Absence of Tolerance to the Anticonvulsant and Neuroprotective Effects of Imidazenil against DFP-Induced Seizure and Neuronal Damage

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a) **Running Title:** Imidazenil is devoid of anticonvulsant tolerance against DFP

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DFP, diisopropyl fluorophosphate ; OP, organophosphates; CWNA, chemical warfare nerve agent; AT, atropine sulphate; 2-PAM, pyridine-2-aldoxime methochloride; BZ, benzodiazepine; IMD, imidazenil; DZ, diazepam; MDZ, midazolam; FJB, fluoro-jade B

Key words: Imidazenil, diazepam, benzodiazepine, organophosphates, neuropathology, seizure, tolerance.

ABSTRACT

The clinical use of diazepam or midazolam to control organophosphate (OP) nerve agent-induced seizure activity is limited by their unwanted effects including sedation, amnesia, withdrawal, and anticonvulsant tolerance. Imidazenil is an imidazo-benzodiazepine derivative with high intrinsic efficacy and selectivity for α2-, α3-, and α5- but low intrinsic efficacy for α1-containing $GABA_A$ receptors. We have previously shown that imidazenil is more efficacious than diazepam at protecting rats and mice from diisopropyl fluorophosphate (DFP)-induced seizures and neuronal damage without producing sedation. In the present study, we compared the tolerance liability of imidazenil and diazepam to attenuate the seizure activity and neurotoxic effects of DFP. Rats received protracted (14 days) oral treatment with increasing doses of imidazenil (1 to 4 mg/kg), diazepam (5 to 20 mg/kg), or vehicle. Eighteen hours after the last dose of the protracted treatment schedule, rats were tested for anticonvulsant tolerance after a 30 min pretreatment with a single test dose of imidazenil (0.5 mg/kg) or diazepam (5 mg/kg) prior to a DFP challenge (1.5 mg/kg). The anticonvulsant (modified Racine score scale) and neuroprotective (fluoro-jade B staining) effects of diazepam were significantly reduced in protracted diazepam-treated animals whereas the effects of imidazenil were not altered in protracted imidazenil-treated animals. The present findings indicate that protracted imidazenil treatment does not produce tolerance to its protective action against the neurotoxic effects of OP exposure.

1. INTRODUCTION:

In an era of increased global risk from terrorist attacks, chemical warfare nerve agent (CWNA) exposure is of great concern (Holstage et al., 1997). Examples of the use of these agents in attacks targeting civilians and military in recent decades include the Japan subway attacks of 1995 (Tochigi et al., 2002; Okumara et al., 2003; Miyaki et al., 2005), the Iraqi-Iranian war of 1998 (Brown and Brix, 1998), and the possible low-level exposure of the US military during the Gulf War in 1991 (Haley et al., 1997, 2000; Couzin, 2004). Moreover, our military combat personnel are confronted on a daily basis with the threat of CWNA exposure during active military operations. The deliberate use of nerve agents in these examples clearly explains why defense and health departments take the threat of CWNA attack or exposure very seriously (Brown and Brix, 1998; Lee, 2003). A major concern with CWNA exposure is the rapid development of self-sustaining status epilepticus which once established, is difficult to treat and is often refractory to all therapies (Martin et al., 1985; Mazarati et al., 1998; McDonough et al., 2010).

The current medical countermeasures against CWNA, including organophosphate (OP) nerve agent poisons, consist of a pre-exposure treatment (when an attack is anticipated) with a reversible AChE inhibitor (usually pyridostigmine bromide) and post-exposure treatment with a muscarinic receptor antagonist [atropine sulfate (AT)] to counteract the acute cholinergic crisis. These treatments are usually accompanied by administration of an oxime [pyridine-2-aldoxime methochloride (2-PAM)] to reactivate CWNA-inhibited acetylcholinesterases. However, these treatments do not protect victims from intense CWNA-induced seizures. Hence, when a CWNA attack is anticipated, preventive treatment with an anticonvulsant benzodiazepine (BZ) [primarily diazepam (DZ) or midazolam (MDZ)] would be desirable to prevent the occurrence of status epilepticus and to mitigate subsequent irreversible neuronal damage (McDonough and Shih, 1997; Lallement et al., 1998; Shih and McDonough, 1999; Newmark, 2004a and

Newmark, 2004b).

