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The Tertiary Oxime Monoisonitrosoacetone Penetrates the Brain, Reactivates Inhibited Acetylcholinesterase, and Saves Lives following Lethal Sarin Intoxication in Guinea Pigs

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14. ABSTRACT

The brain is a critical target for the toxic action of organophosphorus (OP) inhibitors of acetylcholinesterase (AChE) such as the nerve agent sarin. However, current oxime antidotes such as 2-PAM only reactivate OP-inhibited AChE in peripheral tissues. Monoisonitrosoacetone (MINA) is a tertiary oxime that can enter the central nervous system (CNS). The purpose of this study was to investigate whether MINA would be beneficial as a supplemental oxime treatment in preventing lethality and reducing morbidity following lethal sarin intoxication. Guinea pigs were exposed to sarin and treated with atropine sulfate and 2-PAM at one min. Additional 2-PAM or MINA was administered at 1, 3, 5, 15, or 30 min after sarin exposure. Control animals received no additional treatment after one min. Survival and morbidity were assessed at 2 and 24 hours. AChE activity in brain and peripheral tissues was evaluated one hour after MINA or 2-PAM treatment. Microdialysis technique was used to determine partitioning of MINA into the brain. A liquid chromatograph-tandem mass spectrometric method was developed for the analysis of MINA in low volume microdialysis samples. MINA-treated animals exhibited significantly higher survival and lower morbidity compared to 2-PAM-treated animals. 2-PAM was significantly more effective in reactivating AChE in peripheral tissues, but only MINA reactivated inhibited AChE in the CNS. MINA was found in guinea pig brain microdialysate samples beginning at ~10 minutes after administration. The data strongly suggest that a centrally penetrating oxime could provide significant benefit as an adjunct to atropine and 2-PAM treatment for OP intoxication.

15. SUBJECT TERMS

Acetylcholinesterase; Blood brain barrier; In vivo microdialysis; Lethality; Liquid chromatography-tandem mass spectrometry; Monoisonitrosoacetone; Morbidity; Nerve agents; Organophosphorus compounds; Oxime; Pralidoxime chloride; Sarin.

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Abbreviations

ACh = acetylcholine

AChE = acetylcholinesterase

aCSF = artificial cerebrospinal fluid

BBB = blood-brain-barrier

ESI = electrospray ionization

GLM = generalized linear model

CNS = central nervous system

LC = liquid chromatography

LD_{50} = median lethal dose

MINA = monoisonitrosoacetone

MRM = multiple reaction monitoring

NA = nerve agent

OP = organophosphorus compound

2-PAM = pralidoxime chloride

QC = Quality control

RBC = red blood cell

SPE = solid phase extraction

MS/MS = tandem mass spectrometry

WB = whole blood.

Abstract

The brain is a critical target for the toxic action of organophosphorus (OP) inhibitors of acetylcholinesterase (AChE) such as the nerve agent sarin. However, current oxime antidotes such as 2-PAM only reactivate OP-inhibited AChE in peripheral tissues. Monoisonitrosoacetone (MINA) is a tertiary oxime that can enter the central nervous system (CNS). The purpose of this study was to investigate whether MINA would be beneficial as a supplemental oxime treatment in preventing lethality and reducing morbidity following lethal sarin intoxication. Guinea pigs were exposed to sarin and treated with atropine sulfate and 2-PAM at one min. Additional 2-PAM or MINA was administered at 1, 3, 5, 15, or 30 min after sarin exposure. Control animals received no additional treatment after one min. Survival and morbidity were assessed at 2 and 24 hours. AChE activity in brain and peripheral tissues was evaluated one hour after MINA or 2-PAM treatment. Microdialysis technique was used to determine partitioning of MINA into the brain. A liquid chromatograph-tandem mass spectrometric method was developed for the analysis of MINA in low volume microdialysis samples. MINA-treated animals exhibited significantly higher survival and lower morbidity compared to 2-PAM-treated animals. 2-PAM was significantly more effective in reactivating AChE in peripheral tissues, but only MINA reactivated inhibited AChE in the CNS. MINA was found in guinea pig brain microdialysate samples beginning at ~10 minutes after administration. The data strongly suggest that a centrally penetrating oxime could provide significant benefit as an adjunct to atropine and 2-PAM treatment for OP intoxication.

