Central Cholinesterase Reactivation by Oximes Improves Survival and Terminates Seizures Following Nerve Agent Intoxication

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ABSTRACT

The ability of oximes to reactivate organophosphorus nerve agent-inhibited acetylcholinesterase (AChE) activity is critical for protection against chemical warfare nerve agent intoxication. We have studied the capability of the tertiary oximes monoisonitrosoacetone (MINA) and diacetylmonoxime (DAM), in comparison with the quaternary oximes 2-PAM, HLo7 and MMB-4 to reactivate AChE inhibited by sarin (GB) in blood, brain regions (cortex, hippocampus, striatum, midbrain, cerebellum, brainstem, and spinal cord) and peripheral tissues (heart, diaphragm and skeletal muscle) of guinea pigs. Animals were injected subcutaneously (sc) with 1.0 x LD_{50} of GB and treated intramuscularly (im) five min later with one of the oximes (MMB-4 and HLo7 at 58 µmol/kg, im and 2-PAM, MINA and DAM at 145 µmol/kg, im). Sixty min after nerve agent, blood and tissues were collected and prepared for AChE analysis. All animals survived the 60 min after exposure. AChE reactivation in peripheral tissues and blood was zero and in brain regions was marked after treatment with MINA and DAM, whereas AChE reactivation from the quaternary oximes was marked in blood and peripheral tissues, but zero in the brain. In another study, animals were pretreated im with pyridostigmine bromide 30 min prior to challenge sc with 2.0 x LD_{50} of GB, and treated im one min later with a combination of atropine sulfate (2.0 mg/kg) and a varied dose of MINA or DAM. With MINA doses of 20, 26, 35, 46 and 60 mg/kg, 0, 9, 17, 60, and 75%, respectively, of animals never exhibited EEG seizure activity with 43, 64, 75, 90, and 100% survival at 24 hr. With DAM in the dose range from 41 to 231 mg/kg, similar results were obtained. Quaternary oximes did not stop seizures. These data show that the tertiary oximes reactivated AChE in the brain, improved survival and terminated seizures following GB intoxication.

1.0 INTRODUCTION

The potential for exposure to organophosphorus (OP) nerve agents exists on the battlefield (e.g., 1991 Persian Gulf War) and as a terrorist threat to civilian populations (e.g., 1995 Tokyo subway incident). These agents are extremely potent inhibitors of the cholinesterase (ChE) enzymes, which include acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Their toxic effects are due to hyperactivity of the cholinergic system as a result of inhibition of ChE, in particular, AChE, and the subsequent increase in the concentration of the neurotransmitter acetylcholine (ACh) in the brain and periphery (Taylor, 2001). Exposure causes a progression of toxic signs, including hypersecretions, muscle fasciculations, tremor, convulsions, respiratory distress and death (Moore et al., 1995; Taylor, 2001). A combined regimen of prophylaxis and therapy is the

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most effective medical countermeasure for dealing with the threat of nerve agent poisoning to military personnel (Dunn and Sidell, 1989; Moore et al., 1995; Sidell, 1997; Aas, 2003). Pretreatment with carbamate ChE inhibitors, such as pyridostigmine bromide (PB), shields a fraction of ChE in the periphery from irreversible inhibition by the nerve agents (Berry and Davis, 1970; Dirnhuber et al., 1979). In the event of nerve agent poisoning, immediate therapeutic treatment with an anticholinergic drug, such as atropine sulfate, antagonizes the effects of excess ACh at muscarinic receptor sites, and an oxime, such as pyridine-2-aldoxime methylchloride (2-PAM), obidoxime (toxogonin) or HI-6, is used to reactivate any unaged inhibited enzyme (Moore et al., 1995; Aas, 2003).

Atropine sulfate has been universally adopted as the anticholinergic therapy, but countries vary as to their choice of oxime ChE reactivator. Commercially available oximes for OP poisoning refer to compounds that comprise an oxime moiety (RCH=NOH) attached to a quaternary nitrogen pyridinium ring. They reactivate OP-inhibited ChE by dephosphorylating the enzyme active site via interaction with a nearby anionic subsite. Reactivation occurs through nucleophilic attack by the oxime on the phosphorous atom, splitting an oxime-phosphonate away from the active site. The regenerated esteratic site is subsequently able to bind and cleave its normal substrate, ACh. This action of the oximes is considered to be the major mechanism of their antidotal action in reversing the toxic/lethal effects of nerve agents (Maxwell et al., 2006).

2-PAM is the oxime reactivator that is currently issued to U. S. military personnel in an autoinjector (part of the MARK I kit) for emergency buddy treatment of nerve agent exposure. Some countries use different salts (e.g., methanesulphonate) of 2-PAM. Other countries prefer bispyridinium compounds such as obidoxime, TMB-4 or HI-6 as oxime antidotes. Although 2-PAM provides adequate protection against the traditional nerve agent sarin (GB) and VX (Harris and Stitcher, 1983), it is less effective against other traditional nerve agents (e.g., soman) (Fleisher and Harris, 1965; Fleisher et al., 1967). In recent years, several oximes, such as MMB-4, HLo7 and HI-6, have been found to possess much better antidotal capacity than 2-PAM in response to nerve agent intoxication in animal studies (Lundy and Shih, 1983; Harris et al., 1989, 1990; Shih et al., 1991; Shih, 1993; Koplovitz and Stewart, 1994; Dawson, 1994; Kassa 1998; Krummer et al., 2002).

Oximes currently used as medical countermeasures have quaternary structures that are very similar, differing only by the number of pyridinium rings and by the position of the oxime moiety on the ring (Figure 1). The structure of quaternary oximes is positively charged, which renders them unable to cross the blood brain barrier (BBB) and limits their action to only the periphery, thus preventing them from reactivating nerve agent-inhibited AChE within the central nervous system (CNS). The inability of quaternary oximes to enter the brain and reactivate nerve agent-inhibited brain AChE is a major limitation of current oxime therapy.

The brain is a major target for the toxic effects of OP nerve agents. Inhibition of AChE in the brain results in seizures and neuropathology, thus contributing to the incapacitating and lethal effects of these agents (McDonough and Shih, 1997; Shih et al., 2003). The influence of central AChE on protection is reinforced by the reports showing that partially protecting AChE in the CNS with reversible ChE inhibitors, such as physostigmine, prior to nerve agent exposure could improve survival, reduce seizure activity and neuropathology, and lessen behavioral incapacitation following nerve agent exposure (Fosbraey et al., 1992; Wetherell, 1994; Wetherell et al., 2002). It is, therefore, thought by many medical chemical defense scientists that oxime reactivation of nerve agent-inhibited AChE in the CNS would provide significant benefits. However, there have been no systematic studies to investigate this concept.

Monoisonitrosoacetone (MINA) and diacetylmonooxime (DAM) are two tertiary oximes (Figure 1) that had been investigated in the 1950's. Both are highly lipid soluble and readily penetrate the BBB (Cohen and Wiersinga, 1960); they are, therefore, able to reactivate AChE within the CNS (Rutland, 1958; Cohen and Wiersinga, 1960). This could have significant impact in the treatment of the toxic effects of nerve agents. Indeed, when used alone or in combination with atropine sulfate, MINA and DAM were shown to raise the LD_{50} doses of GB in several animal species (Askew, 1956; 1957; Dultz et al., 1957; Rutland, 1958; Myers, 1959; Wills, 1959). Unfortunately, these two tertiary oximes were not pursued further, due to reports that

quaternary pyridinium oximes (e.g., 2-PAM) were several orders of magnitude better reactivators of phosphorylated AChE in human erythrocytes (see review by Hobbiger, 1963).

The present study evaluated the roles of peripheral and central oxime reactivation of nerve agent-inhibited AChE to improve survival and reduce or eliminate other CNS effects of OP nerve agent intoxication in an *in vivo* guinea pig model. To this end two experiments were performed. A reactivation experiment compared several quaternary oximes, 2-PAM, MMB-4 and HLo7, with the tertiary oximes MINA and DAM in their ability *in vivo* to reactivate nerve agent-inhibited AChE activity in blood, peripheral tissues and brain AChE activity. A second, anticonvulsant experiment, compared the capacity of the two tertiary oximes MINA and DAM with the quaternary oximes, 2-PAM, MMB-4 and HLo7, in their ability to prevent/terminate nerve agent-induced seizures and improve survival.

2.0 MATERIALS AND METHODS

2.1 Subjects

Male Hartley guinea pigs (Crl:(HA) BR COBS) weighing 250-300 g were purchased from Charles River Labs (Kingston, NY). They were housed in individual cages in temperature (21 ± 2°C)- and humidity (50 ± 10%)-controlled quarters that were maintained on a 12-h light – dark schedule (with lights on at 0600 h). Laboratory chow and tap water were freely available whenever the animals were in home cages. Animals were quarantined for one week prior to experimentation. The research environment and protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC). In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, in accordance with the stipulation mandated for an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility.

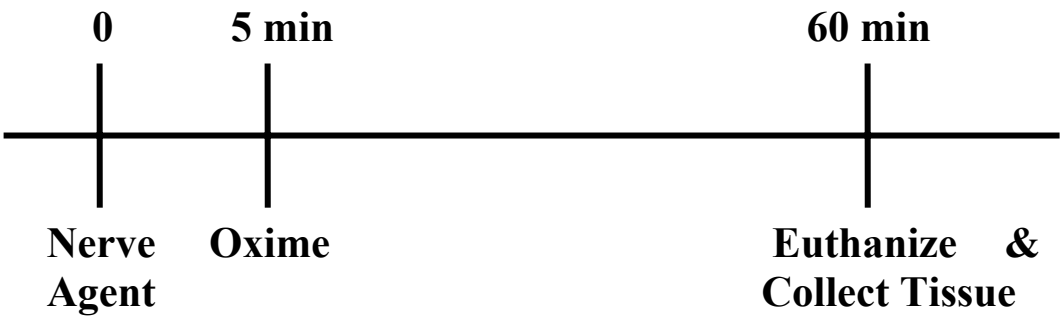
2.2 Materials

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs, Inc. (Berkeley, CA). MINA (Monoisonitrosoacetone; anti-pyruvic aldehyde 1-oxime, 98%), DAM (diacetylmonooxime; 2,3-butanedione monoxime, ≥98%), acetylthiocholine iodide and stropine sulfate were purchased from Sigma-Aldrich (St. Louis, MO). Pyridostigmine bromide (PB) was obtained from Hoffmann-La Roche Inc. (Nutley, NJ) and pyridine-2-aldoxime methylchloride (2-PAM) was purchased from Ayerst Labs, Inc. (New York, NY). HLo7 (1-[[[4-(aminocarbonyl) pyridinio]methoxy]methyl]-2,4-bis[(hydroxyimino)methyl] pyridinium dimethanesulfonate) and MMB-4 (methoxime; 1,1'-methylene bis [4-[(hydroxyimino)methyl]pyridinium] dimethansulfonate) were obtained from the depository at Walter Reed Army Institute of Research (Washington, DC). Bicinchonic acid (BCA) Reagent A (sodium carbonate, sodium bicarbonate, bicinchonic acid and sodium tartrate in 0.1 M sodium hydroxide), BCA Reagent B (4% cupric sulfate), and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Pierce Biotechnology, Inc. (Rockford, Illinois). DTNB was prepared in Tris buffer (0.05M, pH 8.2) to a concentration of 0.424 M. Attane™ (Isofluane, USP) was purchased from Minrad, Inc. (Bethlehem, PA). Buprenorphine HCl was purchased from Reckitt Benckiser Pharmaceuticals, Inc. (Richmond, VA). Heparin sodium was purchased from U.S.P., Inc. (Rockville, MD). The OP chemical warfare nerve agent studied was sarin (GB; isopropylmethylphosphono fluoridate). Sarin was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agent was diluted in ice-cold saline prior to subcutaneous (sc) injection. All oxime compounds were prepared in saline for intramuscular (im) injection. Injection volumes were 0.5 ml/kg for all nerve agents and treatment drugs.

