylinder, which includes crossing quadrants of the cylinder) or immobility (no overt movements other than those required to keep the head above water).

2.7. Cholinesterase assay

Twenty-four additional male Sprague-Dawley rats were treated with peanut oil (n = 9) or DFP (n = 5/time-point) and sacrificed at 24 h, 8 days or 29 days after dosing. Trunk blood was collected into 1.5-ml Eppendorf tubes containing heparin (20 μl, 10,000 units) and immediately mixed by repeated inversion. The tubes were centrifuged at 10,000 rpm using a microcentrifuge, and plasma was collected and stored at -70°C until assay. Cerebellum, frontal cortex, hippocampus and striatum were dissected on ice essentially as described by Glowinski and Iversen [16], and these tissues along with diaphragm (rinsed in ice cold saline and blotted dry) were frozen at -70°C until assay. Thawed tissues were homogenized at 28,000 rpm in 50 mM potassium phosphate buffer, pH 7.4 (1:20, w/v) using a Polytron homogenizer, and total

cholinesterase activity was measured by the radiometric method of Johnson and Russell [22] as described previously using [^{3}H] acetylcholine iodide (1 mM final concentration) as the substrate [39].

2.8. Statistical analyses

Graded functional data were transformed (square root) and analyzed using a repeated measures two-way analysis of variance (ANOVA) with time and treatment as variables, followed by the Bonferroni t-test for multiple comparisons. Body weight and motor activity data were analyzed using a repeated measures two-way ANOVA with time and treatment as variables, followed by the Bonferroni t-test. All other data were analyzed using one-way ANOVA followed by the Tukey's multiple comparisons test. Statistical analyses were all conducted using the GraphPad Prism 4 software. For all data, *p*-values less than 0.05 were considered significant.

3. Results

3.1. Functional signs of toxicity

Figure 1 shows body weight changes following DFP exposure. Compared to control animals (PNO/VEH), DFP (DFP/VEH) caused a significant reduction in body weight at 1 and 6 days after treatment. Compared to the DFP/VEH group, no significant differences in body weight were observed in the DFP/ATR, DFP/URB597, DFP/URB602 or DFP/COMBO groups. No significant treatment-related changes in body weight were noted at 29 days after dosing, however.

Figure 2 shows the influence of atropine, URB597, URB602 or the combination on functional signs of toxicity following DFP exposure. Significant main effects of treatment and time as well as significant interactions were noted with both involuntary movements (A) and SLUD signs (B). DFP elicited involuntary movements from 1-48 h after treatment (DFP/VEH compared to PNO/VEH, Figure 2A). Compared to the DFP/VEH group, involuntary movements were significantly less extensive in the DFP/ATR group at 1, 2 and 4 h after treatment. Significant increases in SLUD signs were also noted following DFP exposure (DFP/VEH) compared to the control (PNO/VEH) group at 1, 2, 8 and 24 h after treatment (Figure 2B). Compared to the DFP/VEH group, SLUD signs were significantly less in the DFP/ATR group at 1, 2 and 24 h and in the DFP/URB597 and DFP/COMBO groups at 8 h after treatment. Interestingly, no lethality was observed in the DFP/VEH, DFP/URB597, DFP/URB602 or DFP/COMBO groups, while 3/13 (23%) rats in the DFP/ATR group died between 8 and 24 h after treatment.

3.2. Motor activity

Figure 3 shows the effects of DFP and post-treatment with vehicle, atropine, URB597, URB602 or the combination on nocturnal motor activity. Ambulation (Figure 3A) was significantly decreased for five days in the DFP/VEH group compared to PNO/VEH controls. Rearing (Figure 3B) was significantly decreased for three days in the DFP/VEH group compared to controls. Compared to DFP only, no significant effects on ambulation or rearing were observed following post-treatment with ATR, URB597, URB602 or the combination.

3.3. Elevated plus maze

Figure 4 shows the effects of DFP and post-treatment with vehicle, atropine, URB597, URB602 or the combination on performance in the elevated plus maze. No significant effects on total number of arm entries (data not shown) or percentage of time spent in the open arms were observed in any treatment group. There was a trend, however, towards a higher percentage of

time spent in the open arms in the DFP/VEH and DFP/URB602 groups at days 6 and 27 and in the DFP/COMBO group at day 27.