Unfortunately, the current use of BZs for prevention and treatment of the neurological consequences of CWNA exposure has significant limitations. The preferred anticonvulsant BZs (DZ and MDZ) at the doses needed to prevent CWNA-induced seizures (Newmark, 2007; Shih et al., 2007; Kadriu et al., 2009, 2011), by acting with high intrinsic efficacy at α1-containing $GABA_A$ receptors, also elicit amnestic, sedative, or hypnotic actions and cardio-respiratory depression effects (Costa and Guidotti., 1996; Costa et al., 2001). Hence, these BZs are incapacitating and cannot be given prophylactically to individuals who have an anticipated risk of CWNA exposure (Tashma et al., 2001). These BZs can be administered immediately after CWNA exposure but are less effective anticonvulsants when administered after the instigation of status epilepticus (Mazarati et al., 1998; McDonough 2010).

In the search for an effective anticonvulsant agent to prevent or treat CWNA-induced seizure without eliciting sedative, amnestic, and cardio-respiratory depressant actions, we discovered imidazenil (IMD) (Giusti et al. 1993; Auta et al., 1994, 2004, 2008; Rump et al., 2000; Pibiri et al., 2008; Kadriu et al., 2009; 2011). This new generation BZ is a non-sedating and long acting (half-life 4-5 hrs in rodents, Giusti et al. 1993) CWNA-anticonvulsant agent with selectivity and high intrinsic allosteric efficacy at GABA receptors expressing α5-, α3-, α2- but low intrinsic efficacy at α1-containing $GABA_A$ receptors (Costa and Guidotti, 1996; Costa et al., 2002; Guidotti et al, 2005).

Further, DZ and MDZ are drugs with serious drawbacks; they have half-lives that are relatively shorter than those for toxic OP compounds that inhibit AChE irreversibly. Because of their unfavorable pharmacokinetic properties, DZ and MDZ treatments must be given repeatedly for up to 14 days depending on the exposure level (Weissmann-Brenner et al. 2002) or must be administered prophylactically to individuals at risk of

exposure to CWNA.

Studies in primates and rodents indicate that subjects treated repeatedly with DZ or MDZ but not IMD developed tolerance to the anticonvulsant actions of these agents (Hayward et al., 1990; Auta et al., 1994, 2008; Ghiani et al., 1994; Zanotti et al., 1996; Shih et al., 2007). Thus, it is important to establish whether repeated administration of equipotent anti-bicuculine doses of DZ and IMD (Auta et al., 2005, 2008) leads to the development of tolerance to the protective actions against OP-induced seizure and neuronal damage.

In the present study, we compared the tolerance liability of IMD to that of DZ with respect to their protective effects against seizures and neuronal damage induced by diisopropyl fluorophosphate (DFP). To this end, we used a previously established rat model of BZ anticonvulsant tolerance (Auta et al., 1994, 2008; Impagnatiello et al., 1996). Rats treated with equipotent anti-bicuculine doses of either IMD or DZ for a protracted period (14 days) were subsequently tested for the protective effects of a single test dose of IMD or DZ against DFP-induced seizures and neuronal damage. The behavioral manifestations of DFP-induced seizure activity were scored continuously for six hrs post-DFP challenge followed by a final 15 min recording at 48 hrs after the DFP challenge. Brain sections were subsequently evaluated histochemically for neuronal degeneration using a fluoro-jade B (FJB) staining procedure.