2.3 Reactivation Experiment (Scheme 1)

One to 3 days prior to the experiment, ~0.5 ml blood was drawn (Vallejo-Freire, 1951) and collected into a 1.0 ml microfuge tube containing 50 µl of heparin sodium (15 units/ml) to determine baseline AChE activity in whole blood (WB) and red blood cells (RBC). On the day of the study, groups of guinea pigs were injected subcutaneously (sc) with either saline (0.5 ml/kg) or a 1.0 x LD_{50} dose of GB (42.0 µg/kg). Five min later, when the inhibition of AChE activity by GB reached maximum (Shih, et al., 2005), saline (0.5 ml/kg), HLo7 (30.2 mg/kg), MMB-4 (26.0 mg/kg), 2-PAM (25.0 mg/kg), MINA (12.63 mg/kg), or DAM (14.66 mg/kg) was given intramuscularly (im). No atropine sulfate therapy was given. Control animals received sc saline (no nerve agents) and im saline (no oximes). There were 8 animals assigned to each treatment group. Additionally, dose-response effects of MINA and DAM were also investigated.

Scheme 1: Reactivation Experiment Time Line



Sixty min after sc saline or GB administration, the animals were deeply anesthetized with isoflurane and euthanized by decapitation. Shortly before anesthesia, the severity of toxic signs of each animal was scored (see below). Blood (~0.5 ml) was collected into a 1.0 ml microfuge tube containing 50 µl of heparin sodium solution (15 U/ml). For the WB samples, 20 µl of blood was diluted 1:25 in 1% Triton–X100 solution. For the RBC samples, the original blood sample was centrifuged for 5 min at 14,000 rpm and 10 µl of the RBC was then diluted 1:50 in 1% Triton–X100 solution. Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord and striatum) and peripheral tissue (diaphragm, heart, and skeletal muscle) were dissected. Brain samples were diluted 1:20, while peripheral samples were diluted 1:5, in 1% Triton-X 100 solution (in water) and then homogenized. The homogenates were then centrifuged (31,000 x g at 4°C; 20 min for brain and 30 min for peripheral tissues) and the supernatant decanted and kept frozen at -80°C until analysis (Shih et al., 2005).

2.3.1 Treatment Dose Rational

The 25 mg/kg dose of 2-PAM approximates the total dose of 2-PAM in 3 autoinjectors (600 mg per injector) given as immediate nerve agent treatment to a 70-75 kg human. The 25 mg/kg dose of 2-PAM is equivalent

to a 145 umol/kg dose, and the initial MINA and DAM doses (12.63 and 14.66 mg/kg, respectively) were matched to this 145 umol/kg dose (molecular weight of MINA = 87.08 and of DAM = 101.10). Later, additional doses of MINA (17.5, 35, 60, or 80 mg/kg, im) and DAM (23, 41, 73, or 128.8 mg/kg, im) were added and examined. In contrast, both MMB-4 and HLo-7 are bispyridinium compounds similar to HI-6, a 58 umol/kg dose was used for MMB-4 and HLo7, based on 3-autoinjector equivalent dose (500 mg per injector) of HI-6 (Clair et al., 2000).

2.3.2 Toxic Signs Test

At 58 min after GB injection, guinea pigs were observed for signs of cholinergic toxicity (Table 1), including secretions (salivation or lacrimation), motor deficits, and general state (activity and coordination). This toxic sign score system for guinea pigs in anti-AChE agent-treated animals was modified from that of Shih and Romano (1988) reported for rats. They were scored for absence [0] or presence [1] of each of the following signs: salivation, lacrimation, and nystagmus. General motor signs were assessed a 0-3 score: normal = 0, fasciculation = 1, tremor = 2, or convulsion = 3. Next, the guinea pig was allowed to walk on the bench top and general state was assessed a 0-3 score: normal = 0, mild uncoordination = 1, impaired movement/with righting reflex = 2, or prostration/no righting reflex = 3. A cumulative score was then calculated by tabulating the salivation, lacrimation, nystagmus, general motor and general state score for each subject. The maximal attainable score was a 9 (Table 1). A cumulative score was categorized as mild intoxication [1-3], moderate intoxication [4-6] and severe intoxication [7-9].

2.3.3 AChE Analysis

The AChE activity was measured spectrophotometrically using a variation of the microplate method modified from Ellman et al. (1961), and a bicinchonic acid (BCA) protein assay was used to obtain protein concentrations in the tissue samples to standardized AChE levels between tissues (Shih, et al., 2005). On the day of AChE analysis, the samples were thawed, and three 7-µl replicates of each tissue sample and three 10-µl replicates of the WB and RBC samples were pipetted into a 96-well UV star microplate (Greiner, Longwood, FL). Twenty µl of deionized water was added to each well containing brain and peripheral tissue samples, and 17 µl of deionized water was added to each WB and RBC sample. Then 200 µl of DTNB (0.424 M, pH 8.2) was added as the chromatophore to each sample well. Each microplate was then incubated for 10 min at 37^{o}C before being placed in the Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA) where it was allowed to shake for 2 min. Immediately after, 30 µl of the substrate acetylthiocholine iodide (51.4 mM) was added to each well. The samples were read at 412 nm (at 20-sec intervals) for 3.5 min, and the activity (umol/ml/min) was determined using Softmax plus 4.3 LS software (Molecular Devices).

2.3.4 Protein Analysis

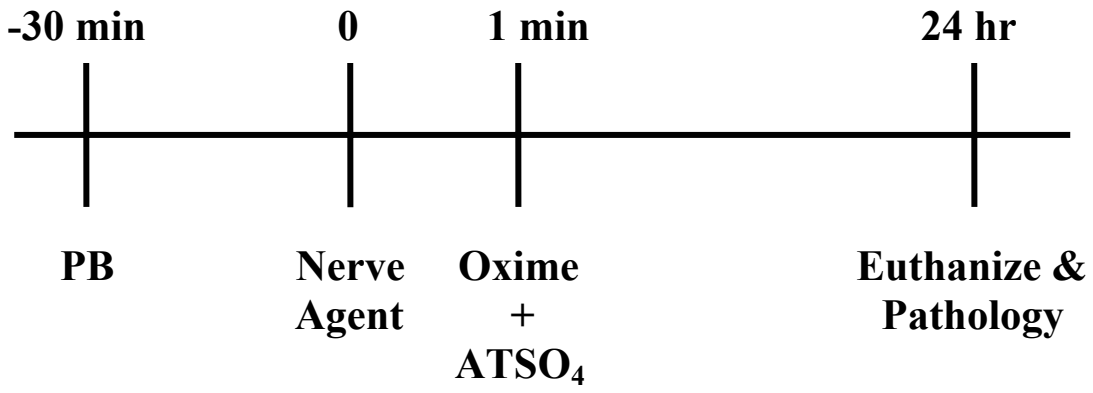
Protein levels in the tissue samples were determined by a bicinchonic acid (BCA) protein assay. The standard curve was created using bovine serum albumin at the following concentrations: 0.5, 0.75, 1.0, 1.5 and 2.0 mg/ml. Three replicates of 10 µl for each brain tissue sample and three replicates of 5 µl for each peripheral tissue sample were added to individual microplate wells. The peripheral tissue samples were further diluted by adding 5 µl of deionized water. Then 200 µl of BCA working reagent was added to each well. The microplates were shaken for 30 sec and then incubated at 37^{o}C for 30 min. The microplates were allowed to cool to room temperature before being read using the Spectramax Plus microplate reader and Softmax Plus 4.3 LS software as described above.

2.4 Anticonvulsant Experiment (Scheme 2)

On the day of study, the animals were placed in a recording chambers and at least 15 min baseline EEG recorded. EEG activities were recorded using CDE 1902 amplifiers and displayed on a computer running

Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, UK). After a 15-min recording of baseline EEG activity, animals received a dose of PB (0.026 mg/kg, im) to produce a 20-30% WB AChE inhibition (Lennox et al., 1985). Thirty min later, animals were challenged with 2 x LD_{50} (sc) GB. One min after nerve agent challenge, animals were treated im with atropine sulfate (2.0 mg/kg) plus HLo7 (30.2 mg/kg), MMB-4 (26.0 mg/kg), 2-PAM (25 mg/kg), MINA (26 – 60 mg/kg), or DAM (82 – 231 mg/kg). The method of probit analysis (Bliss, 1952), using 4-7 doses with 5-6 animals per group, was used to establish an estimated anticonvulsant ED_{50} with 95% fiducial limits for each oxime against GB. Animals were observed continuously for the first hour following exposure and treatment, and periodically thereafter for at least 6 hr. EEG activity was recorded continuously throughout this time and, if the animal survived, for another 30 min at 24 hr after exposure. Seizure onset was operationally defined as the appearance of ≥ 10 sec of rhythmic high amplitude spikes or sharp wave activity in the EEG tracing. Each animal was rated as never having a seizure or having the seizure terminated (OFF) or not terminated (NOT OFF) based on the overall appearance of the EEG record at the end of the experimental day and during the 24-hr observation. (Note: An animal was rated as OFF if a seizure never occurred or if the seizure terminated and the EEG remained normal at all subsequent observation times.) Animals rated as NOT OFF had obvious epileptiform activity that never stopped in response to treatment or reappeared in the EEG record before the end of the 6 hr recording on the day of exposure and/or during the 30 min recording the 24-hr observation. Mortality was recorded 24 hr after nerve agent exposure. Animals that survived 24 hr were euthanized with an overdose of sodium pentobarbital (75 mg/kg, ip) and then perfused through the aorta with saline followed by 10% neutral-buffered formalin for later pathological evaluations (data not presented).

Scheme 2: Anticonvulsant Experiment Time Line



2.4.1 Surgery

For the anticonvulsant study, approximately one week before experimentation the animals were implanted with stainless-steel cortical screw electrodes to record electroencephlographic (EEG) signals. The animals were anesthetized with isoflurane (3% induction, 1.5-2% maintenance; with oxygen) and set in a stereotaxic frame. Three cortical stainless-steel screw electrodes were implanted in the skull: two were placed bilaterally ~3.0 mm lateral from the midline and equidistant between bregma and lambda; the third was placed on the

posterior calvaria as the reference electrode. Stainless-steel wires attached the screws to a miniature connector plug. The electrodes, wires and plug were encased in cranioplastic cement. The incision was sutured; the animal was removed from the frame, given buprenorphine HCl (0.03 mg/kg, SC) for postoperative analgesia and placed on a warming pad for at least 30 min before being returned to the animal quarters (Shih and McDonough, 1999; Shih et al., 2003; 2007).

2.5 Data analysis

AChE activity was expressed initially as µmol/ml/min for blood samples and as µmole/mg protein/min for brain and tissue samples. The enzymatic activities of the treatment groups were then expressed as percentage of the saline-saline control group. Statistical analysis of enzymatic activities and bodyweight change were performed using one-way ANOVAs to compare across treatments. A post hoc Tukey test was used for multiple comparisons. In cases where equal variances could not be assumed a Dunnett C post hoc test was used. Differences in incidence of toxic signs between treatment groups were evaluated using Fisher's Exact Test. Statistical significance is defined as $p<0.05$. Dose-effect curves and the median effective doses (ED_{50}) for anticonvulsant activity of oxime were determined by probit analysis (Bliss, 1952) using 4-7 doses with 5-6 animals per group. A probit regression analysis (SPSS for Windows, Version 14.0, Chicago, IL) was used to estimate the ED_{50} values along with the 95% confidence intervals for each oxime treatment.

3.0 RESULTS

3.1 Reactivation Experiment

3.1.1 Signs of toxicity and lethality

Under the conditions of this study, guinea pigs exposed to a 1.0 x LD_{50} of GB and not receiving any therapy exhibited toxic signs of nerve agent intoxication, such as salivation, rhinorrhea, tremor, muscle fasciculations, and convulsions. Table 2 and Figure 2 display these data. The saline treated controls showed a high incidence (93%; 14 of 15 animals) of toxic signs and the total scores were in the mild to moderate range. Within 60 min three out of ten animals treated with saline had died. Animals treated with 2-PAM, MMB-4 or HLo-7 all showed a similar incidence (100%) of toxic signs that were rated in the mild to moderate range as did the saline controls. The numbers of animals showing signs of nerve agent intoxication were significantly less than the saline controls for those treated with MINA (26%, 10 of 39) or DAM (20%, 8 of 40) (p's < 0.001; Fisher's Exact Test) and the severity of signs were all mild. No animal treated with any quaternary or tertiary oxime died.