3.4. Forced swimming test

Figure 5 shows the effects of DFP and post-treatment with vehicle, atropine, URB597, URB602 or the combination on performance (climbing, swimming and immobility) in the forced swimming test. No significant effects on climbing behavior were observed with DFP exposure at either time-point. Swimming behavior was significantly decreased in the DFP/VEH group compared to the PNO/VEH group at both 8 and 29 days after treatment, however. Conversely, immobility was significantly increased in the DFP/VEH group compared to the PNO/VEH group at both 8 and 29 days after treatment. No post-treatment affected DFP-induced changes in either swimming or immobility at day 8. DFP-induced changes in swimming behavior were significantly reversed in the DFP/URB597 and DFP/COMBO groups at day 29, however. Moreover, DFP-induced changes in immobility were significantly reversed in the DFP/ATR and DFP/COMBO groups at day 29.

3.5. Cholinesterase assay

Table 1 shows the effects of DFP on total cholinesterase activity in diaphragm, plasma and selected brain regions at three different time-points after dosing. Cholinesterase activity in frontal cortex, hippocampus and striatum was significantly decreased in DFP-treated rats compared to controls (PNO group) at all three time-points. Compared to controls, cholinesterase activity in cerebellum, diaphragm and plasma was significantly decreased in DFP-treated rats only at 24 h after dosing.

4. Discussion

Organophosphorus insecticides are used ubiquitously throughout the world to control insect pests. While acute toxicity from these insecticides has been well recognized and characterized, long-term neurological consequences following acute intoxication are reported but much less understood. The anticholinergic drug atropine has been used for decades as the first line of treatment for acute OP intoxication (reviewed in [12]). However, its effects on long-term neurological sequelae following acute intoxications are unclear. Recent studies in our laboratory suggested that enhancing eCB signaling can reduce the functional signs of acute toxicity following OP anticholinesterase exposure [38,39]. We therefore evaluated the possible modulation of long-term neurobehavioral changes following acute DFP intoxication by atropine and selected indirect cannabinomimetics.

DFP (2.5 mg/kg, sc) elicited significant reductions in body weight (Figure 1) and classical signs of cholinergic toxicity (Figure 2). Atropine, URB597, URB602 and a combination of URB597 and URB602 all partially attenuated some of the acute effects of DFP intoxication. Atropine appeared to completely block DFP-induced involuntary movements for the first four hours after dosing, roughly corresponding with the reported plasma half-life of atropine (1.5-4 h; [28]). DFP-treated rats exhibited increased SLUD signs for 24 h after dosing, and atropine, URB597 and the URB597/URB602 combination reduced the severity of SLUD signs at various time-points. Similar to effects on involuntary movements, atropine initially completely blocked the expression of SLUD signs. DFP-treated rats also exhibited marked reduction in nocturnal ambulation and rearing for the first 3-5 days after dosing (Figure 3). None of the post-treatments had any significant effect on DFP-induced changes in motor activity, however.

No lethality was noted in DFP-treated rats post-treated with either vehicle, URB597, URB602 or the combination, while 23% (3/13) of DFP-treated rats given atropine died between the 8 and 24 h observations. While therapeutically effective against acute anticholinesterase

intoxication, atropine is highly toxic with a range of potential adverse effects including agitation, confusion, disorientation, hallucinations, hyperthermia and others. Hypoxic patients are at risk of developing ventricular fibrillation in response to atropine [3]. Hypoxia can be associated with acute OP insecticide intoxication [35], thus extreme caution is obviously necessary under these conditions. While oxygen tension and cardiac function were not monitored in these studies, cardiac complications could have contributed to the lethality noted in DFP-treated rats given atropine.

