Acute imidazenil treatment after the onset of DFP-induced seizure is more effective and longer-lasting than midazolam at preventing seizure activity and brain neuropathology

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Key words: Imidazenil, Benzodiazepine, Organophosphates, Radio telemetry, Neuropathology.
ABSTRACT

Diazepam (DZ), the preferred anticonvulsant benzodiazepine (BZ) for the treatment of organophosphate (OP) nerve agent-induced seizures and neuronal damage has been associated with unwanted effects such as sedation, amnesia, cardio-respiratory depression, anticonvulsant tolerance, and dependence liability. In a search for safer and more effective anticonvulsant BZs against OP-induced seizure and neuronal damage, we have previously shown that imidazenil (IMD), a low intrinsic efficacy positive allosteric modulator of GABA action at $\alpha_1$-, which has high intrinsic efficacy at $\alpha_2$-, $\alpha_3$- and $\alpha_5$-containing GABA<sub>A</sub> receptors, is more potent and longer-lasting than diazepam pretreatment at protecting rats from diisopropyl fluorophosphate (DFP)-induced electrocorticographic (ECoG) seizures and neuronal damage. The effects of IMD were observed at doses that are devoid of sedative, amnestic, and anticonvulsant tolerance actions. In the present study, we compared the anticonvulsant and neuroprotective effects of a combination of atropine (2 mg/kg, ip) and 2-PAM (20 mg/kg, ip) with IMD (0.5 mg/kg, ip) or midazolam (MDZ, 0.5 to 2 mg/kg, ip) administered after the onset of DFP- (1.5 mg/kg, sc) induced seizure activity. The severity of DFP-induced ECoG seizures was assessed by continuous radio telemetry recordings in unrestrained and freely moving rats. Further, the extent of neuronal damage was evaluated using a neuron-specific nuclear protein (NeuN)-immunolabeling and fluoro-jade B (FJB) staining procedure. We report here that IMD is more efficacious and longer-lasting than sedating doses of MDZ in protecting rats from DFP-induced ECoG seizures and neuronal damage.
INTRODUCTION

In the wake of increased terrorist attacks around the globe, organophosphate (OP) nerve agents are a potential threat to civilians and military troops. The insidious use of these agents, e.g, the 1995 Tokyo subway attack (Okumara et al., 2003) or the assaults during the Gulf War, left victims with long-term consequences even several years post exposure to these toxic agents (Yokoyama et al., 1998; Tochigi et al., 2002; Miyaki et al., 2005). Thus, there is an urgent need to develop effective and safe treatment strategies to counteract the toxic effects of nerve agent exposure.

OP nerve agents exert their toxic actions by irreversibly inhibiting acetylcholinesterase (AChE) in the central and peripheral nervous systems, resulting in the accumulation of acetylcholine (ACh) in synaptic clefts. Excessive accumulation of ACh causes hyperstimulation of both nicotinic and muscarinic cholinergic receptors, receptors, which can lead to a sequence of physiological events including bronchoconstriction, cardiac brady-asystole, hypersecretion of secretory glands, respiratory distress, and long-lasting seizure activity (Weinbroum 2004; Carpentier et al, 2001). It is widely accepted that OP-induced seizure activities if not treated in a timely manner, can evolve into status epilepticus. This can lead to irreversible brain damage and long-term neurological, behavioral, and cognitive deficits (Solberg and Belkin, 1997; Brown and Brix, 1998). The mechanisms underlying sustained epileptiform activity may be related to the increased activation of glutamatergic transmission and also to inactivation of GABAergic receptor function (Chen and Wasterlain, 2006).

In the event of OP poisoning, conventional medical therapy includes combined administration of atropine sulfate (AT) to counteract acute muscarinic cholinergic crises,
pralidoxime chloride (2-PAM) to reactivate inhibited AChE, and an anticonvulsant benzodiazepine (BZ) such as diazepam to increase GABA_A receptor function to prevent brain seizures and subsequent neuronal damage (Newmark, 2004; McDonough and Shih, 1997). The combination of AT and 2-PAM alone is used to counteract mild OP intoxication, but this combination is ineffective against moderate-to-severe intoxication, and the addition of an anticonvulsant BZ is necessary to control the progression of seizures into status epilepticus (Harrison et al., 2004., Lallement et al., 1998, Shih and McDonough, 1999). Although a combination treatment including diazepam (DZ) effectively blocks or terminates seizures elicited by OP nerve agents, it does not provide adequate and long-lasting protection from seizure activity and neuronal damage (Hayward et al., 1990; McDonough et al., 1995; Shih et al., 2007, Kadriu et al., 2009). Results obtained from animal studies have shown that for full-blown nerve agent-induced status epilepticus, DZ administered in doses associated with sedation and ataxia (up to 10 mg/kg), does not provide complete protection from OP-induced status epilepticus (Newmark J, 2007).

IMD is a selective positive allosteric modulator that facilitates GABA action with high intrinsic efficacy at α2-, α3- and α5-containing GABA_A receptors (Costa et al., 2002; Guidotti et al., 2005). However, it is virtually inactive at α1- (Guidotti et al., 2005; Costa and Guidotti, 1996; Costa et al., 2002), α4-, and α6- (Knoflach et al., 1996) containing GABA_A receptors. We have demonstrated that the combination of a fixed dose (2mg/kg) of AT with IMD fully protected rats and mice from DFP-induced seizure and mortality (Auta et al., 2004; Rump et al. 2000; Pibiri et al, 2008) in doses that do not elicit sedation or ataxia. More recently, we demonstrated that a 30 min pretreatment with a combination of AT and IMD (0.05 to 0.5 mg/kg), dose-dependently protected rats from DFP-induced seizures and neuronal damage in manner that
is more efficacious and longer-lasting than the combination of AT with increasing doses (0.5 to 5 mg/kg) of DZ.