3.1.2 AChE activity in brain regions

Basal AChE activities in the brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord and striatum are (mean ± SEM) 211.45±6.99, 234.66±6.38, 56.91±1.42, 99.20±1.90, 162.50±2.63, 189.59±7.98, and 389.27±9.15 µmole/mg protein/min, respectively (Table 3). Thus, the AChE activity in striatum was significantly higher than any other region. The next highest AChE activity was found in the cerebellum and brainstem, followed by spinal cord and midbrain, which were significantly higher than hippocampus. Cortex had the lowest AChE activity among all brain regions, approximately one sixth of that of striatum.

3.1.2.1 Effects of GB alone

The ability of GB to inhibit brain regional AChE activity is shown in Table 3. Sixty min following exposure to a 1.0 x LD_{50} dose of GB, AChE activities in the brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord and striatum were inhibited to (mean ± SEM) 19.35±3.58, 10.68±2.13, 10.26±1.50, 23.71±3.06, 13.90±1.91, 29.53±3.57, and 19.80±11.8% of control, respectively (Table 3). The rank orders of AChE

inhibition by GB in brain regions (from high to low) were cerebellum > cortex = midbrain = brainstem > striatum > spinal cord = hippocampus.

3.1.2.2 Effects of oximes

The AChE reactivating results are shown in Table 4. None of the quaternary oximes (2-PAM at 145 umol/kg; MMB-4 and HLo7 at 58 umol/kg) reactivated GB-inhibited AChE activity in any brain regions. The two tertiary oximes (MINA and DAM at 145 umol/kg) reactivated GB-inhibited AChE in the brain and spinal cord with regional specificity. MINA reactivated (i.e., had significantly higher AChE activity when compared with GB-treated group) AChE activity inhibited by GB in brainstem, midbrain, and striatum, while DAM reactivated AChE in cerebellum, midbrain, spinal cord and striatum. Figure 3 shows the typical AChE reactivation effects observed in two brain regions: striatum and midbrain. As the doses of MINA or DAM were increased, a dose-dependent AChE reactivating activity was observed for MINA. DAM, on the other hand, showed dose-dependent AChE reactivation from 14.66 to 41 mg/kg, reaching a plateau at 41 mg/kg. With higher doses of DAM (73 and 128.8 mg/kg), there was a trend of reducing AChE activity, although those AChE activities were still significantly higher than in GB-exposed animals. Figure 4 displays the dose-response reactivating effects of MINA and DAM.

3.1.3 AChE activity in peripheral tissues and blood

Basal AChE activities in diaphragm, heart and skeletal muscle are (mean $\pm$SEM) 14.32$\pm$0.76, 18.23$\pm$0.56, and 10.50$\pm$0.43 µmol/mg protein/min, respectively. In RBC and WB, they are 106.61$\pm$1.86 and 110.05$\pm$3.58 µmol/ml/min, respectively (Table 5). Thus, the rank order from high to low of AChE in the 3 peripheral tissues is heart > diaphragm > skeletal muscle, while in the RBC and WB the AChE activity was about equal.

3.1.3.1 Effects of GB alone

Sixty min following exposure to a 1.0 x LD_{50} dose of GB, AChE activities in the diaphragm, heart, and skeletal muscle were inhibited to (mean $\pm$ SEM) 27.84$\pm$3.53, 17.40$\pm$2.87, and 40.39$\pm$5.67% of control, respectively, and in RBC and WB to 6.65$\pm$2.16 and 8.52$\pm$0.73% of control, respectively (Table 5). Thus, the rank order of AChE inhibition by GB from high to low was heart > diaphragm > skeletal muscle. In RBC and WB, the degree of AChE inhibition by GB was similar.

3.1.3.2 Effects of oximes

The AChE reactivating results are shown in Table 6 and Figures 5-7. In the peripheral tissues and blood, neither tertiary oximes at the145 umol/kg dose (MINA = 12.63 mg/kg and DAM = 14.66 mg/kg) was able to reactivate GB-inhibited AChE (displayed in Figures 5 and 7); however, as shown in Figures 6 and 8 both were able to do so at higher doses (e.g., MINA at 60 or 80 mg/kg and DAM at 41 mg/kg). All three quaternary oximes, on the other hand, were readily able to reactivate GB-inhibited AChE in peripheral tissues and in blood. Figures 5 and 7 show the AChE reactivating effects of equimolar dose of oximes following 1.0 x LD_{50} GB exposure in the diaphragm, heart and skeletal muscle and blood, respectively. In the diaphragm and skeletal muscle (Figure 5), HLo7, MMB-4 and 2-PAM markedly reactivated GB-inhibited AChE, with HLo7 and MMB-4 having significantly greater AChE reactivation than did 2-PAM. In the heart, HLo7, MMB-4 and 2-PAM significantly reactivated AChE activity to a similar degree when compared with the GB-exposed control. In the RBC (Figure 7), MMB-4 reactivated significantly more AChE activity inhibited by GB than did HLo7 and 2-PAM, while in the whole blood, HLo7, MMB-4 and 2-PAM significantly reactivated AChE activity to a similar degree when compared with the GB-exposed control.

3.2 Anticonvulsant Experiment

3.2.1 Seizure Occurrence and Survival at 24 hr

Table 7 shows the incidence of EEG seizure occurrence and the 24-hr survival. In guinea pigs exposed to 2.0 x LD_{50} GB and treated one min later with atropine sulfate (2.0 mg/kg, im) plus any quaternary oxime (2-PAM, MMB-4 or HLo7), all animals (100%) developed continuous seizure activity and 20 - 50% of animals surviving 24 hr. With MINA at doses of 20, 26, 35, 46 and 60 mg/kg, 0, 9, 17, 60, and 75% of animals, respectively, never exhibited EEG seizure activity and 43, 64, 75, 90, and 100% of these animals, respectively, survived 24 hr. Similarly, with DAM at doses of 41, 73, 129 and 231 mg/kg, 0, 17, 67 and 100%, respectively, of animals never exhibited EEG seizure activity and 71, 83, 100 and 100% of these animals, respectively, survived 24 hr.

3.2.2 Seizure Onset

The seizure onset times for 2-PAM-, MMB-4- and HLo7-treated animals were 6.1$\pm$0.4 (n=10), 6.4$\pm$0.4 (n=5), and 6.7$\pm$1.1 (n=5) min after GB exposure, respectively. In those animals that displayed EEG seizure activity, seizure onset times after 2 x LD_{50} of GB are 7.4$\pm$0.4 (n=33) min for MINA and 7.0$\pm$0.8 (n=14) min for DAM treated groups. There was no significant difference in time to seizure onset between these five oxime groups.

3.2.3 Seizure Termination

In animals treated with atropine sulfate (2.0 mg/kg, im) plus 2-PAM, MMB-4 or HLo7 at one min after GB (2.0 x LD_{50}), the EEG seizure activity induced by GB never abated, although at 24 hr the amplitude and frequency of spiking activity were significantly reduced. Figure 9 shows a typical EEG tracing of the ineffectiveness of a quaternary oxime (i.e., MMB-4) to terminate GB-induced seizure activity. In animals treated with MINA at doses that were sufficient to terminate EEG seizure activity, the average termination time was 5.2 min. Figure 10 displays the GB-induced EEG seizure activities (seizure onset at 3.9 min) that were terminated by MINA (35 mg/kg) at 7.5 min after GB administration. For the animals treated with the various doses of DAM, they either failed to develop seizures or, if seizures did develop, they were continuous throughout the recording period as was seen with the quaternary oximes. Figure 11 shows a typical GB-induced EEG seizure (seizure onset at 9.5 min) that was not able to be overcome by DAM at 41 mg/kg, im; note the continued EEG spiking even at 24 hr after treatment. In addition, animals treated with 2-PAM, MMB-4 or HLo7 experienced weight losses that were equivalent to that shown by the GB-exposed saline treated controls. Those animals treated with MINA or DAM, but with seizures terminated, experienced significantly less ($p < 0.001$) body weight loss over the 24-hr survival period than animals with seizures not terminated and animals treated with 2-PAM, MMB-4 or HLo7. These data are displayed in Figure 12.

3.2.4 Anticonvulsant Efficacy

Figure 13 shows the anticonvulsant dose-response effects of MINA and DAM in our anticonvulsant test model (Scheme 2). The anticonvulsant ED_{50} (with 50% confidence limits) for MINA in the presence of atropine sulfate (2.0 mg/kg) against 2.0 x LD_{50} of GB was 36.65 (0.00 – 76.30) mg/kg, im, whereas the anticonvulsant ED_{50} for DAM was determined to be 112.51 (83.78 – 178.87) mg/kg, im. Thus, the anticonvulsant efficacy for MINA was significantly more potent than DAM under similar conditions.

4.0 DISCUSSION

In the present study we investigated the AChE reactivating ability and anticonvulsant capacities of two tertiary oximes, MINA and DAM, and compared their effectiveness to three quaternary oximes, 2-PAM, HLo7 and MMB-4, in GB-exposed guinea pigs. In the reactivation study, animals were challenged with a 1.0 x LD_{50} dose of GB followed 5 min later by treatment with saline (served as control) or a selected dose of an oxime.

CNS and peripheral tissues and blood were collected at 60 min for AChE activity determination. Under the condition of this study, both quaternary and tertiary oximes were able to reactivate GB-inhibited AChE, but in an opposite direction with respect to tissue specificity. In the CNS only the tertiary oximes reactivated GB-inhibited AChE in the brain regions, whereas only quaternary oximes reactivated GB-inhibited AChE in the blood and peripheral tissues. In the anticonvulsant study, guinea pigs were pretreated with PB to inhibit ~20-30% of blood AChE activity 30 min prior to challenge with a 2.0 x LD_{50} of GB. One min later an injection of atropine sulfate (2.0 mg/kg, im) and a dose of oxime were administrated. The EEG activity and mortality rate were followed for up to 6 hr and again at 24 hr after GB exposure. Under the condition of this study, both MINA and DAM, but none of the 3 quaternary oximes, prevented or quickly stopped seizure activity, with MINA displaying a higher potency than DAM. At higher MINA or DAM doses, many animals never developed EEG seizure activity and only lost modest amount of boy weight over the 24-hr survival period.

In the AChE reactivation study, we chose a 145 umol/kg dose of 2-PAM, which is equivalent to 3 auto-injectors of the Mark I nerve agent antidote kit for a 70-kg human. Since the molecular weights of both MINA and DAM are quite low, we also used 145 umol/kg for these two tertiary oximes. On the other hand, since both MMB-4 and HLo7 are bispyridinium compounds similar to HI-6, a 58 umol/kg dose was used for MMB-4 and HLo7, based on 3-autoinjector equivalent dose (500 mg per injector) of HI-6 (Clair et al., 2000). With the dose of 145 umol/kg, both MINA and DAM were not able to reactivate GB-inhibited AChE in blood (RBC and WB) and peripheral tissues (diaphragm, heart and skeletal muscle). The AChE reactivation in blood and peripheral tissues was observed only at higher doses of MINA (60 or 80 mg/kg) and DAM (41 mg/kg, but not 73 or 128.8 mg/kg). The reason for this effect of DAM is not clear, but is probably due to the reported observation that DAM binds initially to carboxylesterase in the plasma of guinea pigs after absorption and reactivates OP-inhibited carboxylesterase, thus reducing its availability to bind and reactivate AChE (Myers, 1959). On the other hand, 2-PAM (at 145 umol/kg), MMB-4 and HLo7 (both at 58 umol/kg) reactivated GB-inhibited AChE in blood and peripheral tissues quite well, with both MMB-4 and HLo7 showing a greater reactivating capacity than that of 2-PAM.