Introduction

Emergency treatment of acute poisoning with organophosphorus (OP) inhibitors of acetylcholinesterase (AChE), including chemical warfare nerve agents (NAs) and pesticides, consists of combined therapy with an anticholinergic such as atropine sulfate, an oxime such as pralidoxime chloride (2-PAM), and a benzodiazepine such as diazepam or midazolam. Atropine antagonizes the effects of excess acetylcholine (ACh) at postsynaptic muscarinic receptors, 2-PAM reactivates the activity of inhibited AChE by de-phosphorylating or de-phosphonylating the OP from the active site of the enzyme, and the benzodiazepine controls convulsions and seizure activities. Atropine and the benzodiazepine are supportive treatments, while the oxime, by reversing the inhibitory action of the OP on AChE, provides specific antidotal efficacy.

In the 1950s, before the discovery of the quaternary pyridinium oximes, a number of uncharged aliphatic oximes and hydroxamic acids were investigated as reactivators of AChE inhibited by OP compounds (Hobbiger, 1963). One of the most promising of these early oximes was monoisonitrosoacetone (MINA) (see Figure 1). MINA was reported to reactivate sarin-inhibited AChE and improve survival (Askew, 1956; 1957; Dultz et al., 1957; Rutland, 1958; Myers, 1959; Wills, 1959; Cohen and Wiersinga, 1960). Although MINA was a much less potent reactivator of sarin-inhibited AChE than 2-PAM, it was more effective than 2-PAM as a sole treatment for sarin intoxication because it entered the central nervous system (CNS) and reactivated brain AChE (Askew, 1957; Rutland, 1958). Recently, the capacity of MINA to reactivate sarin-inhibited AChE in the CNS has been confirmed (Skovira et al., 2010; Shih et al., 2012). Treatment of guinea pigs with MINA 15 minutes after sarin intoxication increased activities of AChE in brain 60 min after treatment by 15-30 percent. Previous experiments showed that using MINA as an immediate treatment alone or as an adjunct to atropine and 2-PAM was more effective against the lethal effects of sarin than was 2-PAM alone or combined atropine plus 2-PAM treatment in guinea pigs (Koplovitz and Schulz, 2007). Additionally, MINA was also able to terminate seizures and reduce the resulting neuropathology when administered after seizure onset. In some experiments, MINA terminated seizures and reduced neuropathology when administered as late as 20-40 minutes after seizure onset (Shih et al., 2010).

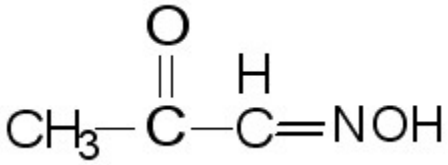


Figure 1. Chemical Structure of MINA

Acute, life-threatening OP poisoning requires prompt and aggressive treatment. The success of treatment depends on the OP compound, route and dose of exposure, the efficacy of the treatment drugs, and the condition of the victim at the time of treatment. In military NA exposure scenarios, treatment with atropine, 2-PAM and diazepam will most likely be given shortly after the onset of symptoms (Moore et al., 1995), because personnel carry first-aid autoinjectors containing these antidotes for emergency treatment. Additional treatment of severely intoxicated individuals with atropine, 2-PAM, and diazepam would almost certainly be given by medics or medical personnel at the time of or after evacuation to control symptoms. Unlike military forces, civilian NA casualties may experience significant delays before treatment with medical countermeasures can be initiated by first responders. This presents many challenges in providing effective life-saving treatment to civilians poisoned with these toxic chemicals. Since oximes are specific antidotes for OP intoxication, this study investigated whether a tertiary oxime such as MINA would be a more effective follow-on (delayed) oxime treatment than a peripherally acting oxime such as 2-PAM in preventing lethality and reducing morbidity following sarin intoxication. The capacity of MINA alone or in combination with 2-PAM to reactivate brain regional and peripheral AChE activity was evaluated. We also applied an *in vivo* microdialysis technique to evaluate the time-course entry of MINA into the CNS by developing a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for detecting a small quantity of MINA in microdialysate samples.

Materials and Methods

Animals: Adult male Hartley guinea pigs (Crl:(HA) BR COBS) weighing 250-400 g were purchased from Charles River Labs, Canada. They were housed in individual cages in temperature (21 ± 2°C) and humidity (50 ± 10%) controlled quarters that were maintained on a 12-hour light – dark schedule (with lights on at 0600 hour). Animals were maintained in quarantine for 5 days after arrival and prior to use in these experiments. Food and water were available *ad libitum*. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. It adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals,* National Research Council, published by the National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended. The animal protocol was approved by the Institute's Animal Care and Use Committee.