2. MATERIALS AND METHODS:

2.1. Animals: Adult male Fisher 344 rats (Harlan, Indianapolis) weighing 250-280 g were housed in groups (three per cage for histological studies) in standard plastic cages (42 x 26 x 20 cm) and maintained on an 11- to 13- hr light-dark cycle (lights from 6:00 A.M to 7:00 P.M). Standard rodent food and tap water were available *ad libitum*. All experiments were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal

Welfare Committee at the University of Illinois at Chicago.

2.2. Drugs: Diazepam (DZ) and imidazenil (IMD) were obtained from Hoffman-La Roche (Nutley, NJ). Atropine sulfate (AT), pyridine-2-aldoxime methochloride (2-PAM), and diisopropyl fluorophosphate (DFP) were obtained from Sigma-Aldrich Co. (St Louis, MO). AT and 2-PAM were dissolved in normal saline (0.9% NaCl). DFP, which is supplied in an oily solution, was freshly diluted in ice-cold saline just prior to administration. For acute administration, DZ or IMD was dissolved in 5-10% DMSO followed by dilution with vehicle that contained 11% polyethylene glycol-400, 50% propylene glycol, and 39% sterile water to a final DMSO concentration of <0.5% (Auta *et al.,* 1995). DZ, IMD, AT, 2-PAM, and vehicle were administered intraperitoneally while DFP was administered subcutaneously (sc) in volumes of 0.1 ml/100 g.

2.3. Schedule for protracted (14 days) BZ treatment

To test for tolerance to the anticonvulsant and neuroprotective effects of DZ or IMD, different groups of rats were treated three times daily (at approximately 9:00 A.M., 2:00 P.M. and 7:00 P.M.) with IMD, DZ, or vehicle for 14 days (see Figure 1). This is a well established rat model method to study BZ anticonvulsant tolerance (Pesold et al., 1997; Auta et al., 2004). For protracted treatment, drugs were suspended in water containing 0.05% Tween-20 and administered in 1 ml volumes by oral gavage. The initial doses of IMD and DZ were selected based on the their comparative anticonvulsant efficacies against DFP-induced seizures (Auta et al., 2005; Kadriu et al, 2009, 2011) and were gradually increased over the 14 day treatment schedule (DZ: days 1–3, 5 mg/kg; days 4–6, 10 mg/kg; days 7–10, 15 mg/kg and days 11–14, 20 mg/kg; IMD: days 1–3, 1 mg/kg, days 4–6, 2 mg/kg, days 7–10, 3 mg/kg, days 11–14, 4 mg/kg) as previously described (Auta et al., 2008) and illustrated in fig. 1. Control rats received vehicle treatment.

2.4. Behavioral manifestations of seizure activity and neuronal damage

To study DFP-induced behavioral manifestation of seizure activity, groups of four rats that received protracted VEH, DZ, or IMD treatment were left drug-free for at least 18 hrs before receiving a 30 min pretreatment with a single intraperitoneal (ip) test dose of DZ (5 mg/kg, ip) or IMD (0.5 mg/kg, ip) prior to administration of a convulsant dose of DFP (1.5 mg/kg, sc) and 2 min later, animals received a combination of AT (2 mg/kg, ip) and 2-PAM (20 mg/kg, ip). Animals were monitored continuously by video camera for the first six hrs and then recorded once again at 48 hrs after DFP acute challenge. After this procedure, rats were killed and brains removed and processed for immunohistological analysis (see Fig.1 for schematic representation).

2.4.1 Modified Racine's Score:

Rats were scored for behavioral manifestations of seizure activity in periods of five min, once every five min for the first 15 min, then once every 15 min up to one hr, then once per hr up to six hrs, and finally for 15 min at 48 hrs after the DFP- challenge.