MDZ, a non-selective and full positive allosteric modulator of GABA action at a variety of GABA$_A$ receptor subtypes (Sieghart, 1995), has recently been considered a possible anticonvulsant replacement for DZ (McDonough et al., 2009). The advantages that have been attributed to MDZ include its rapid bioavailability and the ease of administration by intranasal, sublingual, and intramuscular routes (McDonough et al., 2009). Based on the efficacy of IMD in protecting rats from DFP-induced seizures and neuronal damage, the current study was designed to compare the protective efficacy of IMD to that of MDZ when administered after the onset of DFP-induced seizure activity. We therefore evaluated the effectiveness of a combination of AT and 2-PAM with a single dose of IMD or increasing doses of MDZ to protect rats from DFP-induced electrocorticographic (ECoG) seizure activity and brain neuronal damage. We show here that the administration of a combination of AT and 2-PAM with IMD is more efficacious than the combination AT and 2-PAM with MDZ at protecting rats from DFP-induced ECoG seizure activity and brain neuronal damage.

**MATERIALS AND METHODS**

**Animals:** Adult male Fisher 344 rats (Harlan, Indianapolis) weighing 250-280 g were housed in groups (three per cage for histological studies) or individually (for radio telemetry) in standard plastic cages (42 cm x 26 cm x 20 cm) and maintained on an 11 to 13 hrs light/dark cycle (lights from 6:00 am to 7:00 pm). 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 as approved by the Animal Welfare Committee at the University of Illinois at Chicago.
Drugs: Midazolam (MDZ) was a generous gift from Hoffman La-Roche (Basel, Switzerland). Imidazenil (IMD) was 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 supplied in oily solution was freshly diluted in ice-cold saline just prior to administration. MDZ 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). MDZ, IMD, AT, 2-PAM and vehicle were administered intraperitonially (ip). DFP was administered subcutaneously (sc) in volumes of 0.1 ml/100 g.

Surgical Implantation of Telemetry Probes

The severity and frequency of seizure activities were evaluated by continuous visual observation and radio telemetry recordings. All surgeries were performed under pentobarbital (50 mg/kg, ip) anesthesia using minor modifications of procedures described previously (Kramer and Kinter, 2003; Deveney et al., 1998). Following deep anesthesia, rats were intraabdominally implanted with telemetric PhysiolTel transmitters (Model TL11M2-F40-EET; Data Science International, Arden Hills, MN) to provide telemetric monitoring of the cortical electroencephalogram (ECoG) and electromyogram (EMG) in freely moving and unrestrained rats.

Hair from the flat of the snout between the eyes and across the neck and abdominal area was shaved with a clipper. The skin was swabbed with povidone-iodine. The animal was then placed on a heating pad and a midline incision made from the bridge of the nose to the posterior of the cranium. An abdominal incision (approximately 2-cm) was also made through the linea
alba and the underlying muscle wall along the ventral midline using a scalpel and scissors. The
transmitter was placed in the peritoneal cavity and secured in position by suturing it to the inner
surface of the abdominal wall using a non-absorbable nylon suture. The two cortical and muscle
electrodes were threaded out of the abdominal wall and tunneled subcutaneously along the
abdomen, over the shoulder, and through a 2-cm lateral incision made in the cranium and the
abdominal skin was then sutured. The animal was positioned on a stereotaxis apparatus for
electrode implantation. To ensure optimal placement of the EMG electrodes and to avoid
electrocardiogram interference, leads were sutured bilaterally in the cervico-auricularis muscle
(neck muscle) and the two ECoG cortical electrodes implanted supradurally in 1-mm
circumference holes, each drilled 3-mm from the sagittal suture (midline) and 3-mm anterior to
the lambdoid suture. The electrodes were anchored in to the skull using dental acrylic (Dentsply
Caulk International, Milford, USA). After surgery, animals received a single dose of antibiotic
(gentamycin, 5 mg/kg, im) and an analgesic (carprofen, 5 mg/kg, sc) within three hours of
surgery; rats received additional doses of gentamycin and carprofen 24 hours after surgery.
Animals were allowed initial recovery under a heating lamp and were monitored until they
regained a toe-pinch reflex, then were returned to their individual cages and left for a recovery
period of 10 days prior to experimentation.

**Drug treatment:** After 10 days of recovery from probe implantation, rats were housed in
individual cages and placed on radio telemetry receivers and a 60 min baseline EMG and ECoG
activities recorded. After baseline recordings, rats received acute subcutaneous (sc) injections of
a sub-lethal dose (1.5 mg/kg) of DFP freshly reconstituted in ice-cold saline and 2 min later a
combination of AT (2 mg/kg, ip) and 2-PAM (20 mg/kg, ip). Ten minutes (time to onset of DFP-
induced seizures) after DFP acute challenge separate groups of rats received a single dose of
IMD (0.5 mg/kg, ip) or increasing doses (0.5 to 2 mg/kg, ip) of MDZ or vehicle (see Fig. 1 for schematic representation). The doses for AT, IMD, and DFP were determined in previous experiments (Auta et al., 2004; Kadriu et al., 2009). On the day of each experiment, the behavior and characteristic ECoG activity of each animal were closely monitored for the onset, termination, and re-appearance of seizure activity following acute DFP challenge and subsequent treatment with a combination of AT and 2-PAM with IMD, MDZ, or vehicle. Radio telemetric ECoG activities were continuously recorded and monitored for at least eight hours (between 10 am and 6 pm) after which uninterrupted recording continued for 24 hours after drug treatment.