No statistically significant effects on elevated plus maze performance were observed in any treatment group (Figure 4). There was a trend, however, for DFP-treated rats to spend a higher percentage of time in the open arms compared to controls at days 6 and 27. Schulz and coworkers [54] reported that repeated exposures to methyl parathion in rats increased open arm time in the elevated plus maze. In contrast, low acute exposures to sarin and soman were reported to decrease time spent in the open arms [56]. Similarly, Assini et al. [2] reported that malathion (100 mg/kg) decreased open arm time while another laboratory [61] reported no effect of malathion (150 mg/kg) in elevated plus maze performance. Thus, it appears that under some conditions OP toxicants may elicit anxiogenic or anxiolytic responses, as modeled by elevated plus maze performance. Obviously, many factors could potentially influence behavioral outcome in this test across studies including the different OPs evaluated, dose levels, environmental conditions and others.

DFP-treated rats exhibited decreased swimming behavior and increased immobility in the forced swimming test at both time-points evaluated (i.e., 8 and 29 days after dosing; Figure 5), suggesting a depression-like response. Both atropine and URB597 significantly reversed these changes at day 29, possibly through different mechanisms (i.e., atropine decreased immobility whereas URB597 increased swimming behavior). Although URB602 had no effect on the behavioral changes elicited by DFP in the forced swimming test, the combination of URB597 and URB602 increased swimming behavior and decreased immobility at day 29. Atropine was previously reported to decrease immobility in the forced swimming test [30]. URB597 had potent antidepressant-like effects in both the mouse tail-suspension and rat forced swimming tests [1,17]. In fact, the effects of URB597 in the forced swimming test resemble those produced by serotonergic antidepressants [41]. Gobbi et al. [17] showed that URB597 increased firing in serotonergic neurons in the dorsal raphe nucleus, a region long implicated in the pathogenesis of depression (reviewed in [33]). It must be stressed, however, that the effects of atropine and URB597 in these cited studies were pharmacological in nature, i.e., they were evident relatively shortly after dosing. The modulation of forced swimming behavior noted in our studies was protracted, however, occurring days to weeks after DFP challenge with immediate post-treatment using atropine, URB597 or the combination of URB597 and URB602. It is not uncommon for xenobiotics to influence some measures of activity in the forced swimming test but not others. Antidepressants which selectively inhibit norepinephrine reuptake (e.g., desipramine) reduce immobility and selectively increase climbing without affecting swimming [29]. In contrast, selective serotonin reuptake inhibitors (e.g., fluoxetine) reduce immobility but increase swimming without affecting climbing. Thus, the effects of atropine and the cannabinomimetics noted here may involve contributions from both noradrenergic and serotonergic signaling.

A number of studies have reported that some OPs, including DFP, can inhibit eCB-degrading enzymes [40,43-45]. In fact, our laboratory previously reported that the dosage of DFP used in this study (2.5 mg/kg, sc) inhibits hippocampal FAAH activity by 42% at 24 h after dosing [39]. Given that URB597 is a FAAH inhibitor, it may seem counterintuitive to propose this type of agent to ameliorate the adverse effects of OP exposure. URB597 is a much more potent FAAH inhibitor *in vivo*, however [13]. Moreover, Nomura et al. [40] argued that the effects of OPs on FAAH activity contrast markedly with those produced by pharmacological or genetic

disruption of FAAH. These authors suggested that OP-induced cannabinergic alterations may be more likely attributable to the blockade of 2-AG degradation or possibly the disruption of both 2-AG and AEA metabolism. Our laboratory previously reported that DFP was a more potent *in vitro* inhibitor of hippocampal MAGL activity than FAAH [39], thus alterations in 2-AG and not AEA levels may be more likely a result of DFP exposure. Furthermore, the significant reduction in DFP-induced immobility elicited by the combination of URB597 and URB602 (Figure 5B) suggests that combined inhibition of the degradation of both 2-AG and AEA could be beneficial.

Hungund et al. [19] demonstrated an up-regulation of CB1 receptor density in the dorsolateral prefrontal cortex of persons with major depression, suggesting a possible role for the CB1 receptor in the pathophysiology of depression. In addition, Hill et al. [18] reported reduced serum 2-AG levels in drug-free females diagnosed with major depression, with levels of 2-AG negatively correlated to the duration of depressive episodes. Moreover, CB1 knockout mice display behavioral alterations related to depression including anhedonia, anxiety, enhanced stress responsiveness and feeding dysregulation [7,32,50]. Taken together, these findings suggest that the neuropsychological consequences of acute OP intoxication are sensitive to early modulation by drugs that enhance eCB signaling *via* either the blockade of AEA hydrolysis or the disruption of both AEA and 2-AG metabolism. Knowledge of how acute OP intoxication may lead to changes in affect could be useful in treating OP poisonings as well as in understanding the neurochemical mechanisms of depression.