DFP-induced behavioral manifestations of seizures were scored using a six-grade modified Racine scoring system (Racine, 1972; Lüttjohann et al., 2009). Briefly, a score of 0 represents no response (normal rat movement); score 1, ear and facial twitching; score 2, myoclonic jerks, without rearing; score 3, myoclonic jerks with rearing and bilateral forelimb clonus; score 4, tonic-clonic seizure; and score 5, generalized tonic-clonic seizure with loss of postural control. A seizure episode was defined as the time window from seizure onset to recovery from the attack.

In previous experiments using continuous radiotelemetry recordings to monitor and quantify DFP-induced electroencephalographic (EEG) seizure activity, we established a good correlation between the behavioral signs of seizure and EEG seizure activity (Kadriu et al. 2009, 2011).

To study the tolerance liabilities of IMD and DZ with respect to their protective actions

against DFP-induced seizures, we used a modified Racine scale score instead of EEG-telemetry for the following reasons: 1) The acute or repeated daily oral route of drug administration for protracted periods that we used in previous experiments to study BZ tolerance (Auta et al., 1994, 2004; Impagnatiello et al. 1996) will not be practicable once animals have been implanted with radio telemetry probes because three times daily restraining and handling of animals during oral drug treatment might damage electrodes and telemetry probes. 2) Following implantation of radio telemetry probes animals require 10-14 days to recover from their surgeries before the experimental procedures. Furthermore, the onset of withdrawal signs following discontinuation of protracted treatment is about 48 hr (Auta et al., 1994). For these reasons it is difficult to assess BZ's anticonvulsant tolerance using quantitative EEG analysis in animals implanted with radio telemetry probes.

2.4.2 Fluoro-jade B staining: To identify degenerating neurons in the brain, we used histochemical staining with FJB. This staining procedure is a sensitive and reliable marker for the neuronal damage that results from OP-poisoning and traumatic brain injury (Schmued et al., 1997 and Hopkins, 2000). We studied neuronal damage in piriform cortex, CA1 hippocampus, and amygdala since we have previously shown that these brain areas are sensitive to the neurotoxic effects of prolonged DFP-induced seizure activity (Kadriu, 2011).

Briefly, 25 μm brain sections were treated with 1% NaOH in 80% ethanol for five min and then hydrated in graded ethanol and distilled water. Sections were then incubated in 0.06 % potassium permanganate solution for 15 min, followed by a quick rinse and incubation in 0.001% FJB freshly prepared working solution. The slides were then rinsed and kept on a slide warmer set at approximately 45ºC until fully dried and then cleared by immersion in xylene for one min before cover slipping with DPX (Sigma-Aldrich Co., St. Louis, MO) and a non-aqueous, non-fluorescent plastic mounting

medium. Sections were examined using a Zeiss fluorescence microscope with a blue (450-490) excitation light by using the filter designed for visualizing fluorescein or FITC, which was suitable for FJB staining. Fluorescent images were captured using an AxioVision 4.6 (Zeiss) and an AxioCam Camera. For each brain area; five to six sections were taken and fluorescent-labeled cells were counted randomly with a bi-dimensional cell counting method at 40X objective in a square area of 100 x 100 μm. The final composites were processed using PowerPoint (Microsoft).

2.5 Statistical Analysis: The behavioral manifestations of seizure activity (modified Racine's score) were analyzed by Kruskal-Wallis ANOVA on ranks followed by Student-Neuman-Keuls multiple range comparison. Neuronal counts for FJB staining were analyzed by one-way repeated measures ANOVA and *post hoc* significance tests using Duncan's test with multiple range comparison. The p values equal to or less than critical values of 0.05 and 0.001 were considered statistically significant.