To study neuronal degeneration, separate groups of six rats received acute DFP (1.5 mg/kg, sc) challenge and 2 min later a combination of AT (2 mg/kg, ip) and 2-PAM (20 mg/kg, ip). Ten minutes after the DFP acute challenge, rats received a single dose (0.5 mg/kg, ip) of IMD or increasing doses (0.5-2 mg/kg, ip) of MDZ (see Fig.1 for schematic representation). Forty-eight hours after DFP acute challenge, rats were killed and brains removed and processed (see the details below) for immunohistological analysis. Neuronal damage or degeneration was evaluated in CA1 & CA3 hippocampus, layers II (MC LII) and III (MC LIII) motor cortex, amygdala, and layer II pyriform cortex (PC LII).

**Electrocorticographic (ECoG) seizure data acquisition and spectral analysis:** The implanted radio transmitter allows electrocorticography (ECoG), electromyography (EMG), and activity monitoring in unrestrained and freely moving rats housed in their home cages. Signals detected by the telemetry transmitter were transmitted to a radio-receiver board (RPC-1 receiver plates, Data Science International, Arden Hills, MN) placed underneath rat home cages. The signals captured by the receiver-boards were routed via a data exchange matrix (DSI) to a PC and sampled at a rate of 500 Hz using DSI analog software (Dataquest A.R.T. 4.1) with a filter cut-
off of 50 Hz. ECoG and EMG traces were visually and manually analyzed for seizure activity. Power spectrum density (frequency ranges: $\delta$, 1-4 Hz; $\theta$, 4.8 Hz; $\alpha$, 8-12 Hz; $\beta_1$, 12-16 Hz; $\beta_2$ 16-32 Hz; $\gamma$, 32-50 Hz) analysis of ECoG traces was performed using a computer terminal with Dataquest A.R.T. system. The ECoG tracings were processed and analyzed as previously described (Fitzgerald et al., 2003). In brief, raw EEG signals were routed to a digital storage oscilloscope with fast Fourier transformation [FFT; a logarithmic display of signal power (dBV) versus a linear display of frequency (Hz)] and averaging capabilities to record and process electrocorticograms. The raw ECoG signal was continuously digitized every 0.004 sec (sample rate, 250/sec) using a 1-MHz band pass filter. The results for the selected ECoG tracings were computer generated as periodograms showing the time-relationships of the power spectra (absolute values in mV$^2$/Hz) and the relative contribution of each frequency range to the total power signal. Off-line calculations were performed to average the values obtained for 60 sec sampled segments of ECoG tracings for the respective frequency ranges. FFTs for 60 sec segments of raw signals were sampled at 30 min after vehicle treatment, 10 min after DFP + AT + 2-PAM, and at 5, 30 and 180 min after IMD (0.5 mg/kg) or MDZ (0.5 to 2.0 mg/kg) acute treatment. This computerized frequency analysis by FFT is faster and less subjective than visual scoring of ECoG tracings and provides an objective quantitative method for evaluating ECoG tracings.

Neuronal Damage

1. NeuN immunohistochemistry
The neuronal nuclei antigen (NeuN) is a very specific protein that is highly expressed in differentiated neurons. Thus, we used NeuN immunohistochemistry to identify neuronal loss and to quantify total number of neurons in various rat brain areas.

Briefly, NeuN studies were carried out by washing free floating coronal sections (20 µm) two times for 20 min in TBS buffer (0.15 M NaCl; 0.1 M Tris-HCl, pH=7.5) containing 0.3 % (v/v) H₂O₂ to block the endogenous peroxidases at room temperature. This was followed by a 30 min incubation in TBS-BSA [TBS containing 1% w/v BSA and 3% normal goat serum (NGS)] at room temperature to block non-specific antibody binding. The sections were then incubated overnight with 1:500 anti-NeuN antiserum (primary antibody; Chemicon, Temecula, CA) diluted in 1% NGS in TBS at 4°C. After three 10 min washes with 1% NGS in TBS, the sections were incubated with 1:250 biotinylated anti-mouse IgG (secondary antibody; Vector Laboratories, Burlingame, CA) for 1 h in 1% NGS in TBS at room temperature. NeuN-immuno-staining was visualized using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and 3-3-diaminobenzidine tetra hydrochloride (DAB; Vector Kit).

NeuN-immunopositive cells were counted with 40X-objective in CA1 & CA3 hippocampus, MC LII, MC LIII, amygdala, and PC LII. For each brain area, five to six sections were chosen (one every four slices) and the number of NeuN-immunopositive cells were counted using a two-dimensional cell counting method in a 100 x 100 µm square. NeuN counting was performed blindly in three randomly selected squares in each of the five to six sections; thus a total of 15-18 squares per sample were counted. The number of NeuN-immunopositive cells per mm² for each section counted was calculated by averaging the counts obtained from the respective brain areas.
2. Fluoro-Jade B staining (FJB): To identify degenerating neurons and their processes in the brain slices we used FJB staining. This staining procedure is a sensitive and reliable marker for neuronal degeneration that results from OP-poisoning and traumatic brain injury (Schmued et al., 1997 and Hopkins, 2000).