Chemicals: Sarin (>95% purity) was obtained from the US Army Combat Capabilities Development Command Chemical Biological Center, Aberdeen Proving Ground, Maryland. Gravimetric concentrations of approximately 2 mg/mL were prepared in normal saline and stored at -80°C until use. Atropine sulfate and pralidoxime (2-PAM) chloride were purchased from Spectrum Chemicals, New Brunswick, NJ. MINA (monoisonitrosoacetone) was purchased from Sigma-Aldrich, St. Louis, MO. Atropine sulfate, 2-PAM, and MINA were freshly prepared in normal saline at the time of use. Volume of injection was 0.5 mL/kg.

Experimental Design:

Survival Studies: Animals were challenged, subcutaneously (s.c.), with 129 µg/kg sarin between the shoulder blades. This dose is 3 times the 24-hour median lethal dose (LD_{50}) and 1.8 times more than the dose of sarin that will kill 100% of the animals. One minute after sarin challenge each animal was treated intramuscularly (i.m.) in a hind limb with 0.5 mg/kg atropine sulfate plus 25.0 mg/kg 2-PAM. The atropine dose was the human equivalent of 6 mg (3 autoinjectors) based on the body surface area formula guidance of the Food and Drug Administration (FDA, 2005). The 2-PAM dose was the human mg/kg equivalent of 1,800 mg (3 autoinjectors). Groups of animals (n = 10) received one additional oxime treatment consisting of either 2-PAM (25.0 mg/kg) or MINA (56.0 mg/kg) given at 1, 3, 5, 15 or 30 min after sarin challenge (10 groups total). This MINA dose given 15 min after sarin challenge results in an average of 15-20% reactivation of CNS AChE after 1 hour (Skovira et al., 2010; Shih et al., 2012). The additional oxime treatment was injected in the opposite hind limb. A control group of animals (n =20) received no additional oxime treatment. Morbidity and lethality were assessed at 2 and 24 hours after sarin challenge. Morbidity was assessed by generating a morbidity score for each surviving animal. The morbidity scores ranged from 0 to 4 and were defined as follows: 0 = normal looking animal; 1 = minor signs (e.g., rough coat, fine tremors) of intoxication but animals is upright and ambulatory; 2 = animal is upright but lethargic, will move with prodding and may have mild to moderate signs (tremors, ataxia) of intoxication; 3 = animal is prone, not ambulatory, conscious, able to support head and may have mild to moderate signs (tremors) of intoxication; and 4 = animal is prostrate, unable to support head, with mild to severe overt signs of intoxication. Animals that died were given a score of 5. Body weight at 24 hours after sarin exposure was determined and compared to pre-nerve agent challenge weights.

AChE Reactivation Studies: One to 3 days prior to the experiment, control blood samples (~0.5 mL) were drawn using the toenail clip method (Vallejo-Freire, 1951) and collected into a 1.0-mL microfuge tube containing 50 µL of heparin sodium (15 U/mL) to determine baseline AChE activity in whole blood (WB) and red blood cells (RBC). On the day of the study, guinea pigs were pretreated with atropine methyl nitrate (2.0 mg/kg, i.m.) 15 min prior to sarin exposure to minimize peripheral toxic effects (i.e., clear the airway from mucus secretion). Atropine methyl nitrate is a muscarinic receptor blocker that does not affect AChE activity. Animals were injected s.c. with either saline (0.5 mL/kg) or a 1.0 x LD_{50} dose of sarin (42.0 µg/kg). Fifteen minutes after sarin exposure, saline (0.5 mL/kg), 2-PAM (25.0 mg/kg), MINA (56.0 mg/kg) or 2-PAM (25.0 mg/kg) + MINA (56.0 mg/kg) was given i.m. Control animals received s.c. saline (no nerve agent) and i.m. saline (no oximes).

Sixty minutes after s.c. saline or sarin administration, the animals were deeply anesthetized with isoflurane and euthanized by decapitation. 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 (v:v) 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 packed 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 (v:v), while peripheral tissue samples were diluted 1:5 in 1% Triton-X100 solution and then homogenized. The homogenates were then centrifuged (31,000 x g at 4^{o}C; 20 min for brain and 30 min for peripheral tissues), and the supernatant was decanted and kept frozen at -80^{o}C until AChE activity and protein concentration analyses according to the methods described elsewhere (Shih et al. 2005).