3. RESULTS

3.1. Anticonvulsant tolerance develops after protracted (14 days) DZ but not IMD treatment

DFP (1.5 mg/kg)-challenge followed two min later by a combination of AT (2 mg/kg) and 2-PAM (20mg/kg) resulted in severe seizure activity in 100% of rats that received vehicle (VEH) pretreatment (Fig 2). The onset of the behavioral manifestations of DFP-induced seizure activity ranged between five and ten min and was preceded by predictable and consistent behavioral signs including bouts of chewing activity and intermittent head tremors. These initial behavioral signs were followed by whole body tremors, jerky motions, and Straub tail movements that rapidly progressed to explosive tonic-clonic motor convulsions and finally into status epilepticus. Despite the severity of the

behavioral manifestation of seizure activity, the majority of rats (96 %) survived. Seizure intensity decreased in severity during the 72 hr following DFP-acute challenge.

To evaluate tolerance to the anticonvulsant effects of IMD and DZ against DFP-induced seizures, groups of rats that received protracted (14 days) VEH, IMD, or DZ treatment and were left drug free for 18 hrs prior to receiving a 30 min intraperitoneal pretreatment with IMD (0.5 mg/kg) or DZ (5 mg/kg) before the DFP (1.5 mg/kg, s.c.) acute challenge. Two min after DFP treatment, rats received a combination of AT (2 mg/kg) and 2-PAM (20 mg/kg) and were scored for behavioral manifestations of DFP-induced seizure activity.

During the first hour following DFP acute treatment, the severity of the behavioral manifestations of DFP-induced seizure activity in protracted VEH-treated rats was reduced to a similar extent by single test dose of IMD or DZ pretreatment. However, after the first hour, 0.5 mg/kg of IMD was more effective at attenuating DFP-induced behavioral manifestations of seizures than 5 mg/kg of DZ (Fig 2). Protracted VEH-treated rats that received the same single test dose of IMD showed reduced signs of seizure activity six hrs after DFP administration and almost complete recovery 48 hrs after DFP treatment. In contrast, protracted VEH-treated rats that received DZ (5 mg/kg) exhibited signs of intermittent body tremors and myoclonic jerks that were more evident approximately 2 hrs after the DZ injection and continued through all the behavioral observation period (Fig 2).

The Racine scores for protracted DZ-treated rats that received a single test of DZ prior to DFP challenge were not significantly different (see statistical analyses in legend of Fig 2) from the Racine scores of the control group (protracted VEH-treated rats) that received VEH pretreatment prior to DFP challenge (Fig 2), indicating the emergence of tolerance to the anticonvulsant effects of DZ. In contrast, protracted IMD-treated rats that received a 30 min pretreatment with a single test dose of IMD prior to DFP acute

challenge showed statistically lower Racine scores compared to the control group that received VEH pretreatment prior to DFP challenge. Moreover, protracted IMD and VEH-treated rats that received a 30 min pretreatment with a test dose of IMD showed similar Racine scores, suggesting the lack of tolerance to the anticonvulsant action of IMD against DFP-induced seizure activity. These data suggest that protracted DZ treatment resulted in anticonvulsant tolerance whereas protracted IMD treatment fails to induce anticonvulsant tolerance.

3.2. Fluoro-jade B (FJB) studies:

We used FJB staining to evaluate the extent of neuronal damage resulting from sustained (48 hrs) DFP-induced seizure activity. In Fig. 3, we show representative photomicrographs of FJB-positive cells in the piriform cortex of protracted VEH, DZ, or IMD-treated rats that received 30 min pretreatment with VEH and a single test dose of IMD or DZ prior to DFP challenge, respectively. We have previously shown that neurons of the piriform cortex are particularly susceptible to neurodegeneration after 48 hrs of sustained DFP-induced seizure activity and this brain area also shows marked protection following DZ or IMD pretreatment (Kadriu et al., 2009, 2011).