Briefly, sections were treated with 1% NaOH in 80% ethanol for five minutes and then hydrated in graded ethanol and distilled water. Section were then incubated in 0.06 % potassium permangate 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 45°C until fully dried and then cleared by immersion in xyline for 1 min before cover slipping with DPX (Sigma-Aldrich Co., St. Louis, MO) and a non-aqueous non-fluorescent plastic mounting media.

Analysis: Sections were examined using a Zeiss fluorescent microscope with a blue (450-490) excitation light by using the filter designed for visualizing fluorescein or FITC, which was suitable for fluoro-jade B 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).

Statistical Analysis

The neuronal counts for FJB stain, NeuN, and power spectrum density analysis of ECoG recordings were analyzed using a one-way repeated analysis of variance (ANOVA) and post hoc analysis of variance was determined by the Duncan’s test with multiple range comparison. The p values less than critical values .05 and .01 were considered statistically significant.
RESULTS

Effects of imidazenil (IMD) or midazolam (MDZ) on DFP-induced seizures

a) Behavioral Observations

Following acute DFP (1.5 mg/kg, sc) challenge and treatment with a combination of AT (2 mg/kg, ip) and 2-PAM (20 mg/kg, ip) (Fig. 1), the incidence of seizure activity which began 5 to 8 min post-DFP administration was 100%. Seizure activity was preceded by predictable and consistent behavioral signs including bouts of chewing activity and intermittent head tremors. These behavioral signs were followed by whole body tremors, jerky motions, and Straub tail which rapidly progressed to explosive tonic-clonic motor convulsions, and finally into status epilepticus. Despite the severity of the seizure activity, the majority of rats (96%) survived. Seizure intensity and severity decreased over the next 8 to 24 h after acute DFP challenge.

The severity of seizure activity was reduced (absence of convulsive-like tremors, clonic jerks) in the first 30 min to 1 h in rats that received a combination including IMD (0.5 mg/kg, ip) or MDZ (1 or 2 mg/kg, ip) 10 min after DFP-acute challenge (Fig. 1). However, only IMD was still effective 3 h after DFP acute treatment. Moreover, rats that received IMD showed no noticeable signs of seizure activity 18 h after acute DFP challenge and appeared to have fully recovered 36 h after DFP treatment. In contrast, rats that received the combination with MDZ (2 mg/kg) exhibited body tremors, intermittent clonic jerks, dacryorrhea, and signs of respiratory distress 18 h after DFP treatment.

b) ECoG power spectra density analysis

In Fig. 2 we show 25 sec representative electrocorticographic (ECoG) traces and the corresponding electromyographic (EMG) activity recorded at baseline (panel A), 6 min after the
DFP acute challenge (panel B), 30 and 180 min after 0.5 mg/kg of IMD (panel C and E, respectively), and 30 and 180 min after 2 mg/kg MDZ (panel D and F, respectively) treatment (see Fig. 1 for treatment scheme). When compared to the low-amplitude high-frequency waveform activity recorded at baseline (panel A), DFP-induced ECoG seizure activity is characterized by repetitive and continuous high-frequency large-amplitude activity (panel B). It is noteworthy that this DFP-induced characteristic waveform activity is not altered by treatment with AT and 2-PAM. At 30 min post-MDZ (panel C) or -IMD (panel D) acute challenge, ECoG activity is characterized by low-amplitude high-frequency waveform activity and is similar to that observed at baseline. Interestingly, at 180 min after IMD (panel E) treatment, ECoG waveform activity is similar to that observed at baseline. In contrast, at 180 min after MDZ treatment, ECoG activity is characterized by high-frequency large-amplitude waveform activity similar to that observed after DFP-induced ECoG seizure activity. The results suggest that while IMD and MDZ were both effective in attenuating DFP-induced ECoG seizure activity 30 min post treatment, IMD but not MDZ was still effective 180 min after acute DFP challenge.

To quantify the effects of the various treatments, we performed power spectra density [frequency ranges: $\delta$ (1-4 Hz); $\theta$ (4-8 Hz); $\alpha$ (8-12 Hz); $\beta_1$ (12-16 Hz); $\beta_2$ (16-32 Hz); $\gamma$ (32-50 Hz)] analysis of EEG tracings sampled at 30 min post vehicle, 10 min post-DFP acute challenge and at 5, 30 and 180 min post IMD or MDZ treatment. This analysis shows that DFP-induced ECoG seizure activity is associated with remarkable and consistent changes in power spectra densities for $\delta$ (1-4 Hz), $\beta_1$ (12-16 Hz), and $\beta_2$ (16-32 Hz) frequencies compared with vehicle treatment (Fig. 3). However, at 5 and 30 min after IMD treatment, DFP-induced increases in power spectra density for $\delta, \beta_1$, and $\beta_2$ frequencies were significantly attenuated. Except for the increase in power spectra density for the $\beta_2$ frequency, IMD attenuation of DFP-induced
increases in power spectra density for all three frequencies was sustained even by 180 min after IMD treatment. It is interesting to note here that IMD-induced attenuation of DFP-induced increases in power spectra density for the $\delta$ frequency was not only sustained over 180 min but the magnitude of this effect continued to decrease in a time-dependent manner after IMD acute challenge. In contrast to IMD, MDZ treatment was only effective against DFP-induced increase in the power spectra density for the $\delta$ frequency at 5 and 30 min after treatment. Moreover, the effect of MDZ on the $\delta$ frequency returned to DFP-induced levels at 180 min after MDZ treatment.