As can be expected 2-PAM, MMB-4 and HLo7 did not show any AChE reactivation in the CNS, since they do not penetrate the BBB due to their quaternary structure and limited lipid solubility. On the other hand, MINA and DAM are tertiary structures and highly lipid soluble and, at higher doses, significantly reactivated GB-inhibited AChE in all the brain regions (including spinal cord) studied. At dose of 145 umol/kg, MINA reactivated AChE activity significantly in brainstem, midbrain, and striatum, while DAM significantly reactivated the AChE activity in midbrain, striatum, spinal cord, and cerebellum. The differences in the regional specificity of these two tertiary oximes are not understood, but may be due to their individual distribution profile in brain regions. However, at higher doses the AChE reactivating capacity in the CNS is highly significant for both MINA and DAM, with MINA more potent than DAM.

Another noteworthy observation in the reactivation study was that, while MINA produced a dose-dependent increase in brain regional AChE reactivation from 12.6 to 80 mg/kg, the reactivation profile of DAM reached a plateau at 41 mg/kg in all tissues. From the 73 to 128.8 mg/kg doses of DAM, the AChE activities were reduced, although the overall AChE activity was still significantly higher than those inhibited by GB. The phenomenon produced by DAM in the CNS is not clear. Even though the CNS AChE reactivation produced by DAM was not quite dose-dependent, animals treated with increasingly higher doses of both MINA and DAM clearly displayed less signs of GB-induced cholinergic toxicity than observed in those animals treated with 2-PAM, MMB-4 or HLo7. These observations were confirmed by the results of our anticonvulsant study.

In the anticonvulsant study, both MINA and DAM produced anticonvulsant effects with MINA having greater potency than DAM. When MINA or DAM was administered along with 2.0 mg/kg atropine sulfate in PB-pretreated guinea pigs, EEG seizure activities induced by 2.0 x LD_{50} of GB was prevented or quickly arrested. As the doses of MINA or DAM were increased, greater percentages of animals never exhibited EEG seizure activity. If seizure activity did occur, it was spontaneously terminated within minutes

(averaged 5.2 min). Thus, increasing the doses of MINA or DAM reduced seizure occurrence and increased the propensity for seizure termination. Additionally, it was observed that increases in the dose of MINA or DAM enhanced survival and minimized overnight weight loss. On the other hand, the quaternary oximes, 2-PAM, MMB-4 and HLo7, had no effect on GB-induced seizure activity and, while preventing lethality, did not protect against the GB-induced body weight loss of ~35%.

MINA and DAM had been investigated as potential oximes for the treatment of nerve agent exposure in the 1950's (Askew, 1956; 1957; Dultz et al., 1957; Rutland, 1958; Myers, 1959; Wills, 1959). In view of current pharmacological data, it was unfortunate that these two tertiary oximes were not pursued further, because of the reports that quaternary pyridinium oximes were several orders of magnitude better reactivators of phosphrylated AChE in human erythrocytes (see review by Hobbiger, 1963). Both are lipid soluble and can readily penetrate the blood brain barrier (Cohen and Wiersinga, 1960), as was confirmed with the reactivation study and this reactivation has significantly beneficial functional consequences as shown with the anticonvulsant study. When MINA or DAM was administrated at high doses one min after 2.0 x LD_{50} of GB, many of the animals never expressed EEG seizure activity, or the bursting seizure activity could be quickly stopped. These tertiary oximes are able to reactivate AChE within the CNS (Rutland, 1958; Cohen and Wiersinga, 1960). Our present data not only supported these earlier findings, but further showed that effective reactivating central AChE can increase survival and possibly prevent seizure and associated neuropathology. Askew (1956, 1957) showed in the late 1950's that when used alone or in combination with atropine sulfate, MINA and DAM markedly raised the LD_{50} doses of GB in mice, rats, guinea pigs, and rabbits. In our anticonvulsant study, we confirmed that both MINA and DAM increased survival in guinea pigs. We also showed that both MINA and DAM possessed the anticonvulsant effectiveness against GB, with MINA being more potent in this respect. It has been our observations (McDonough and Shih, 1997; McDonough et al., 1999, 2000; Shih et al., 2003; 2007) that if nerve agent-induced seizure activity can be eliminated rapidly with any number of different effective anticonvulsant treatment drugs, there will be less brain pathology and enhanced probability of survival. It is reasonable to predict that with MINA or DAM included in a therapeutic regimen for nerve agent poisoning, significantly less brain pathology and associated behavioral abnormalities might be seen in survivors.

In conclusion, these two experiments clearly show that tertiary oximes (DAM and MINA) reactivated ChE in the brain, reduced toxic signs, improved survival and terminated seizures following GB intoxication. The current results support the notion that central AChE reactivation or preservation of CNS AChE activity following nerve agent intoxication is critical in the medical management of nerve agent intoxication (Fosbraey et al., 1992; Wetherell, 1994; Wetherell et al., 2002). Thus, tertiary oximes with high lipid solubility could be an excellent adjunct to current pretreatment and therapy regimens for medical management of nerve agent poisoning (Sidell, 1997; Moore et al., 1995).

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Table 1: Toxic Sign Scores of Guinea Pigs Following Nerve Agent Exposure*

Toxic Signs	Score	Level/degree of toxicity (Total scores)
Salivation (1)	1	Mild (1-3)
Lacrimation (1)	1	
Nystagmus(1)	1	
General Motor (0-3)		Moderate (4-6)
Fasciculation	1	
Tremor	2	
Convulsion	3	
General State (0-3)		Severe (7-9)
Mild uncoordination	1	
Impaired movement	2	
Prostration	3	

* Guinea pigs were scored for absence [0] or presence of salivation [1], lacrimation [1], nystagmus [1], and the general motor deficits, which ranged from 1-3, for the presence of fasciculation [1], tremor [2], or convulsion [3]. Additionally, the guinea pig was allowed to walk on the bench top and the general state scores, which ranged from 0-3, were recorded with the following ranks: normal [0], mild uncoordination [1], impaired movement/with righting reflex [2], or prostration/without righting reflex [3]. A cumulative score was then calculated by tabulating the salivation, lacrimation, nystagmus, general motor and general state scores for each subject. The maximal attainable score was a 9. A cumulative score was categorized as mild intoxication [1-3], moderate intoxication [4-6] or severe intoxication [7-9]. Modified for guinea pig from Shih and Romano (1988).

Table 2: Incidence and Severity of Toxic Sign Scores in Guinea Pigs Exposed to Sarin and Treated with Various Oximes*

Treatment (mg/kg)	# Animals Showing Signs	Ave. Score
Saline	14/15	4.07
HLo7 (30.2)	8/8	4.13
MMB-4 (26)	7/7	3.43
2-PAM (25)	8/8	2.75
MINA (12.63)	0/7	0.00
MINA (17.5)	2/8	0.88
MINA (35)	2/8	0.25
MINA (60)	5/8	0.75
MINA (80)	1/8	0.13
DAM (14.66)	0/8	0.00
DAM (23)	1/8	0.13
DAM (41)	4/8	0.88
DAM (73)	3/8	0.38
DAM (128.8)	0/8	0.00

*Guinea pigs were injected subcutaneously with saline (0.5 ml/kg) or a 1.0 x LD_{50} dose of sarin, followed 5 min later with intramuscular saline (as control) or a specified dose of an oxime. Toxic sign scores (based on Table 1) were taken at 58 min after sarin exposure for each animal and averaged for the same treatment group.

Table 3: AChE Activity in Brain Regions and Spinal Cord in Control and Sarin-Intoxicated Guinea Pigs*

Tissue	Brainstem	Cerebellum	Cortex	Hippo	Midbrain	Spinal Cord	Striatum
Control AChE Activity (µmole/mg protein/min)#, mean ± SEM							
Control	211.45 ±6.99	234.66 ±6.38	56.91 ±1.42	99.20 ±1.90	162.50 ±2.63	189.59 ±7.98	389.27 ±9.15
% of Control AChE Activity at 60 min#, mean ± SEM							
Sarin	19.35 ±3.58	10.68 ±2.13	10.26 ±1.50	23.71 ±3.06	13.90 ±1.91	29.53 ±3.57	19.80 ±11.84

*Guinea pigs were injected subcutaneously with saline (0.5 ml/kg) or a 1.0 x LD_{50} dose of the nerve agent sarin. Brain regions and spinal cord were collected at 60 min after treatment.

#Control AChE activity was expressed as µmole/mg protein/min for brain samples. The AChE activity in sarin-exposed group was expressed as percentage of saline-treated control group. Value shown are mean ± S.E.M. with group size N= 37 for control and 9 for sarin, respectively.

Table 4: AChE Activity in Brain Regions of Guinea Pigs Exposed to Sarin (GB) Followed by Oxime Therapy

	% of AChE Activity#						
	Brainstem	**Cerebellum**	**Cortex**	**Hippocampus**	**Mid Brain**	**Spinal Cord**	**Striatum**
GB/Saline	19.35	10.68	10.26	23.71	13.90	29.53	19.80
145 µmol/kg							
MINA (12.63 mg/kg)	39.05*	26.99	20.41	36.96	33.24*	48.62	47.46*
DAM (14.66 mg/kg)	37.47	27.61*	23.52	37.17	31.90*	50.75*	41.85*
2-PAM (25 mg/kg)	20.75	13.77	15.60	22.78	15.01	25.89	22.01
58 µmol/kg							
HLo7 (30.2 mg/kg)	16.55	11.77	13.42	27.00	14.52	28.09	20.17
MMB-4 (26 mg/kg)	21.67	13.29	13.10	30.38	15.81	32.87	28.46

AChE activity as % of normal baseline activity in each brain region. Guinea pigs were injected subcutaneously with a 1.0 x LD_{50} dose of sarin (GB) and followed 5 min later with intramuscular saline (as control) or a specified dose of oxime. Brain regions and spinal cord were collected at 60 min after treatment. Group size N = 9 for GB/Saline and 7-8 for oxime treatments.

* $p<0.05$ when compared with GB/saline control.

Table 5: AChE Activity in Peripheral Tissues and Blood in Control and Sarin-Intoxicated Guinea Pigs*

Control AChE Activity#, mean ± SEM					
Tissue	Diaphragm	Heart	Skeletal Muscle	RBC	WB
Control	14.32±0.76	18.23±0.56	10.50±0.43	106.61±1.86	110.05±3.58
% of Control AChE Activity at 60 min#, mean ± SEM					
Sarin	27.84±3.53	17.40±2.87	40.39±5.67	6.65±2.16	8.52±0.73

*Guinea pigs were injected subcutaneously with saline (0.5 ml/kg) or a 1.0 x LD_{50} dose of the nerve agent sarin. Peripheral tissues (diaphragm, heart and skeletal muscle) and blood (red blood cells [RBC] and whole blood [WB]) were collected at 60 min after treatment.

#Control AChE activity was expressed as µmole/mg protein/min for tissue samples and as µmole/ml/min for blood samples. The AChE activity in sarin-exposed group was expressed as percentage of saline-treated control group. Value shown are mean ±S.E.M.with group size N= 37 for control and N= 9 for sarin, respectively.

Table 6: AChE Activity in Peripheral Tissues and Blood of Guinea Pigs Exposed to Sarin (GB) Followed by Oxime Therapy

% of AChE Activity#					
	Diaphragm	**Heart**	**Skeletal**	**RBC**	**WB**
GB/Saline	27.84	17.40	40.39	6.65	8.52
145 µmol/kg					
MINA (12.63 mg/kg)	32.44	16.08	41.82	5.14	11.10
DAM (14.66 mg/kg)	32.65	16.19	43.03	4.94	8.58
2-PAM (25 mg/kg)	62.48*	61.70*	70.23*	52.52*	61.78*
58 µmol/kg					
HLo7 (30.2 mg/kg)	80.10*	58.68*	104.78*	52.15*	66.78*
MMB-4 (26 mg/kg)	90.09*	56.19*	88.73*	75.34*	83.13*

AChE activity as % of normal baseline activity in each peripheral tissue or blood (red blood cells [RBC] and whole blood [WB]). Guinea pigs were injected subcutaneously with a 1.0 x LD_{50} dose of sarin (GB) and followed 5 min later with intramuscular saline (as control) or a specified dose of oxime. Peripheral tissues (diaphragm, heart and skeletal muscle) and blood (red blood cells [RBC] and whole blood [WB]) were collected at 60 min after treatment. Group size N = 9 for GB/Saline and 7-8 for oxime treatments.