In Table 1, we show that in protracted VEH-treated rats, 48 hrs of sustained DFP-induced seizure activity resulted in significant increases in the number of FJB-positive neurons in the piriform cortex, CA1 hippocampus, and amygdala. However, in protracted VEH-treated rats that received a single test dose of IMD (0.5 mg/kg, i.p.) 30 min before DFP challenge, there was an ~72%, 56%, and 62% reduction in the number of FJB-positive neurons in the CA1 hippocampal region, amygdala, and piriform cortex, respectively (Table 1). Furthermore, in protracted VEH-treated rats, a single test dose of DZ (5 mg/kg) administered 30 min before DFP treatment resulted in an ~40% , 32% and 46% decrease in FJB-positive neurons in the CA1 hippocampus, amygdala, and piriform cortex, respectively (Table 1). In protracted VEH- and IMD-treated mice, the same

single test dose of IMD (0.5 mg/kg) produced a similar magnitude of protection from DFP-induced neuronal damage in piriform cortex, amygdala, and in CA1 hippocampus. In contrast, in the protracted DZ-treated group, the same single test dose of DZ (5 mg/kg) failed to produce a significant decrease in the number of FJB-positive stained neurons induced by DFP challenge in all the brain areas studied (Table 1 & Fig 3). These data suggests that protracted DZ but not IMD treatment resulted in loss of protective action against DFP-induced neuronal damage, most likely due to loss of anticonvulsant action following protracted DZ treatment.

4. Discussion

Data from humans (Haley et al., 1997, 2000; Couzin; 2004; Yamasue et al., 2007) and rodents show that survivors of life-threatening exposures to CWNA are likely to experience long-term neurological consequences (Abdel-Raman et al., 2002) including brain damage (Yamasue et al., 2007) as well as behavioral changes and cognitive deficits (Fullerton and Ursano., 1990; Brown and Brix, 1998, Pibiri et al. 2008).

The current antidotal therapy for CWNA poisoning on the battlefield includes pretreatment with pyridostigmine bromide and a posttreatment with atropine, oxime, and anticonvulsant BZs (such as DZ or MDZ) to reduce the short- and long-term after effects of seizure activity. Anticonvulsant drugs like DZ or MDZ can arrest OP-induced seizures when administered shortly prior to or immediately after CWNA exposure (Lipp, 1972; Martin et al., 1985; McDonough et al., 1989; Shih, 1990; Hayward et al., 1990; Jones et al., 2002). However, due to their relative short half life, the effectiveness of these BZs wanes after a relatively short time (Mazarati et al., 1998; McDonough et al., 2010; Kadriu 2009; 2011), thus allowing OP-induced seizures to recur and limiting clinical application of BZs for post-exposure treatment or as prophylactic anticonvulsant agents. Hence,

there is need for repeated large doses of these preferred anticonvulsant BZs to control OP-induced seizures and neuropathologies.

The use of DZ or MDZ in large and repeated doses induces severe sedation (2001; Mohler et al., 2001), depression of cardio-respiratory centers (Norris, 1999; Nordt and Clark, 1997; Ogutu et al., 2002; Chin et al., 2008), and anticonvulsant tolerance as shown here and by others (Costa et al., 2001; van Rijnsoever et al., 2004). Thus, to develop an effective and safe anti-CWNA treatment there is a need for an anticonvulsant and neuroprotective compound with a half-life significantly longer than that of DZ or MDZ. Such a compound should be capable of preventing seizures without causing cardio-respiratory depression, sedation, and anticonvulsant tolerance and should be able to increase the window of opportunity to prevent or minimize the neurological consequences of prolonged CWNA-induced seizures and also to augment the beneficial effects of currently used compounds such as AT and 2-PAM.