***In Vivo* Brain Partitioning Studies:** Guinea pigs were anesthetized with isoflurane and surgically implanted with microdialysis guide cannulae using standard aseptic surgical techniques (O'Donnell et al., 2010b, Acon-Chen et al., 2016). Anesthetized guinea pigs were placed in a Kopf stereotaxic frame, and guide cannulae (15 mm in length; part no. MD2251; Bioanalytical Systems, W. Lafayette, IN) were targeted stereotaxically at the caudate nucleus (+11.4 mm anterior, +3.6 mm lateral, -4.6 mm ventral to the skull surface) based on the atlas of Luparello (1967) using a device-defined zero coordinate (Kopf electrode angle calibrator [model 935]). Guide cannulae were then anchored to the skulls with dental acrylic. Subjects were allowed to recover for 6 - 10 days before the day of the experiment.

On the day of the experiment, animals were randomly divided into 2 groups: one group received MINA alone (MINA group), and the other group received sarin and was treated with MINA (sarin+MINA group). Each animal was placed in an individual collection chamber (23 cm deep x 31 cm wide x 45 cm high). A brain microdialysis probe (0.34 mm OD, BAS BR-2 [part no. MD-2200]) was inserted into the guide cannula. The probe was perfused at a constant rate of 2.0 µL/min with an artificial cerebrospinal fluid (aCSF) containing 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1.2 mM Na_2HPO_4, 0.27 mM NaH_2PO_4, 74 mM glucose (pH 7.4), and a 2.5-hr normalization period was applied. After the normalization period, continuous collection of dialysate samples at 10-min intervals was initiated, resulting in a total volume of 20 µL in each vial (O'Donnell et al., 2010a, b, 2011). Dialysate samples were collected for 60 min (served as baseline) prior to injection of either saline (0.5 mL/kg, s.c.) or a 1.0 x LD_{50} dose of sarin (42.0 µg/kg, s.c.). Ten minutes after saline or sarin exposure MINA at 22.0, 56.0 or 139.0 mg/kg i.m. was administered. Sample collection was continued for 240 min after MINA administration. After collection, the dialysate samples were frozen and kept at –0°C until analysis for MINA by a newly developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (described below). After the experiment, animals were deeply anesthetized with pentobarbital sodium (75 - 100 mg/kg, intraperitoneally [i.p.]) and perfused with saline followed by 10% neutral buffered formalin for verification of probe location.

Development of an Analytical Method for MINA in Microdialysate Samples: *In vivo* microdialysis is a technique that can be applied to assess levels of endogenous/exogenous chemicals in peripheral tissues or in the brain. As such it provides a convenient tool to determine the ability of exogenously administered compounds to cross the blood-brain barrier (BBB) in live animals. However, one drawback of this procedure is that it provides low sample volume. Relatively small (<20 µL) sample volumes can pose problems for analytical method development if sample preparation involves numerous steps for isolation of the analyte. This necessitates the development of analytical methods to identify and quantify relevant compounds at the appropriate levels. We applied a commercially available solid phase extraction (SPE)

technology called micro-elution (Waters, Milford, MA), which eliminates the need for evaporation and reconstitution steps (Grumbach et al., 2004). The micro-elution SPE technique was optimized for sample clean-up, followed by LC-MS/MS for the identification and quantification of MINA in aCSF. Subsequently, this method was applied to *in vivo* microdialysis samples obtained from guinea pigs, which had been intramuscularly dosed with three different dose levels of MINA, to determine its practicality.

Sample/standard preparation: Calibration curves were prepared by making serial dilutions of MINA in aCSF. Concentrations of matrix-matched standards ranged from 3.03 to 3100 ng/mL. Quality control (QC) samples were prepared at 18.2, 291, and 2330 ng/mL to evaluate analytical figures of merit. An internal standard (2,3-butanedione monoxime), added at a concentration of 84.7 ng/mL, was used to normalize samples and standards. Total sample volume, after internal standard addition, was 17 µL. Samples were then basified with ammonium hydroxide solution (5% v/v, 7.5 µL) before cleanup. Solid phase extraction (SPE) was performed on samples and standards using a µElution 96-well plate format (Waters, Milford, MA) with a weak cation exchange stationary phase, although the mechanism used for cleanup was reversed phase. The sorbent was conditioned with 200 µL deionized water, followed by sample loading, washed with 100 µL deionized water, and eluted with 125 µL 100% LC/MS grade methanol.