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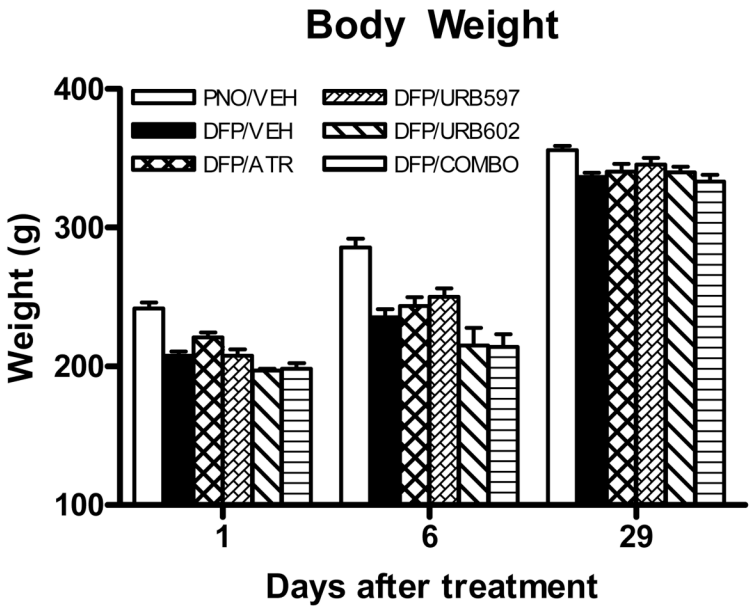

Figure 1. Effects of DFP and post-treatment with vehicle, atropine, URB597, URB602 or the combination of URB597 and URB602 on body weight

Rats were injected with DFP and immediately treated with vehicle (DFP/VEH, n = 17), atropine (DFP/ATR, n = 10), URB597 (DFP/URB597, n = 8), URB602 (DFP/URB602, n = 5) or the combination (DFP/COMBO, n = 5). Control rats (PNO/VEH, n = 9) were injected with peanut oil and immediately treated with vehicle. Body weight was recorded at 1, 6 and 29 days post-injection, and data are expressed as mean + standard error of the mean (SEM). All DFP-treated groups showed significant body weight reductions at both 1 and 6 days after dosing, while no significant treatment-related differences were noted at day 29.