It is widely accepted that seizures resulting from OP nerve agent exposure most be treated in a timely manner to avoid progression into status epilepticus. In addition, prolonged status epilepticus can lead to irreversible brain damage and long-term neurological, behavioral and cognitive deficits (Brown and Brix, 1998; Solberg and Belkin, 1997). Thus, by facilitating GABA-gated chloride influx at $GABA_A$ receptors, anticonvulsant BZs such as diazepam and midazolam prevent OP-induces seizure activity and subsequent neuronal damage (McDonough and Shih, 1997; Newmark, 2004a and Newmark, 2004b). In a previous study using continuous radio telemetry recordings to monitor and quantify the severity of DFP-induced electrocorticographic seizure activity and fluoro-jade B (FJB) staining to evaluate the extent of neuronal damage, we (Kadriu et al 2009, 2011) demonstrated that a 30 min pretreatment with IMD (0.05 to 0.5 mg/kg) dose-dependently protected rats from DFP-induced electrocorticographic seizure activity and neuronal damage in a manner that is more

efficacious and longer-lasting than pretreatment with increasing doses (0.5 to 5 mg/kg) of DZ. These results suggest that IMD, which is virtually devoid of sedative and amnestic effects (Giusti et al., 1993; Rump et al., 2000; Auta et al., 1995, 2010), can be used to treat or given preemptively to persons who are at risk of OP exposure without concerns for the motor and cognitive impairment that are drawbacks with the currently preferred anticonvulsant BZs.

Using Racine behavioral scores to rate the severity of the behavioral manifestations of DFP-induced seizure activity, we confirm here that IMD (Rump et al., 2000; Auta et al., 2004; Pibiri et al., 2008; Kadriu et al., 2009, 2011) is more efficacious than DZ in attenuating OP-induced seizures and neuronal damage in rats. Most importantly, IMD protects against DFP-induced seizures at a dose that does not induce sedation or tolerance (Giusti et al. 1993; Auta et al., 1994, 2008, 2010). Unlike the protective effects of DZ or MDZ that are short-lasting allowing DFP-induced seizures to recur, IMD's protective effects persist for longer periods (Kadriu et al., 2009, 2011) and thus prevent the recurrence of DFP-induced seizure activity and the consequent neuronal damage. Although diazepam is rapidly metabolized into oxazepam and desmethyl diazepam which have relatively longer elimination half-lifes, the short duration of action for diazepam might be due to its short elimination half-life (Riss et al., 2008).

Several brain areas, including the motor and piriform cortex, hippocampus, and amygdala play critical roles in the initiation, propagation, and maintenance of CWNA-induced seizure activity (Piredda and Gale, 1985; McDonough et al., 1987; Loscher and Ebert, 1996; Bertram et al., 1998). Using histological analysis with FJB staining, we confirm here that in these susceptible brain areas, neurons are extremely sensitive to the neurotoxic effects of DFP. Furthermore, pretreatment with a dose of IMD, which attenuates DFP-induced seizure activity, resulted in remarkable neuronal protection in the piriform cortex, hippocampus, and amygdala of both protracted vehicle- and IMD-

treated animals. In contrast, a single test dose of DZ, which is equipotent to that of DZ at protecting from DFP-induced seizure activity during first two hr after DFP treatment, showed modest protection from DFP-induced neuronal damage (Table 1). Moreover, rats that received protracted DZ treatment and were tested with a single test dose of DZ prior to DFP exposure actually showed a severe level of neuronal damage with virtually no protection against DFP-induced neurotoxicity.

To explain the specific decrease in sensitivity to DZ after chronic DZ treatment, it has been suggested that chronic DZ treatment alters α and γ $GABA_A$ receptor subunit expression or uncouples α and γ receptor subunit interactions (Costa et al. 2001, Hu and Ticku, 1994; Roca et al., 1990). Our data are not inconsistent with this idea with respect to DZ tolerance. We have previously reported that chronic DZ treatment down-regulates the expression of α1 and γ2 subunits of $GABA_A$ receptors (Impagniatelio et al., 1996; Auta et al., 2008) with a consequent uncoupling of the allosteric modulation between GABA and BZ recognition sites (Roca et al. 1990; Hu and Ticku 1994; Ali and Olsen, 2001). In contrast, IMD, a non-sedating BZ recognition site ligand that acts with high intrinsic selective efficacy at α2-, α3- and α5- $GABA_A$ receptor subunits but is virtually inactive at α1-containing $GABA_A$ receptors, fails to down-regulate the expression of α1, γ2, or any other α $GABA_A$ receptor subunit and is devoid of tolerance and dependence liabilities (Giusti et al., 1993; Auta et al., 1994; Auta et al., 2004, 2005, Impagnatiello et al. 1996) although it still elicits strong anticonvulsant and anxiolytic properties.