**NeuN immuno-labeling:**

To evaluate whether sustained DFP-induced seizure activity leads to neuronal damage, we used NeuN-immunolabelling, which has been used as biomarker for neuronal damage (Gavrielt et al, 1992; Davoli et al, 2002; Igarashi et al, 2001). In **Table 1 and Fig 4**, we show that 48 h of sustained DFP-induced seizure activity resulted in a significant decrease in the total number of NeuN immuno-positive neurons in the CA1 hippocampus (40%), CA3 hippocampus (21%), motor cortex layer II (MC LII) (36%), and motor cortex layer III (MC LIII) (35%), pyriform cortex layer II (PC LII) (36%) and amygdala (44%) compared to their respective vehicle-treated groups. These results also indicate that the CA1 hippocampus, MC LII & LIII, PC LII, and amygdala are more sensitive to the neurotoxic effects of DFP than the CA3 hippocampus. Most importantly, IMD (0.5mg/kg) significantly attenuated DFP-induced decreases in the number of NeuN immunopositive neurons in all brain areas studied. In contrast, significant protective effects for MDZ against DFP-induced neuronal loss were only observed in MC LII, PC LII, and amygdala with the 2mg/kg dose. Most importantly, lower doses of MDZ (0.5 and 1.0 mg/kg) failed to show significant protective effects against DFP-induced decreases
in the number of NeuN immunopositive neurons in most of the brain areas studied. In Fig 4, we show representative photomicrographs of immunohistological staining in the CA1 hippocampus obtained from vehicle, DFP (1.5 mg/kg), IMD (0.5 mg/kg), and MDZ (2.0 mg/kg) treated rat brains. NeuN-immunopositive neurons are intensely stained dark (DAB detection) and loss of immunoreactivity is shown as light-dark stain.

**Fluoro-jade B studies:** We used fluoro-jade B (FJB) staining to investigate ongoing neuronal degeneration after 48 h of sustained DFP-induced seizure. In Table 2, we show counts for the number of FJB-positive cells in the six brain areas studied, while in Fig. 5 we show representative photomicrographs for vehicle, DFP (1.5 mg/kg, sc), IMD (0.5 mg/kg, ip), or MDZ (0.5 and 2.0 mg/kg, ip) taken from CA1 hippocampus and amygdala. The data show that 48 h of sustained DFP-induced seizure activity increased the number of FJB-positive cells in all brain areas examined. Counts for the number of FJB positive cells (Table 2) show that prolonged DFP-induced seizure activity resulted in 12, 10, 7, 8, 6, and 7-fold increases in the number of FJB positive cells in the CA1 and CA3 hippocampus, amygdala, MC LII & LIII and PC LII, respectively. Most importantly, 0.5 mg/kg of IMD administered 10 min post DFP-acute challenge and after the onset of seizure activity significantly attenuated DFP-induced neuronal degeneration in all the brain areas examined. Although a dose-dependent effect of MDZ was observed in all the six brain areas examined, only the highest dose (2 mg/kg) resulted in a statistically significant attenuation of DFP-induced neuronal degeneration in all brain regions except the CA3 hippocampus.

**DISCUSSION**

A close association between sustained centrally-mediated ECoG seizure activity and brain neuropathologies that accompany exposure to OP agents has been well established
(Gunderson et al., 1992; McDonough and Shih, 1993, 1997; Tryphonas et al., 1996; Hayward et al., 1990). Furthermore, it has been hypothesized that during repeated seizure activity or status epilepticus there is decreased activity of GABA inhibitory tone (Ben-Ari, 2006). Thus, the enhancement of GABA_A receptor-mediated neurotransmission by benzodiazepines and congeners that allosterically facilitate GABA–gated chloride currents at various GABA_A receptor subtypes has been used to control OP-induced seizures (McDonough and Shih, 1993; 1997, Shih et al., 2003; 2007).

Studies in humans and rodents have demonstrated that after prolonged seizure activity, drugs like diazepam or midazolam show refractoriness in their efficacy to control seizure activity as a result of the rapid functional desensitization of GABA_A receptors that occurs during status epilepticus (Kapur et al., 1989; Walton et al., 1988, McDonough et al., 2010). Consequently, these positive allosteric modulators of GABA-action at GABA_A receptors do not effectively protect against OP-nerve agent-induced status epilepticus, brain neuropathologies, and the associated behavioral and cognitive deficits (Hayward et al., 1990; McDonough et al., 1995; Lallement et al., 1999). Most importantly, in the large doses used to treat OP-nerve agent poisoning, they produce further depression of the already compromised cardio-respiratory center activity (Gillis et al., 1989; Nordt and Clark, 1997; Ogutu et al., 2002).