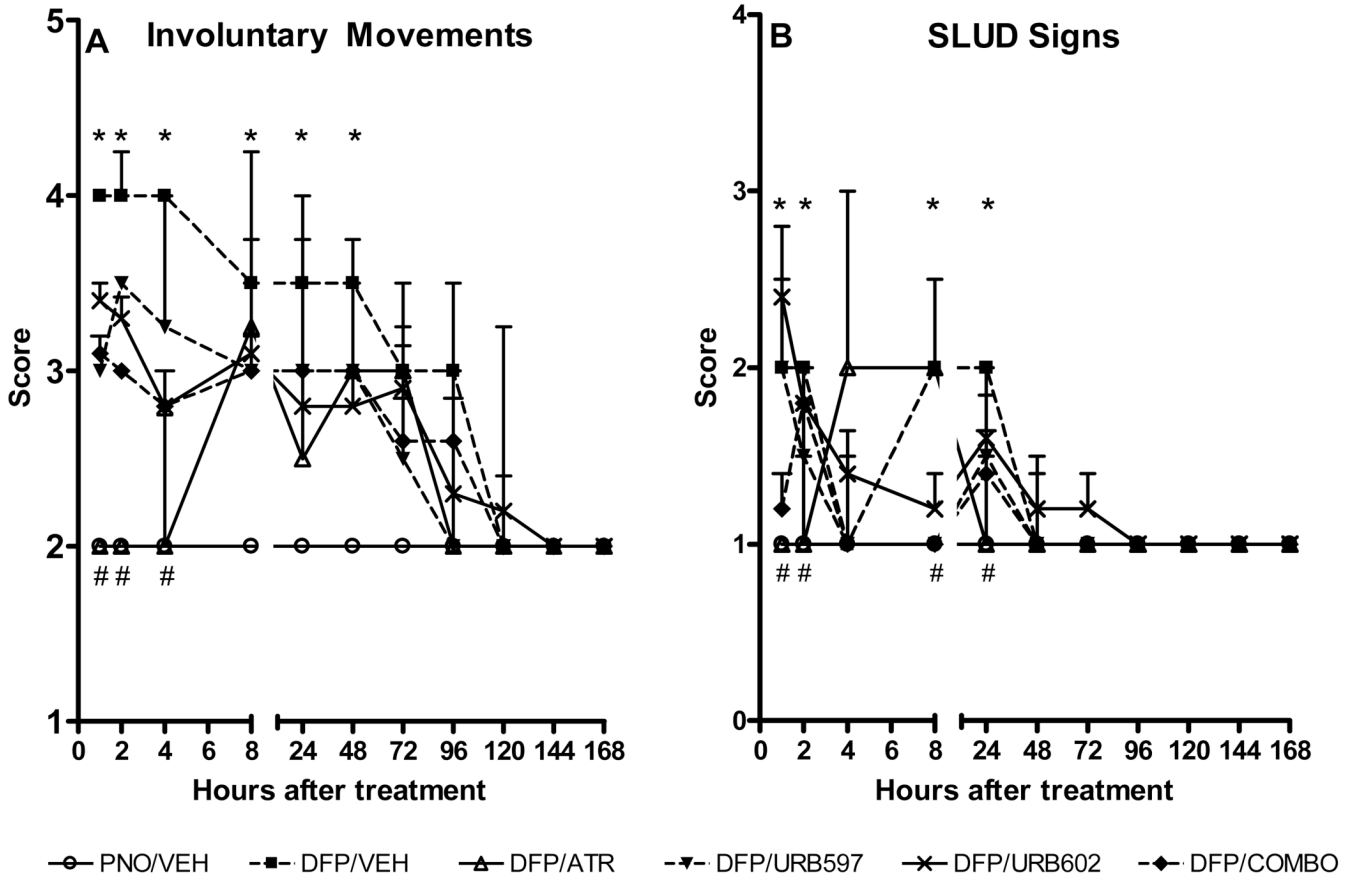

Figure 2. Effects of post-treatment with vehicle, atropine, URB597, URB602 or the combination of URB597 and URB602 on functional signs of toxicity elicited by DFP

Rats were injected with DFP and immediately treated with vehicle (DFP/VEH, n = 17), atropine (DFP/ATR, n = 10), URB597 (DFP/URB597, n = 8), URB602 (DFP/URB602, n = 5) or the combination (DFP/COMBO, n = 5). Control rats (PNO/VEH, n = 9) were injected with peanut oil and immediately treated with vehicle. Functional signs of toxicity [i.e., involuntary movements (A) and SLUD signs (B)] were measured at 1, 2, 4 and 8 h post-injection as well as daily for seven consecutive days. Data are expressed as median + interquartile ratio. An asterisk indicates a significant difference compared to the PNO/VEH group, and a pound sign indicates a significant difference compared to the DFP/VEH group.