Recent mutational investigations of different subunits of $GABA_A$ receptors have advanced our understanding of the role of various $GABA_A$ receptor subunits in the regulation of $GABA_A$ receptor function in response to agonists, antagonists, or inverse allosteric modulators. For example, in knock-in mice in which the α1-$GABA_A$ receptors have been rendered insensitive to the motor depressant and amnestic actions of BZs,

DZ still elicits anxiolytic and anticonvulsant actions, most likely through its positive modulatory actions at α2-, α3-, and α5-containing $GABA_A$ receptors (Mohler et al. 2001, Rudolph et al, 1999; Tobler et al 2001).

This unique pharmacological profile of IMD makes it an ideal agent to be used as therapeutic or prophylactic treatment against OP nerve agents. In addition, our findings confirm that long-term activation of $GABA_A$ receptors (Auta et al., 2008), particularly those containing $\alpha 1$ subunits but not the $\alpha 5$ receptor subunit, is crucial for the induction of anticonvulsant tolerance.

FIGURE LEGENDS:

Figure 1: Schematic illustration of protracted BZ treatment.

Figure 2: Racine scores for behavioral manifestations of DFP-induced seizure activity. Protracted (14 days) diazepam (DZ) but not imidazenil (IMD) treatment (Fig 1) produces tolerance to its anticonvulsant action against DFP-induced seizures. Statistical analysis: Each point is the mean ± SE of four rats per group. We compared the different experimental groups using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Student-Newman-Keuls multiple comparisons.

Tim	Min 0		Min 10	Min	Min	Min	1	2 h	3 h	4 h	5 h	6 h	~48
H	0.7	0.7	12.	15.	15.	16.	16.	16.	15.	16.	16.	16.	16.
p	0.94	0.10	0.01	0.004	0.004	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.002

Student-Newman-Keuls multiple comparisons: *p<0.05 when protracted VEH + DFP-treated rats were compared with VEH + DZ, VEH + IMD and protracted IMD + IMD-treated rats. #p<0.05 when protracted DZ + DZ-treated rats were compared with VEH + DZ, VEH + IMD and protracted IMD + IMD-treated rats.

Figure 3: Absence of tolerance to the neuroprotective effects of IMD against DFP-induced neuronal damage.

Rats received protracted treatment (14 days) with vehicle (VEH), increasing doses of IMD or DZ (illustrated in Fig 1) and were left drug free for 18 hrs before receiving i.p. pretreatment with single (acute) test dose of DZ (5 mg/kg), IMD (0.5 mg/kg) or VEH 30 min prior to DFP (1.5 mg/kg, s.c.) challenge. Two min after DFP treatment, animals received a combination of AT (2 mg/kg, i.p.) and 2-PAM (20 mg/kg, i.p.) and were killed 48 hrs after DFP challenge.

Representative photomicrographs of FJB stained neurons in protracted VEH + acute VEH (panel A); protracted VEH + DFP challenge (panel B); protracted VEH + acute DZ pretreatment before DFP challenge (panel C); protracted DZ + acute DZ

pretreatment before DFP challenge (Panel D); protracted VEH + acute IMD pretreatment before DFP challenge (panel E) and protracted IMD + acute IMD pretreatment before DFP challenge (panel F). Panels A, B, C, D, E, and F represent photomicrographs taken with 10X (objective) and the inserts are 40X magnification of the respective photomicrographs.

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The authors declare no conflict of interest.

LITERATURE:

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