In the present study, we show that a non-sedating dose of IMD provided a long-lasting and effective protection from DFP-induced seizure and neuronal damage whereas the protection by higher and sedating doses of MDZ was relatively moderate and short-lasting. Although at higher doses, MDZ elicited a rapid but short-lasting control of DFP-induced seizure activity, it fails to effectively protect rats from DFP-induced neuronal damage that occurs at a later time. ECoG power spectral density analysis also shows that administration of IMD or MDZ attenuated
DFP-mediated increases in power spectra density for the delta (δ) frequency at 5 and 30 min after DFP-acute challenge. However, the attenuation of the δ frequency by IMD but not MDZ lasted beyond 180 minutes.

It is widely accepted that the neuropathological consequences of OP-poisoning is related to the severity and duration of seizure activity (Weinbroum, 2004; Lallement et al., 1998). In addition, the concurrent and sustained changes in relative power spectra density for the δ frequency that occur during prolonged seizure-activity have been correlated with the neuropathological changes that occur in the brain during OP-poisoning (Carpentier et al., 2001). Thus, one can surmise that the differences in the effectiveness of these BZs in attenuating DFP-induced neuronal damage may be due to differences in the duration of action in suppressing DFP-induced increases in the relative power spectra density for the δ frequency. These differences could also be related to the differences in the pharmacokinetic properties of IMD and MDZ. MDZ is less efficacious than IMD, most likely because of its relatively short duration of action in suppressing the propagation of DFP-induced seizure activity. This view is supported by evidence suggesting that continued limbic seizures lasting for about 1 h produce moderate brain damage; whereas 3 to 4 h of status epilepticus induces extensive brain damage (Lemos and Cavalhiero, 1995; Fujikawa, 1996). Thus, the preferred anticonvulsant agent for cases of OP-poisoning must not only possess a fast onset and prolonged duration of action but also should be devoid of unwanted side effects including sedation, amnesia, cardio-respiratory depression, and anticonvulsant tolerance liability. Doses of diazepam or MDZ that are effective in controlling seizures induced by nerve agents such as tabun, sarin, soman, cyclosarin, or VX (Shih and McDonough, 2000; Shih et al., 2003) also induce profound sedation, myorelaxation, amnesia, and respiratory depression. In contrast, doses of IMD that effectively attenuate DFP-induced
seizures and neuronal damage fail to induce sedation, myorelaxation, anticonvulsant tolerance, amnesia (Giusti et al., 1993; Auta et al., 1994; 2000), or to decrease body temperature (Schwartz-Bloom et al., 1998). Most importantly, our results show that unlike DZ or MDZ, IMD provides long-lasting protection from DFP-induced seizures and neuronal damage when administered after the onset of DFP-induced seizure activity. Thus, IMD might be an ideal anticonvulsant benzodiazepine to protect from OP nerve agent-induced seizure activity and neuronal damage because it is virtually devoid of the aforementioned side effects and has a prolonged duration of action.

In summary, we have shown that even when administered after the onset of DFP-induced seizures, IMD, a non-sedating and potent anticonvulsant BZ, provides long-lasting protection from DFP-induced seizures and neuronal damage at doses that fail to induce sedation or amnesia (Giusti et al., 1993; Auta et al., 2000). In contrast to IMD, only a high and sedating dose of MDZ provided short-lasting protection from DFP-induced seizures and moderate protection from DFP-induced neuronal damage in two of the five brain areas examined. Furthermore, the longer duration of action of IMD in suppressing the propagation of DFP-induced seizures activity and the concurrent attenuation of relative power spectra density of the δ frequency prevented the progression of DFP-induced seizure into status epilepticus and thus neuronal damage. We have also demonstrated that early and effective control of the propagation of OP-induced seizure activity with efficacious and long-lasting anticonvulsant BZs can minimize the extent of OP-nerve agent-induced seizures and the associated brain neuropathology.

FUNDING:
This work was supported by SBIR grant: ‘[W81XWH-05-C-0125] to Neupharm Inc. (513 Central Avenue, Chicago IL) subcontracted to the Board of Trustees of the University of Illinois (Department of Psychiatry).

The authors declare no conflict of interest.

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**Figure legends:**

**Fig 1.** Schematic representation of drug treatment protocol for radiotelemetry and neuronal damage studies.

**Fig 2.** Representative radio telemetric ECoG and corresponding EMG tracings showing 25 s segments recorded after vehicle (panel A), 6 min after the combination of AT (2 mg/kg) and 2-PAM (20 mg/kg) with 1.5 mg/kg of DFP (panel B), 30 and 180 min after 0.5 mg/kg of IMD (panel C and E, respectively) and 30 and 180 min after 2 mg/kg of MDZ (panel D and F, respectively). The electrocorticographic trace shows repetitive seizure activity (repetitive and continuous high-frequency large-amplitude activity) following DFP treatment. At 180 min after IMD acute treatment, note the characteristic low-amplitude high-frequency discharge, which is similar to vehicle treatment. In contrast, 180 min after MDZ treatment, ECoG activity is characterized by high-frequency large-amplitude seizure activity similar to that observed during DFP-induced seizure activity.
Fig 3. IMD is more potent and longer lasting than MDZ in attenuating DFP-induced ECoG power spectra density changes. Power spectra density (PSD) analysis was performed for the following frequency ranges: δ (1-4 Hz), β1 (12–16 Hz) and β2 (16–32 Hz). These analyses were performed on 60 s segments of ECoG tracings sampled 30 min after vehicle treatment, 10 min after DFP-acute challenge in the presence of 2 mg/kg of AT and 20 mg/kg of 2-PAM, and 5 min, 30 min, and 180 min after acute IMD (0.5 mg/kg) or MDZ (2 mg/kg) treatment. Each bar is the mean ± SE of four animals per group. Threshold for statistical significance was p<0.05. * VEH vs. all treatments, # DFP vs. all treatments.