* $p<0.05$ when compared with GB/saline control.

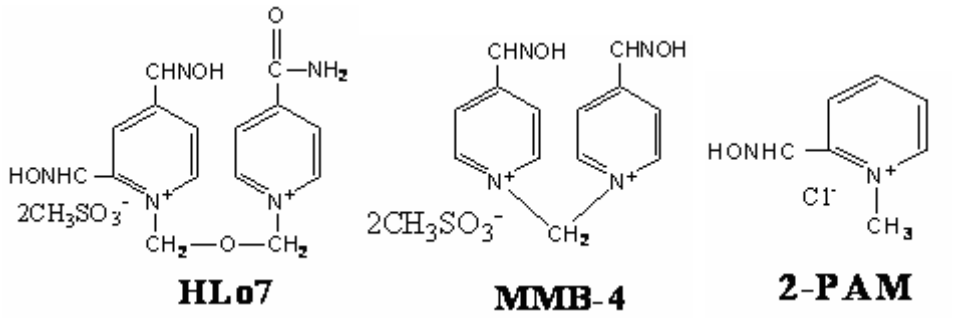
Table 7: Effects of Oxime on Sarin-Induced Seizure Occurrence, Termination and Survival

Treatment (mg/kg)	Never Seized	Seizure "OFF"	Survival (24 hr)
2-PAM (25)	0/10 (0%)	0/10 (0%)	5/10 (50%)
MMB-4 (26)	0/5 (0%)	0/5 (0%)	2/5 (40%)
HLo7 (30.2)	0/5 (0%)	0/5 (0%)	1/5 (20%)
MINA (20)	0/7 (0%)	0/7 (0%)	3/7 (43%)
(26)	1/11 (9%)	1/11(9%)	7/11 (64%)
(35)	2/12 (17%)	7/12 (58%)	9/12 (75%)
(46)	6/10 (60%)	9/10 (90%)	9/10 (90%)
(60)	9/12 (75%)	11/12 (92%)	12/12 (100%)
DAM (41)	0/7 (0%)	0/7 (0%)	5/7 (71%)
(73)	1/6 (17%)	1/6 (17%)	5/6 (83%)
(129)	4/6 (67%)	4/6 (67%)	6/6 (100%)
(231)	6/6 (100%)	6/6 (100%)	6/6 (100%)

* Guinea pigs were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to sarin (2 x LD_{50}, sc) and followed 1 min later by atropine sulfate (2.0 mg/kg, im) and a dose of an oxime (im). EEG seizure onset, termination and 24-hr survival were recorded.

Figure 1: Chemical Structures of Oximes

Quaternary Oximes



Tertiary Oximes

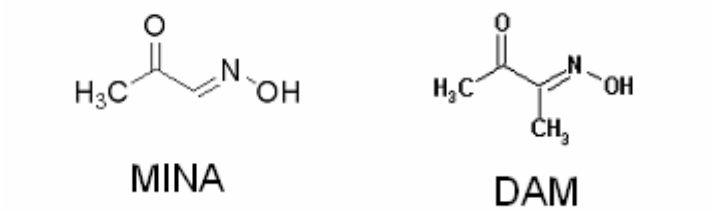


Figure 1. Chemical structures of quaternary and tertiary oximes studied.

Figure 2: Toxic Sign Scores

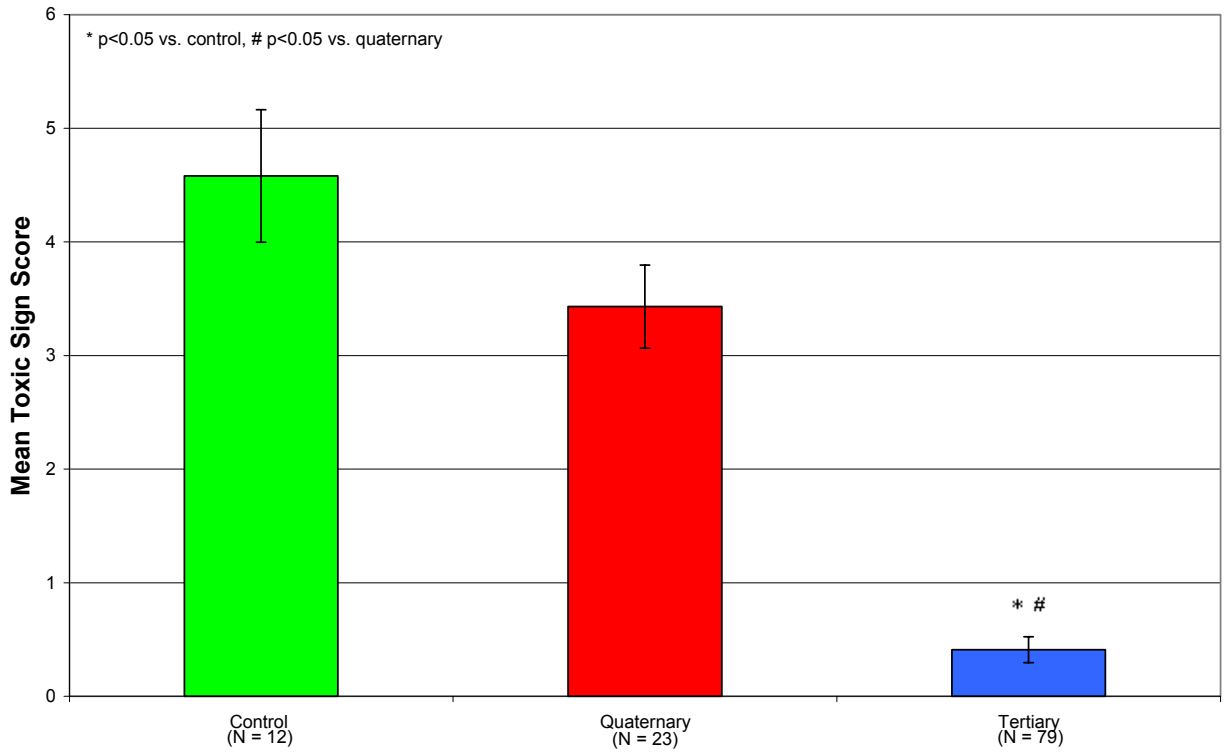


Figure 2. The overall toxic sign scores in animals exposed to a 1.0 x LD_{50} dose of sarin and treated 5 min later with saline (served as control), quaternary (2-PAM, MMB-4 and HLo7) or tertiary (DAM and MINA) oximes. Toxic signs were scored at 58 min after sarin administration in survivors.
[*]P<0.05 compared with saline control group.
[#] P<0.05 compared with quaternary oxime group.

Figure 3: AChE Activity in Striatum and Midbrain of Guinea Pigs: Equimolar Doses

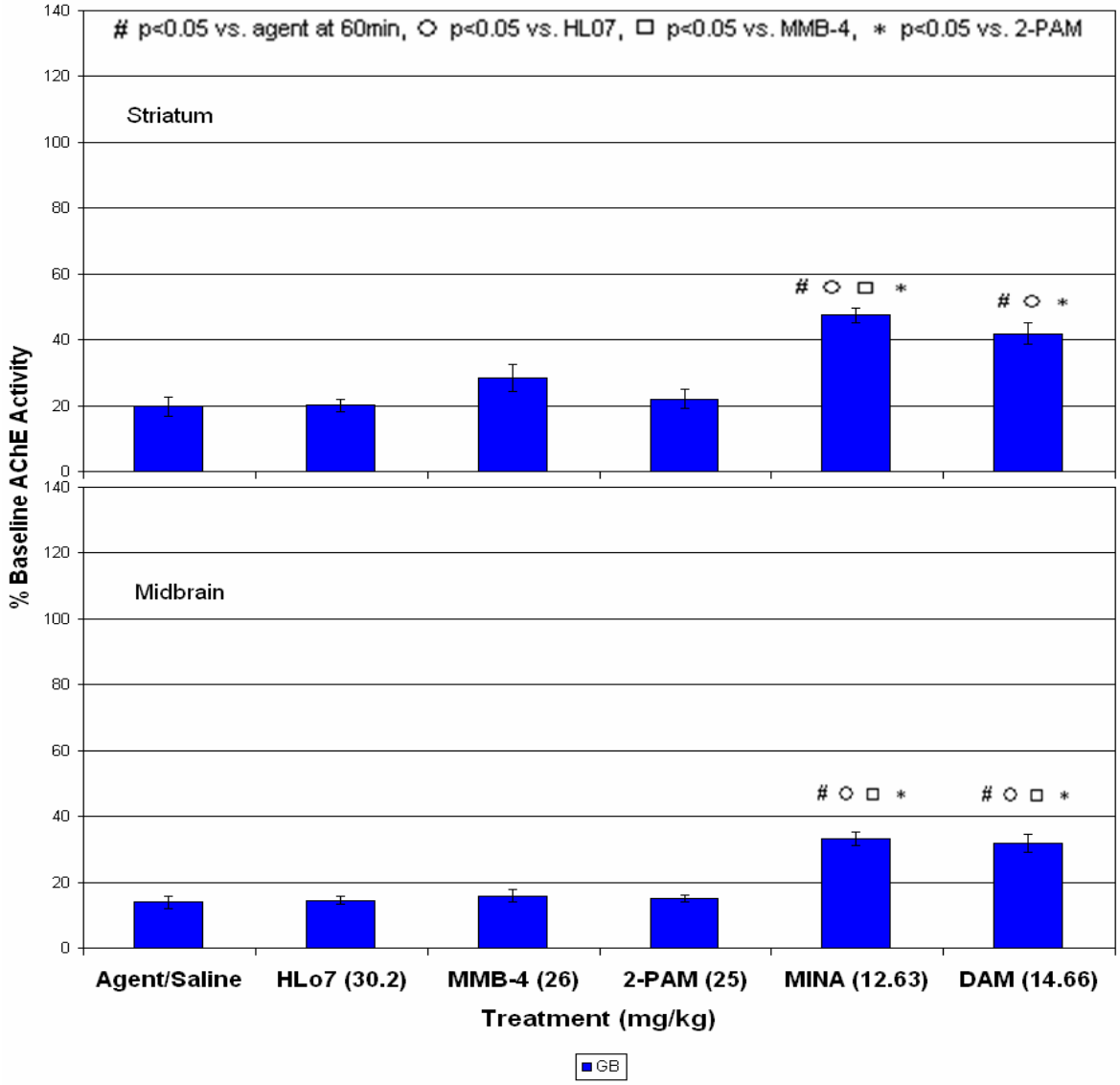


Figure 3. AChE activity in striatum and midbrain regions of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) followed 5 min later treated intramuscularly with saline (as control), 58 µmol/kg of HLo7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Brain tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.
[#] p<0.05 compared with agent/saline group (control).
[○] p<0.05 compared with HLo7 group.
[□] p<0.05 compared with MMB-4 group.
[*] p<0.05 compared with 2-PAM group.