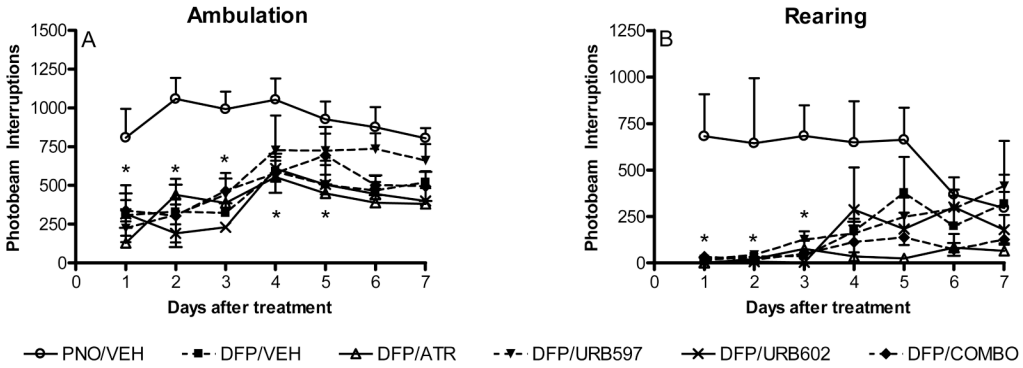

Figure 3. Effects of DFP and post-treatment with vehicle, atropine, URB597, URB602 or the combination of URB597 and URB602 on nocturnal motor activity

Rats were injected with DFP and immediately treated with vehicle (DFP/VEH, n = 7), atropine (DFP/ATR, n = 3), URB597 (DFP/URB597, n = 6), URB602 (DFP/URB602, n = 5) or the combination (DFP/COMBO, n = 5). Control rats (PNO/VEH, n = 6) were injected with peanut oil and immediately treated with vehicle. Nocturnal motor activity [i.e., ambulation (A) and rearing (B)] was measured daily for seven consecutive days post-injection. Data are expressed as mean + SEM. An asterisk indicates a significant difference between the PNO/VEH and DFP/VEH groups.

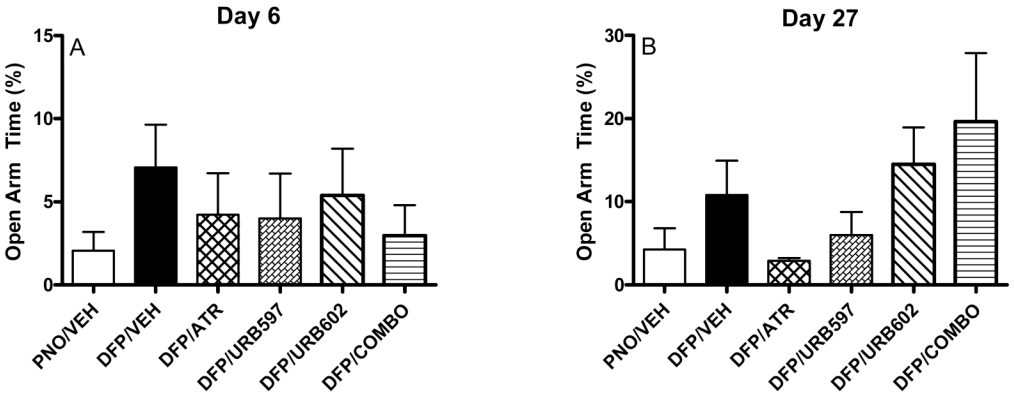

Figure 4. Effects of DFP and post-treatment with vehicle, atropine, URB597, URB602 or the combination of URB597 and URB602 on performance in the elevated plus maze

A) Rats were injected with DFP and immediately treated with vehicle (DFP/VEH, n = 15), atropine (DFP/ATR, n = 9), URB597 (DFP/URB597, n = 8), URB602 (DFP/URB602, n = 5) or the combination (DFP/COMBO, n = 5). Control rats (PNO/VEH, n = 9) were injected with peanut oil and immediately treated with vehicle. Performance in the elevated plus maze (i.e., percentage of time spent in the open arms) was then measured at six days after dosing. B) Rats were injected with DFP and immediately treated with vehicle (DFP/VEH, n = 9), atropine (DFP/ATR, n = 3), URB597 (DFP/URB597, n = 8), URB602 (DFP/URB602, n = 5) or the combination (DFP/COMBO, n = 5). Control rats (PNO/VEH, n = 9) were injected with peanut oil and immediately treated with vehicle. Percentage of time spent in the open arms was then measured at 27 days after dosing. Data are expressed as mean + SEM.