Fig 4. Representative NeuN-immunopositive neurons in CA1 hippocampus 48 hours after DFP-acute challenge (panel C and D), 0.5 mg/kg of IMD (panel E and F) or 2 mg/kg of MDZ (panel G and H) administered after the onset of DFP-induced seizure and in the presence of 2 mg/kg of AT and 20 mg/kg of 2-PAM. Photomicrographs of NeuN stained CA 1 hippocampal neurons at different (50 and 400) magnifications, respectively. Panels A, C, E, and G represent CA1 hippocampal brain slices taken with 50 magnification (5X objective), whereas the panels B, D, F and H are 400 magnification (taken with 40X objective) corresponding to images from the CA1 hippocampal coronal section, respectively.

Fig 5. Representative photomicrographs of fluoro-jade B stained CA 1 hippocampus and amygdala neurons obtained from vehicle (panel A and B), DFP (panel C and D), DFP + 0.5 mg/kg of IMD (panel E and F), DFP + 0.5 mg/kg of MDZ (panel G and H) and DFP + 2.0 mg/kg of MDZ (panel I and J) treated rats, all in the presence of combination of AT (2mg/kg) and 2-PAM (20 mg/kg). Panels A, C, E, G and I represent CA1 hippocampus brain slices whereas panels B, D, F, H, and J represent amygdala sections taken with 400 magnifications, respectively.
Table 1: Quantification of NeuN immunopositive cells in different rat brain regions 48 h after Vehicle, DFP, DFP + IMD or DFP + MDZ treatment

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Vehicle</th>
<th>DFP</th>
<th>DFP + IMD</th>
<th>DFP + MDZ 0.5</th>
<th>DFP + MDZ 1.0</th>
<th>DFP + MDZ 2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Motor Cortex</strong></td>
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</tr>
<tr>
<td>Layer II (MC LII)</td>
<td>2.92 ± 0.10</td>
<td>1.96 ± 0.11</td>
<td>2.77 ± 0.10</td>
<td>2.16 ± 0.17</td>
<td>2.25 ± 0.22</td>
<td>2.52 ± 0.05</td>
</tr>
<tr>
<td><strong>Motor Cortex</strong></td>
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<tr>
<td>Layer III (MC)</td>
<td>1.80 ± 0.16</td>
<td>1.14 ± 0.08</td>
<td>1.74 ± 0.09</td>
<td>1.10 ± 0.03</td>
<td>1.14 ± 0.09</td>
<td>1.28 ± 0.02</td>
</tr>
<tr>
<td><strong>Pyriform Cortex</strong></td>
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</tr>
<tr>
<td>Layer II (PC LII)</td>
<td>2.40 ± 0.05</td>
<td>1.53 ± 0.02</td>
<td>2.05 ± 0.06</td>
<td>1.46 ± 0.06</td>
<td>1.68 ± 0.05</td>
<td>1.72 ± 0.04</td>
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<tr>
<td><strong>CA1</strong></td>
<td></td>
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<tr>
<td>Hippocampus</td>
<td>3.27 ± 0.05</td>
<td>2.32 ± 0.34</td>
<td>3.22 ± 0.21</td>
<td>2.26 ± 0.09</td>
<td>2.46 ± 0.14</td>
<td>2.46 ± 0.12</td>
</tr>
<tr>
<td><strong>CA3</strong></td>
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<td></td>
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</tr>
<tr>
<td>Hippocampus</td>
<td>1.98 ± 0.02</td>
<td>1.59 ± 0.04</td>
<td>1.90 ± 0.12</td>
<td>1.40 ± 0.05</td>
<td>1.52 ± 0.04</td>
<td>1.67 ± 0.05</td>
</tr>
<tr>
<td><strong>Amygdala</strong></td>
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<tr>
<td></td>
<td>2.08 ± 0.08</td>
<td>1.24 ± 0.12</td>
<td>1.89 ± 0.14</td>
<td>1.21 ± 0.05</td>
<td>1.46 ± 0.04</td>
<td>1.61 ± 0.02</td>
</tr>
</tbody>
</table>

Rat received an acute subcutaneous injection of DFP (1.5 mg/kg) and 2 min later a combination of 2 mg/kg, ip of atropine (AT) and 20 mg/kg, ip of pralidoxime chloride (2-PAM). Ten minutes later after DFP-acute challenge separate group of rats received a single dose 0.5 mg/kg of imidazenido (IMD) or increasing doses of 0.5-2.0 mg/kg of midazolam (MDZ) or vehicle and were sacrificed 48 h after DFP-acute administration. Results are expressed as mean ± SD of the number of NeuN immunopositive cells. Rats were sacrificed 48 h after DFP administration. p<0.05 compared with the respective DFP treated groups (One Way ANOVA followed by Duncan’s Multiple Comparison) for each brain region studied. * DFP vs. vehicle; # DFP vs. treatment.
### Tabel 2: Quantification of fluoro-jade B positive cells in different rat brain regions 48 h after Vehicle, DFP, DFP + IMD or DFP + MDZ treatment