Figure 4: AChE Activity in Striatum and Midbrain of Guinea Pigs: Dose-Respose

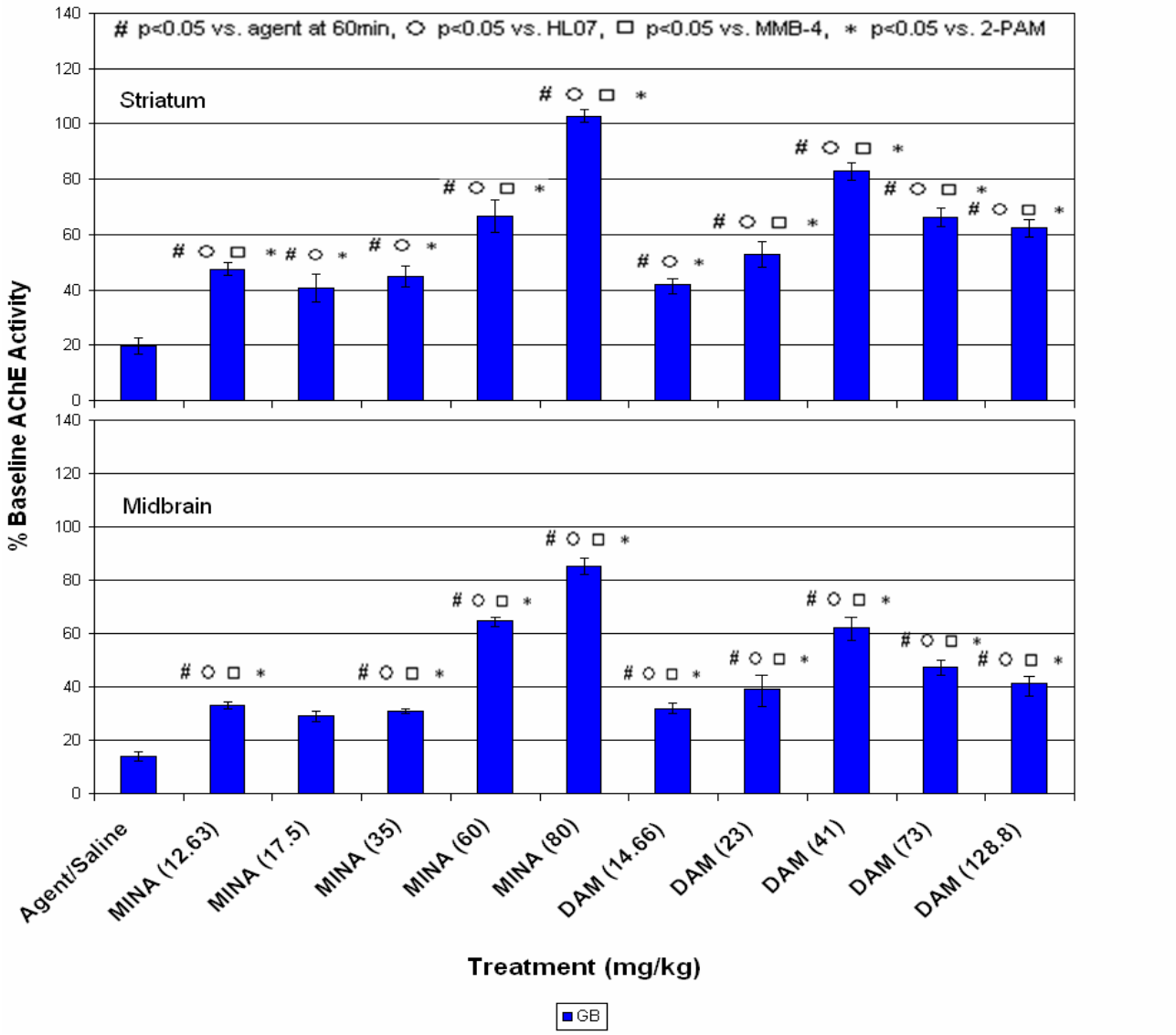


Figure 4. AChE activity in striatum and midbrain regions of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) followed 5 min later treated intramuscularly with saline (as control) or various doses of MINA or DAM. Brain tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.
[#] p<0.05 compared with agent/saline group (control).
[○] p<0.05 compared with HLo7 group.
[□] p<0.05 compared with MMB-4 group.
[*] p<0.05 compared with 2-PAM group.

Figure 5: AChE Activity in Diaphragm, heart and skeletal muscle of Guinea Pigs: Equimolar Doses

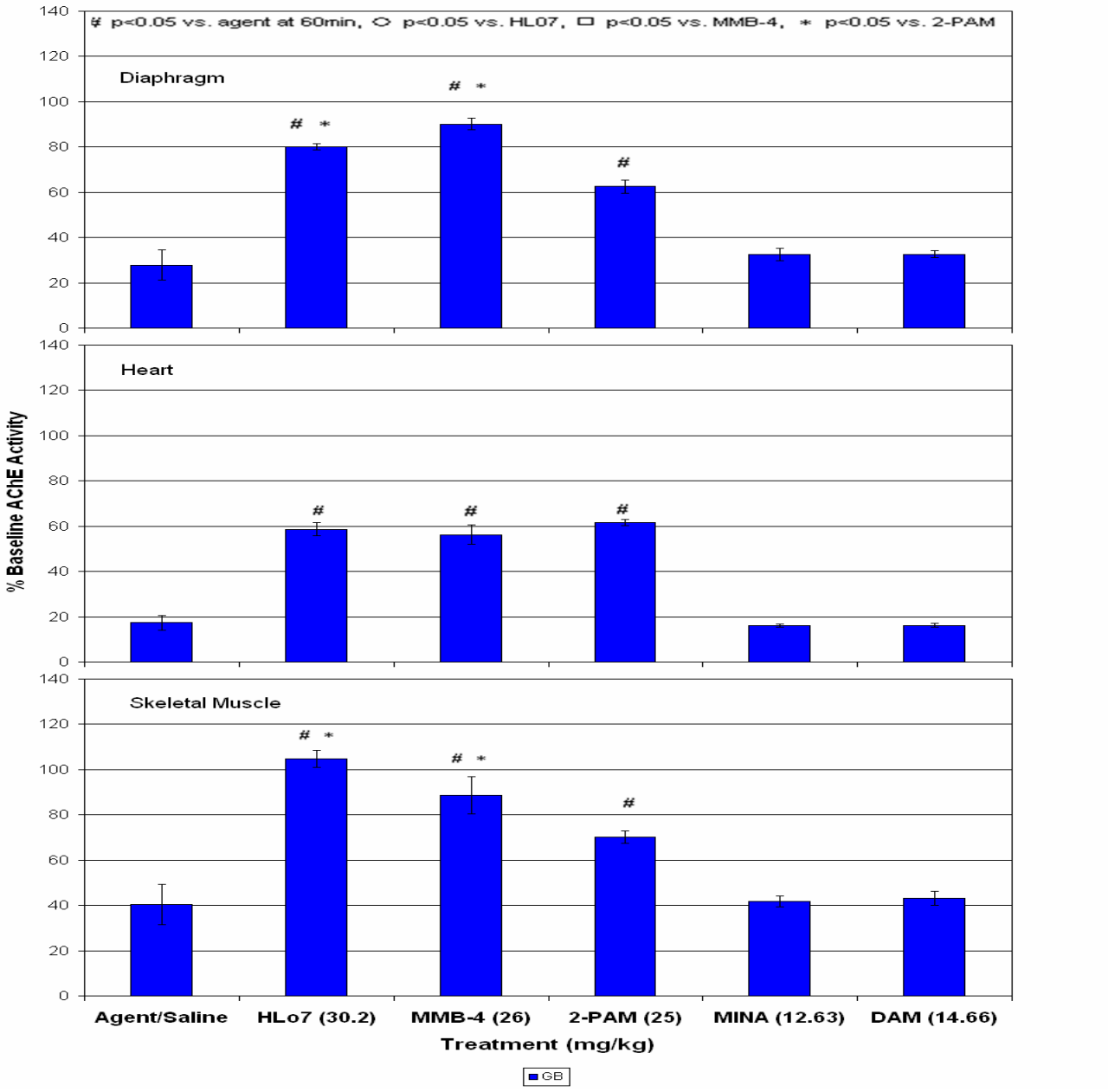


Figure 5. AChE activity in the diaphragm, heart and skeletal muscle of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) followed 5 min later treated intramuscularly with saline (as control), 58 µmol/kg of HLo7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.
[#] p<0.05 compared with agent/saline group (control).
[○] p<0.05 compared with HLo7 group.
[□] p<0.05 compared with MMB-4 group.
[*] p<0.05 compared with 2-PAM group.

Figure 6: AChE Activity in Diaphragm, heart and skeletal muscle of Guinea Pigs: Dose-Respose

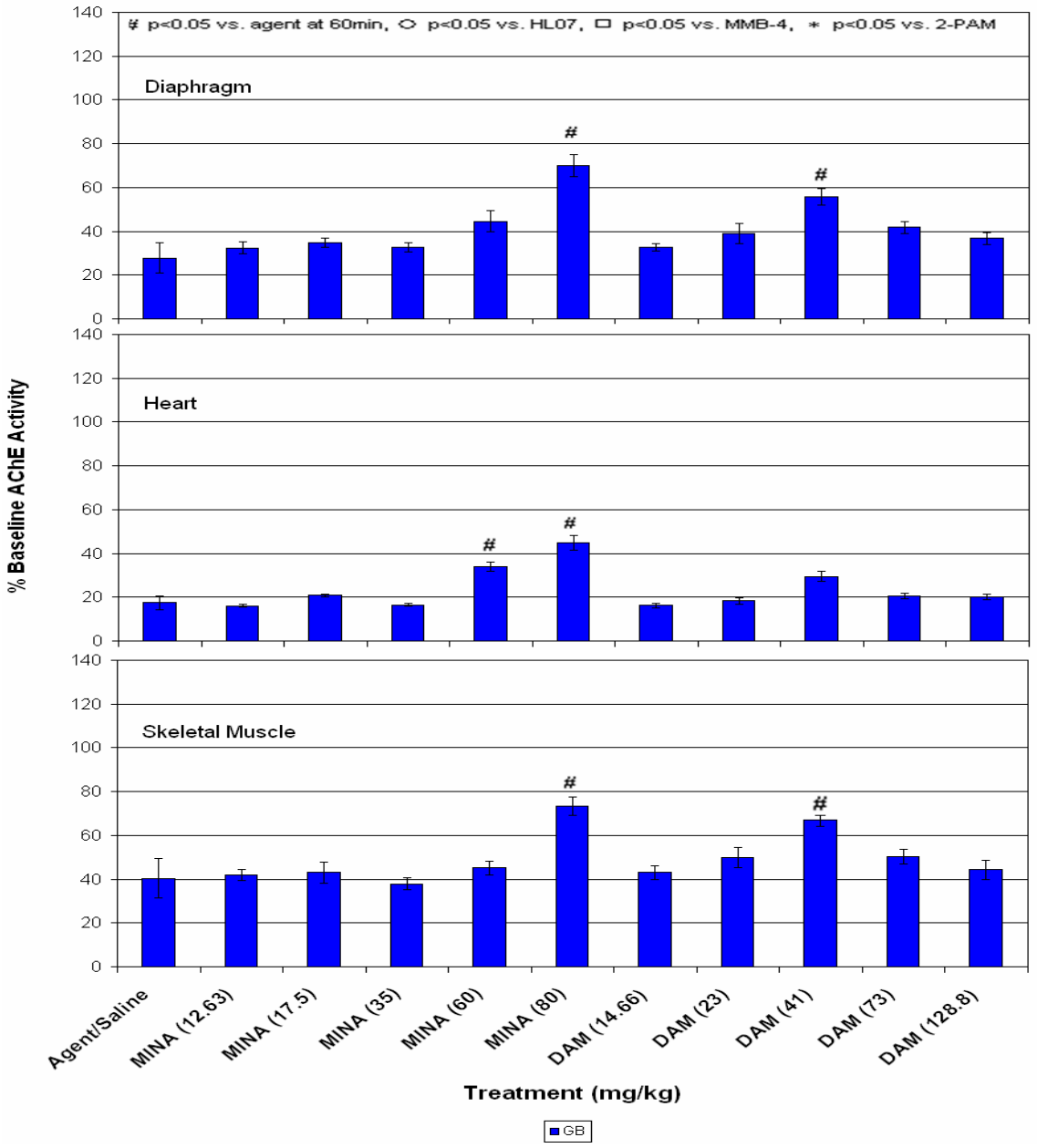


Figure 6. AChE activity in the diaphragm, heart and skeletal muscle of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) followed 5 min later treated intramuscularly with saline (as control), or various doses of MINA or DAM. Tissues were collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.
[#] p<0.05 compared with agent/saline group (control).
[○] p<0.05 compared with HLo7 group.
[□] p<0.05 compared with MMB-4 group.
[*] p<0.05 compared with 2-PAM group.