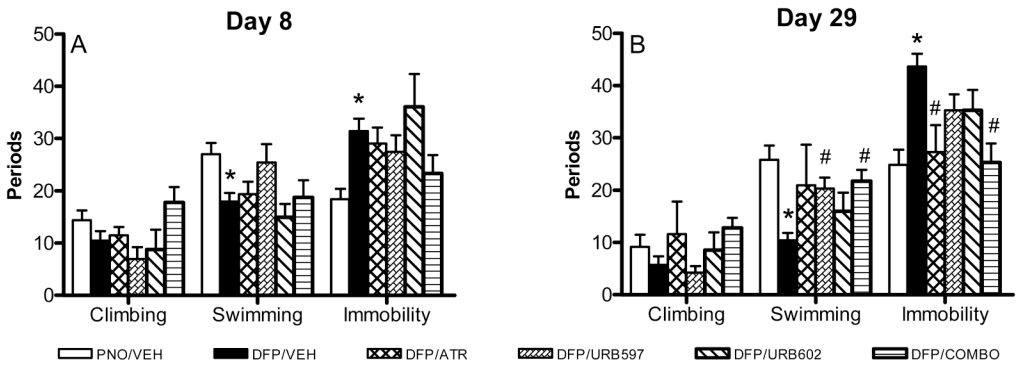

Figure 5. Effects of DFP and post-treatment with vehicle, atropine, URB597, URB602 or the combination of URB597 and URB602 on performance in the forced swimming test

A) Rats were injected with DFP and immediately treated with vehicle (DFP/VEH, n = 17), atropine (DFP/ATR, n = 10), URB597 (DFP/URB597, n = 8), URB602 (DFP/URB602, n = 5) or the combination (DFP/COMBO, n = 5). Control rats (PNO/VEH, n = 9) were injected with peanut oil and immediately treated with vehicle. Performance in the forced swimming test (i.e., time spent climbing, swimming or immobile) was then measured at eight days after dosing. B) Rats were injected with DFP and immediately treated with vehicle (DFP/VEH, n = 9), atropine (DFP/ATR, n = 3), URB597 (DFP/URB597, n = 8), URB602 (DFP/URB602, n = 5) or the combination (DFP/COMBO, n = 5). Control rats (PNO/VEH, n = 9) were injected with peanut oil and immediately treated with vehicle. Performance in the forced swimming test was then measured at 29 days after dosing. Data are expressed as mean + SEM. An asterisk indicates a significant difference compared to the PNO/VEH group, and a pound sign indicates a significant difference compared to the DFP/VEH group.

Table 1

Effects of DFP on total cholinesterase activity

Rats were treated with peanut oil (PNO; n = 9) or DFP (n = 5/time-point) and sacrificed at 24 h, 8 days or 29 days after dosing. Various tissue samples along with plasma were collected from the rats and stored at -70°C until assay. Values in parenthesis represent percent of PNO. An asterisk indicates a significant difference between PNO- and DFP-treated tissues.

Tissue	PNO	DFP-24 hours	DFP-8 days	DFP-29 days
Cerebellum [a]	17.3 ± 1.0	4.7 ± 0.9 (27)*	14.2 ± 1.8 (82)	18.3 ± 0.8 (106)
Striatum [a]	391.2 ± 9.1	30.9 ± 4.5 (8)*	148.4 ± 5.0 (38)*	287.4 ± 5.5 (73)*
Frontal Cortex [a]	128.8 ± 7.6	12.5 ± 1.6 (10)*	62.8 ± 7.3 (49)*	80.6 ± 8.6 (63)*
Hippocampus [a]	36.8 ± 2.2	5.0 ± 0.1 (14)*	14.6 ± 1.4 (40)*	26.4 ± 1.1 (72)*
Plasma [b]	408.1 ± 10.8	155.3 ± 16.8 (38)*	452.3 ± 31.0 (111)	432.2 ± 23.6 (106)
Diaphragm [a]	5.3 ± 0.6	0.87 ± 0.05 (16)*	4.9 ± 0.2 (92)	3.7 ± 0.8 (70)

[a] Cholinesterase data is reported as nmol acetylcholine hydrolyzed/min/mg protein (mean ± SEM).

[b] Cholinesterase data is reported as nmol acetylcholine hydrolyzed/min/ml plasma (mean ± SEM).