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Number Fluoro-Jade B positive cells/mm² X 10^3</th>
<th>Vehicle</th>
<th>DFP</th>
<th>DFP + IMD</th>
<th>DFP + MDZ 0.5</th>
<th>DFP + MDZ 1.0</th>
<th>DFP + MDZ 2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motor Cortex Layer II (MC LII)</td>
<td>0.32 ± 0.05</td>
<td>2.64 ± 0.06*</td>
<td>0.70 ± 0.06#</td>
<td>2.48 ± 0.14</td>
<td>2.12 ± 0.12#</td>
<td>1.64 ± 0.11#</td>
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<tr>
<td>Motor Cortex Layer III (MC LIII)</td>
<td>0.33 ± 0.05</td>
<td>1.95 ± 0.08*</td>
<td>0.62 ± 0.03#</td>
<td>1.94 ± 0.10</td>
<td>1.73 ± 0.11#</td>
<td>1.21 ± 0.09#</td>
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</tr>
<tr>
<td>Pyriform Cortex Layer II (PC LII)</td>
<td>0.29 ± 0.04</td>
<td>2.03 ± 0.26*</td>
<td>0.99 ± 0.14#</td>
<td>2.23 ± 0.17</td>
<td>1.87 ± 0.14#</td>
<td>1.56 ± 0.09#</td>
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<tr>
<td>CA1 Hippocampus</td>
<td>0.23 ± 0.05</td>
<td>2.79 ± 0.12*</td>
<td>0.63 ± 0.04#</td>
<td>1.74 ± 0.15#</td>
<td>1.66 ± 0.12#</td>
<td>1.29 ± 0.11#</td>
<td></td>
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<tr>
<td>CA3 Hippocampus</td>
<td>0.18 ± 0.02</td>
<td>1.76 ± 0.16*</td>
<td>0.64 ± 0.10#</td>
<td>1.67 ± 0.12</td>
<td>1.60 ± 0.14</td>
<td>1.46 ± 0.09</td>
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</tr>
<tr>
<td>Amygdala</td>
<td>0.34 ± 0.07</td>
<td>2.53 ± 0.06*</td>
<td>0.87 ± 0.06#</td>
<td>2.32 ± 0.19</td>
<td>1.89 ± 0.13#</td>
<td>1.47 ± 0.16#</td>
<td></td>
</tr>
</tbody>
</table>

Rat received an acute subcutaneous injection of DFP (1.5 mg/kg) and 2 min later a combination of 2 mg/kg, i.p of atropine (AT) and 20 mg/kg,i.p of pralidoxime chloride (2-PAM). Ten minutes later after DFP-acute challenge separate group of rats received a single dose 0.5 mg/kg of imidazenil (IMD) or increasing doses of 0.5-2.0 mg/kg of midazolam (MDZ) or vehicle and were sacrificed 48 h after DFP-acute administration. Results are expressed as mean ± SD of the number of NeuN immunopositive cells. Rats were sacrificed 48 h after DFP administration. * p<0.05 compared with the respective DFP treated groups (One Way ANOVA followed by Duncan’s Multiple Comparison) for each brain region studied. * DFP vs. vehicle; # DFP vs. treatment.
Fig 1. Schematic representation of drug treatment protocol for radiotelemetry and neuronal damage studies.

177x52mm (300 x 300 DPI)
Fig 2. Representative radio telemetric ECoG and corresponding EMG tracings showing 25 s segments recorded after vehicle (panel A), 6 min after the combination of AT (2 mg/kg) and 2-PAM (20 mg/kg) with 1.5 mg/kg of DFP (panel B), 30 and 180 min after 0.5 mg/kg of IMD (panel C and E, respectively) and 30 and 180 min after 2 mg/kg of MDZ (panel D and F, respectively). The electrocorticographic trace shows repetitive seizure activity (repetitive and continuous high-frequency large-amplitude activity) following DFP treatment. At 180 min after IMD acute treatment, note the characteristic low-amplitude high-frequency discharge, which is similar to vehicle treatment. In contrast, 180 min after MDZ treatment, ECoG activity is characterized by high-frequency large-amplitude seizure activity similar to that observed during DFP-induced seizure activity.

127x99mm (300 x 300 DPI)
Fig. 3. These analyses were performed on 60 sec segments of ECoG tracings sampled at 60 sec tracing. Each bar is the mean ± SE of 4 animals per group. Threshold for significant difference was p<0.05. * VEH vs. all treatment; # DFP vs. all treatment.

177x136mm (600 x 600 DPI)
Fig 4. Representative NeuN-immunopositive neurons in CA1 hippocampus 48 hours after DFP-acute challenge (panel C and D), 0.5 mg/kg of IMD (panel E and F) or 2 mg/kg of MDZ (panel G and H) administered after the onset of DFP-induced seizure and in the presence of 2 mg/kg of AT and 20 mg/kg of 2-PAM. Photomicrographs of NeuN stained CA1 hippocampal neurons at different (50 and 400) magnifications, respectively. Panels A, C, E, and G represent CA1 hippocampal brain slices taken with 50 magnification (5X objective), whereas the panels B, D, F, and H are 400 magnification (taken with 40X objective) corresponding to images from the CA1 hippocampal coronal section, respectively.
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127x258mm (300 x 300 DPI)