Figure 7: AChE Activity in the Blood of Guinea Pigs: Equimolar Doses

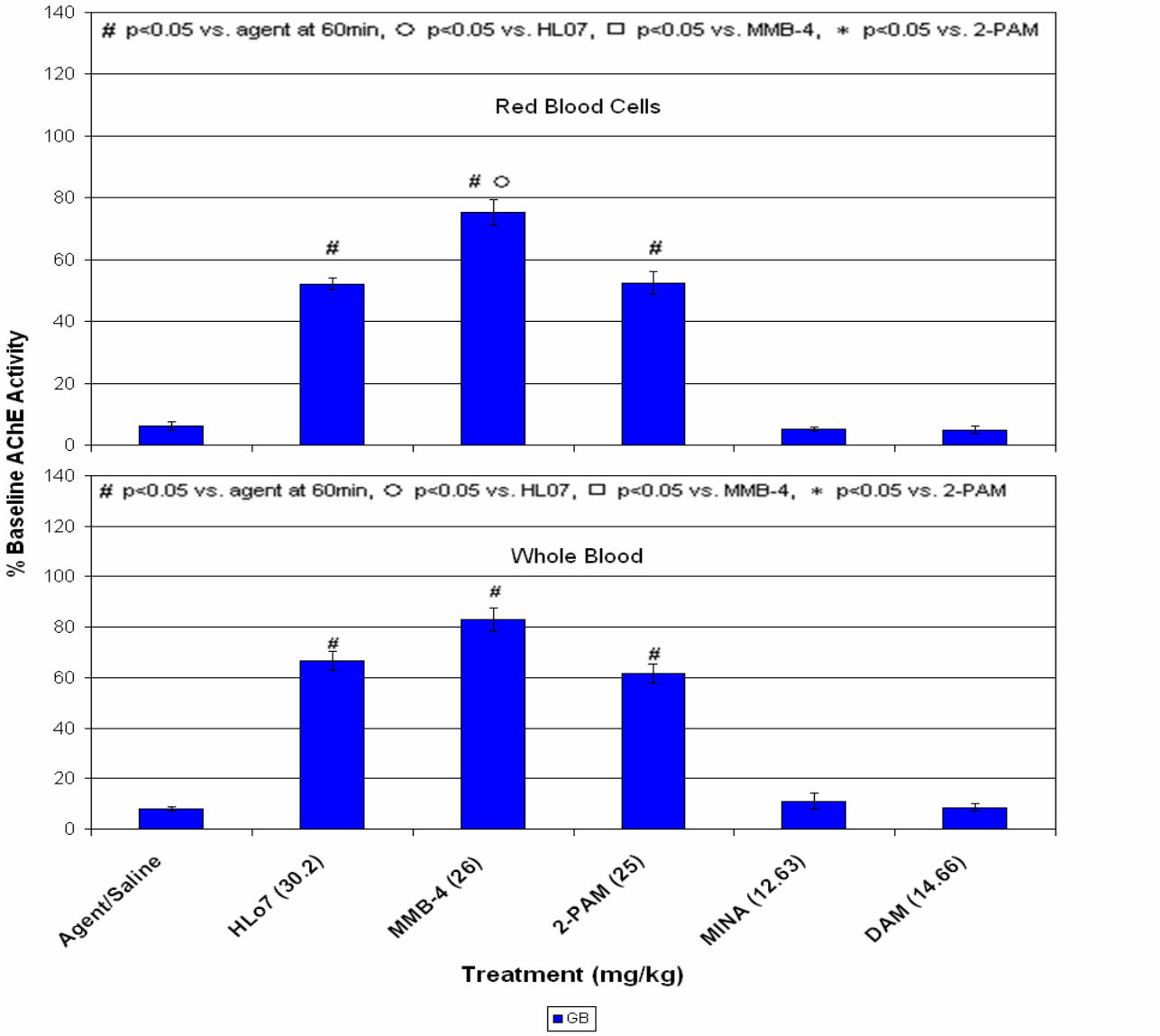


Figure 7. AChE activity in the red blood cell (RBC) and whole blood (WB) of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) followed 5 min later treated intramuscularly with saline (as control), 58 µmol/kg of HLo7 and MMB-4, or 145 µmol/kg of 2-PAM, MINA and DAM. Blood was collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.
[#] p<0.05 compared with agent/saline group (control).
[○] p<0.05 compared with HLo7 group.
[□] p<0.05 compared with MMB-4 group.
[*] p<0.05 compared with 2-PAM group.

Figure 7: AChE Activity in the Blood of Guinea Pigs: Equimolar Doses

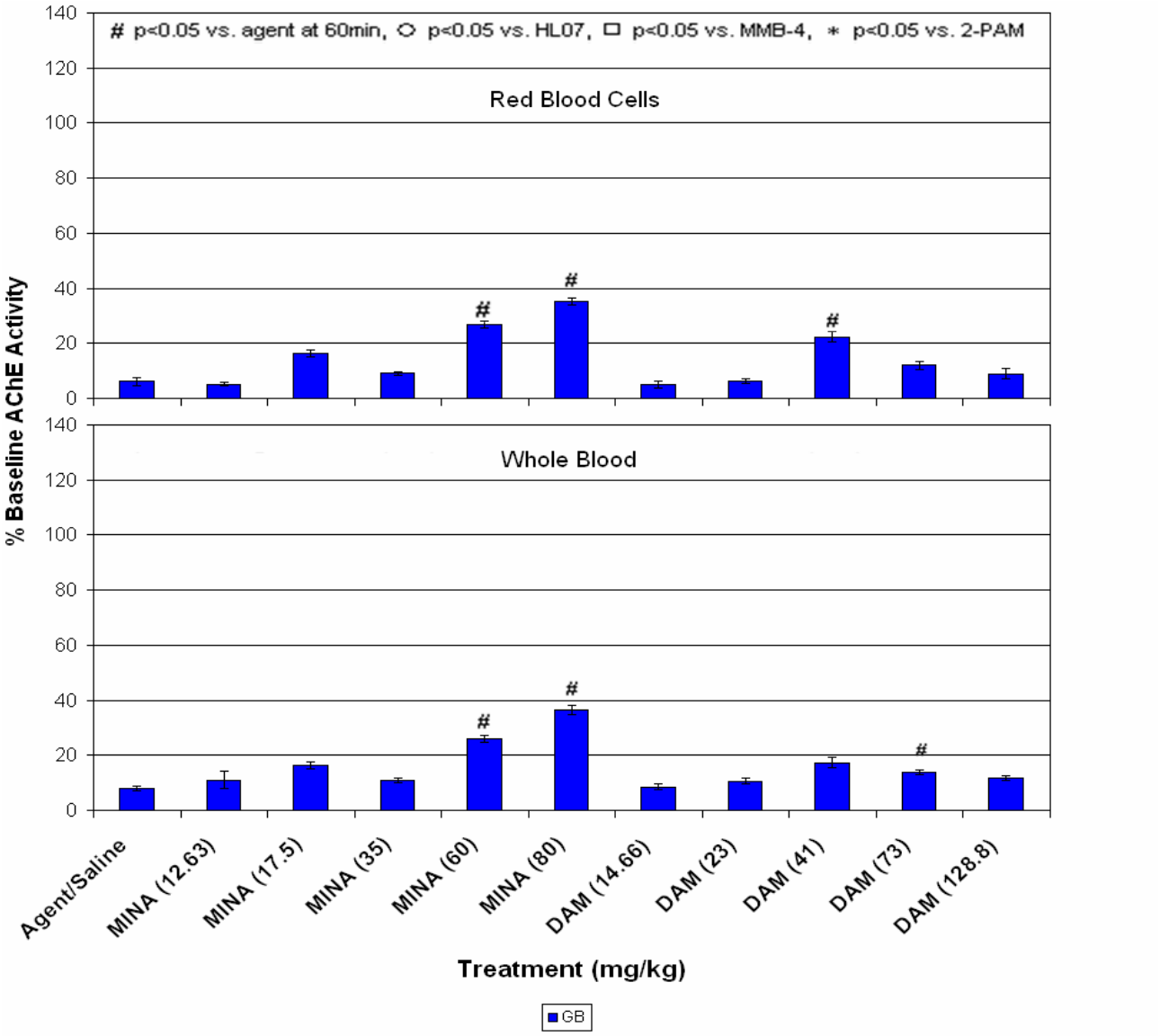


Figure 8. AChE activity in the red blood cell (RBC) and whole blood (WB) of the guinea pigs exposed to sarin (1.0 x LD_{50}, sc) followed 5 min later treated intramuscularly with saline (as control), or various doses of MINA and DAM. Blood was collected at 60 min after sarin exposure. AChE activities were expressed as mean % baseline activity ± SEM with N= 8 in each treatment group.
[#] p<0.05 compared with agent/saline group (control).
[○] p<0.05 compared with HLo7 group.
[□] p<0.05 compared with MMB-4 group.
[*] p<0.05 compared with 2-PAM group.

Figure 9: EEG tracings of Guinea Pig Treated with MMB-4

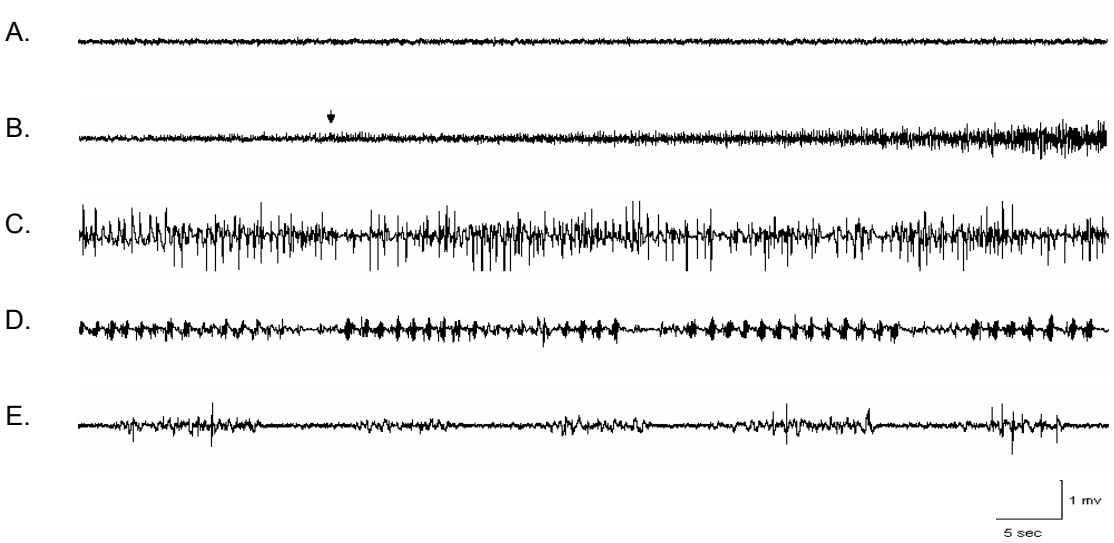


Figure 9. An example of MMB-4 (26 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide 30 min prior to sarin (2 x LD_{50}) exposure and treated with MMB-4 and atropine sulfate (2.0 mg/kg) 1 min postexposure. (A) Baseline EEG activity. (B) Seizure onset at 6.5 min postexposure (arrow). (C-E) Seizure activity at 1, 4 and 24 hr, respectively, after onset.

Figure 10: EEG tracings of Guinea Pig Treated with MINA

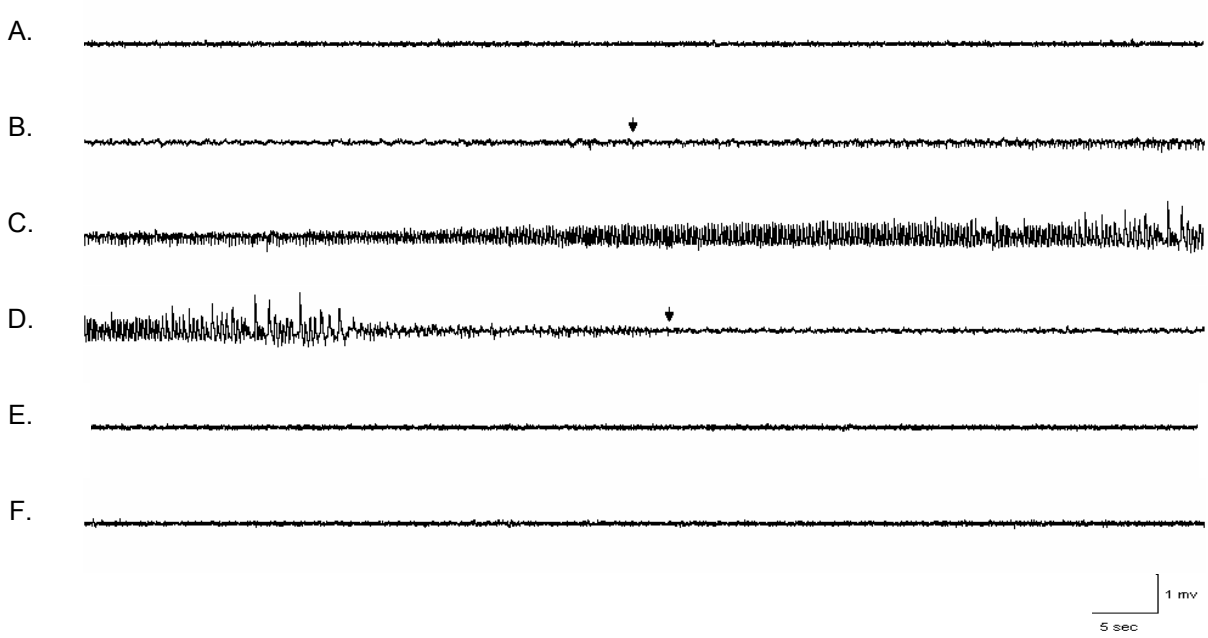


Figure 10. An example of MINA (35 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to sarin (2 x LD_{50}, sc) exposure and treated intramuscularly with MINA and atropine sulfate (2.0 mg/kg, im) 1 min postexposure. (A) Baseline EEG activity. (B) Seizure onset (arrow) at 3.9 min postexposure. (C) Peak seizure activity. (D) Seizure termination at 7.5 min after onset. (E and F) EEG recordings at 4 and 24 hr, respectively, show no signs of seizure activity.

Figure 11: EEG tracings of Guinea Pig Treated with DAM

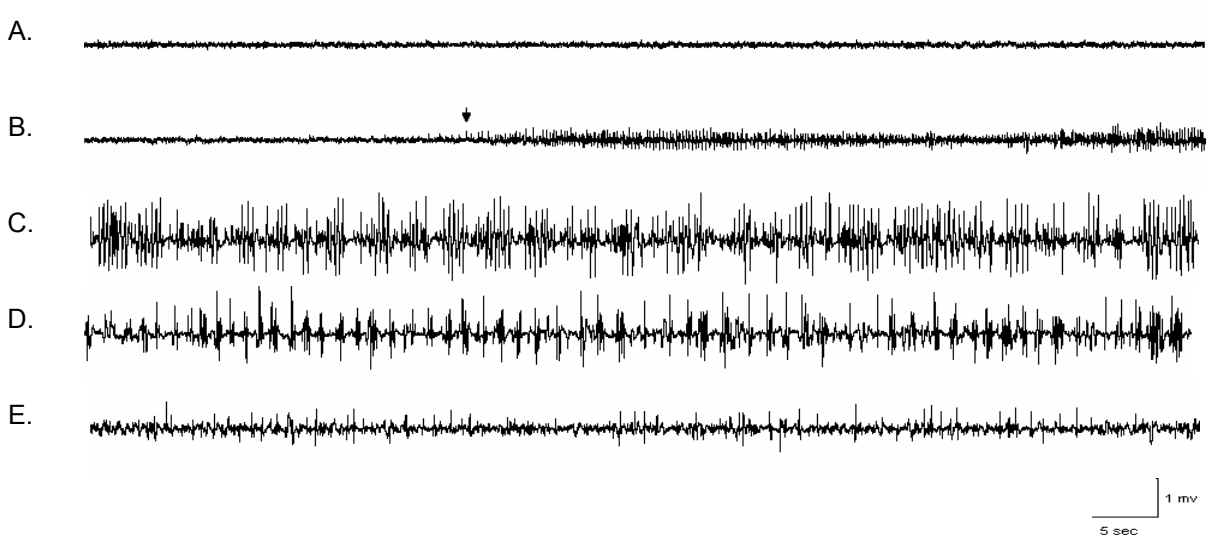
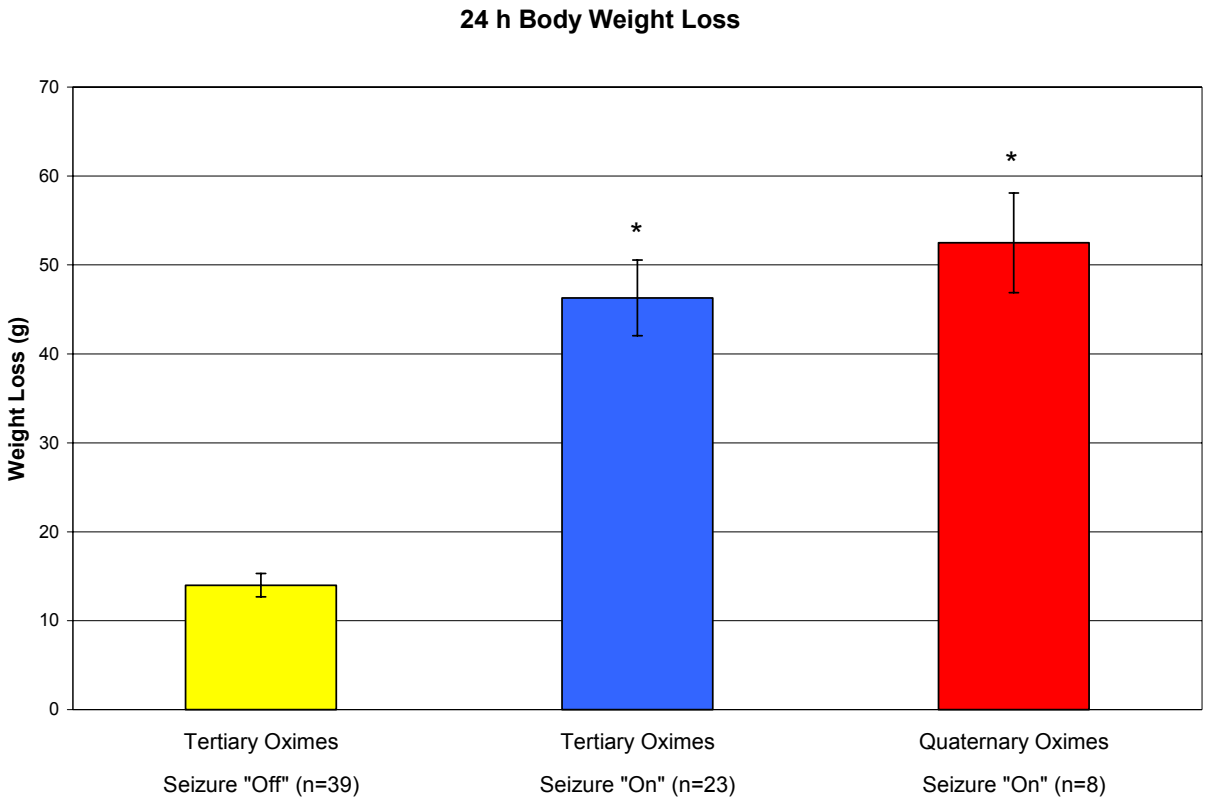


Figure 11. An example of DAM (41 mg/kg) treatment on sarin-induced EEG seizure activity. Animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to sarin (2 x LD_{50}, sc) exposure and treated intramuscularly with DAM and atropine sulphate (2.0 mg/kg) 1 min postexposure. (A) Baseline EEG activity. (B) Seizure onset (arrow) at 9.5 min postexposure. (C-E) Seizure activity at 1, 4 and 24 hr, respectively, after onset.

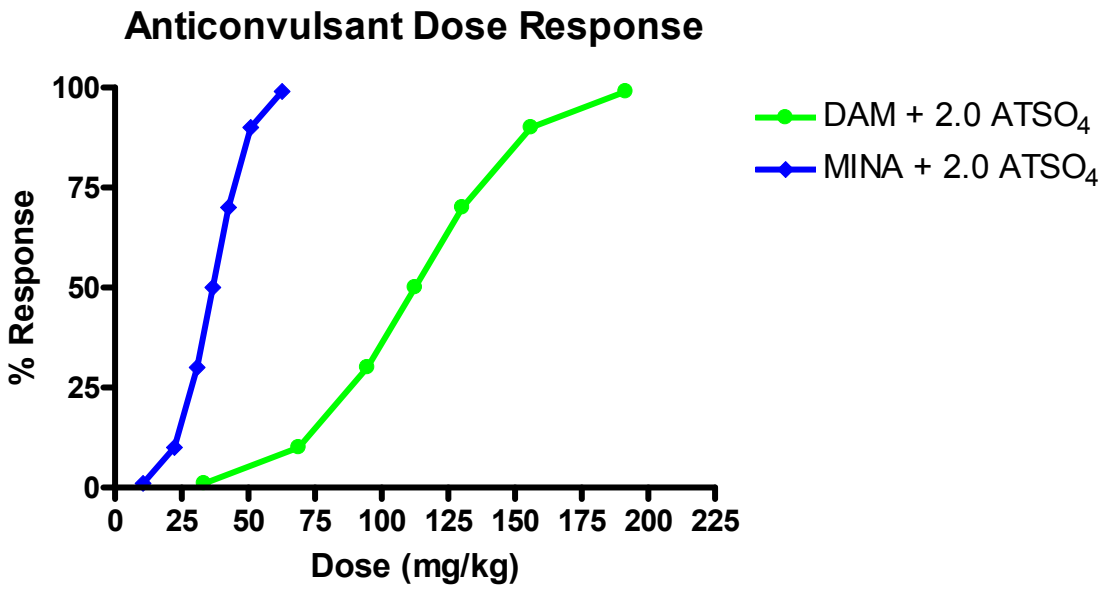
Figure 12: Body Weight Loss in 24 Hours



	Seizure "Off" (n=39) Tertiary Oximes	Seizure "On" (n=23) Tertiary Oximes	Seizure "On" (n=8) Quaternary Oximes
mean	14	46.3	52.5
N	39	23	8
S.D.	8.2	20.4	15.9
SEM	1.3	4.2	5.6

Figure 12. The overnight body weight loss in animals survived a 2.0 x LD_{50} dose of sarin, with EEG seizure activity terminated (seizure off) or not terminated (seizure on) by tertiary or quaternary oxime treatments. All animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) at 30 min prior to and treated intramuscularly with atropine sulfate (2.0 mg/kg) and an oxime at one min after sarin challenge. Body weight of each animal was recorded before sarin challenge and at 24 hr after sarin exposure in survivors.
*$P<0.05$ compared with tertiary oximes seizure off group.

Figure 13: Anticonvulsant Dose Response Curves for DAM and MINA



Summary Of Anticonvulsant ED_{50} Doses

Model	Dose (mg/kg)
MINA + $ATSO_4$ (2.0 mg/kg)	36.65 (0.00 - 76.30)
DAM + $ATSO_4$ (2.0 mg/kg)	112.51 (83.78 - 178.87)

Figure 13. The dose-response curves for prevention/termination of seizures by MINA and DAM following sarin (2 x LD_{50}, sc) challenge in guinea pigs. All animals were pretreated with pyridostigmine bromide (0.026 mg/kg, im) at 30 min prior to and treated intramuscularly with atropine sulfate ($ATSO_4$; 2.0 mg/kg) and an oxime at one min after sarin challenge.