LC-MS/MS method: An Agilent (Santa Clara, CA) 1260 Infinity binary HPLC pump equipped with an autosampler was used for online separation. The chromatographic method was a simple isocratic run with 100% methanol as mobile phase, pumped at 250 µL/min for 4 minutes. This method was simply used to remove any residual interferents that may have been present in the samples. The stationary phase used for separation was a Phenomenex (Torrance, CA) Luna silica column (150 x 2 mm, 3 µm particle). A SCIEX (Foster City, CA) 4000 QTrap mass spectrometer with a Turbo V electrospray ionization (ESI) source was employed for compound detection, operated in multiple reaction monitoring (MRM) mode. The ESI source conditions were as follows: curtain gas = 20 psi, ionspray voltage = -4500 V, temperature = 600°C, nebulizing gas = 55 psi, drying gas = 25 psi, interface heater = on, collision gas = high. The vacuum region conditions were as follows: declustering potential = -40 V, entrance potential = -11 V. Two MRM transitions were monitored for compound identification and quantification: *m/z* 86 → 59 (collision potential = -12 V, collision cell exit potential = -23 V) and *m/z* 86 → 41 (collision potential = -42 V, collision cell exit potential = -5 V). The MRM transition monitored for the internal standard was *m/z* 101 → 59 (collision potential = -15 V, collision cell exit potential = -9 V). Data were processed using Analyst software version 1.6.0 (SCIEX, Foster City, CA). To extend the dynamic range of the method, a quadratic fit was applied to calibration curves with no weighting. A semi-arbitrary upper limit of quantification was set at a level wherein precision and accuracy data remained acceptable experimentally.

Precision and accuracy: Both inter-day and intra-day precision and accuracy of the analytical method were investigated. Quality control (QC) samples were prepared in aCSF for each study on the day of analysis at three different concentrations: QC-high (QCH) at 2330 ng/mL, QC-middle (QCM) at 291 ng/mL, and QC-low (QCL) at 18.2 ng/mL. Calibration curves were also prepared, as described above, on the day of analysis. For inter-day precision and

accuracy, one calibration curve and one replicate of each QC sample were prepared and analyzed each day for five consecutive days. For intra-day precision and accuracy, one calibration curve and five replicates of each QC sample were analyzed in the same day.

The LC-MS/MS method was applied to the analysis of brain microdialysate samples in animals receiving intramuscular doses of MINA (22.0, 56.0, or 139 mg/kg) alone or following exposure to sarin. Data were processed using Analyst software version 1.6.0 (SCIEX, Foster City, CA), and MINA concentrations in each of the dialysate samples were calculated. Figures were created in GraphPad Prism software version 6 (San Diego, CA).

Data Analysis:

Survival Studies: The 2- and 24-hour lethality proportions for the treatment groups and various treatment times were compared using the Marascuillo procedure for multiple proportions (Marascuillo, 1966). A generalized linear model (GLM) was used to analyze the morbidity scores and changes in weight (McCulloch, 2001). An animal that died was given a morbidity score of 5 to maintain balance in the analysis. A two-factor GLM was used to compare the atropine sulfate/2-PAM/2-PAM- and atropine sulfate/2-PAM/MINA-treated groups and the times administered with respect to morbidity scores at 2 and 24 hours and weight change at 24 hours. This was followed by a one-factor GLM comparing all treatment groups (treatment at each time was considered a unique group) for morbidity scores and percent weight change. Morbidity scores had an ordinal response and used a multinomal distribution and cumulative logit link function in the GLM. Percent weight change used a normal distribution and an identity link function in the GLM. SPSS version 20 was used for the GLM analyses.

AChE Reactivation Studies: AChE activity was initially expressed as µmol substrate hydrolysed/mL/min for RBC and WB and then converted to percentage of the individual animal's baseline AChE value that was obtained one to three days prior to the day of experiment. In peripheral tissues and brain regions, the AChE activity was initially expressed as µmol substrate hydrolyzed/g protein/min and then expressed as percentage of the saline-treated control AChE value obtained on the day of experiment. The enzymatic activities of the treatment groups were then expressed as percentage of the saline/saline control group (mean ± SEM % of control value). Statistical analysis of enzymatic activities was performed using a one-way ANOVA to compare across treatments. A Tukey's test was used for multiple comparisons. Statistical significance was defined as $p < 0.05$. The significantly increased AChE activity (via reactivation) in oxime-treated groups was expressed as the % recovery of the saline/saline-treated control baseline activity above that of the remaining AChE activity in nerve agent-inhibited group (i.e., the % of control AChE activity in nerve agent-exposed and oxime-treated group minus the % of control AChE activity in nerve agent-exposed and saline-treated group).

Analytical Method Precision and Accuracy: The inter-day and intra-day data were analyzed using Analyst software version 1.6.0 (SCIEX, Foster City, CA). Experimentally derived sample concentrations were compared against known analyte concentrations to determine assay

accuracy. Precision was expressed as the percent coefficient of variation (% CV, also known as % relative standard deviation) for each experiment.

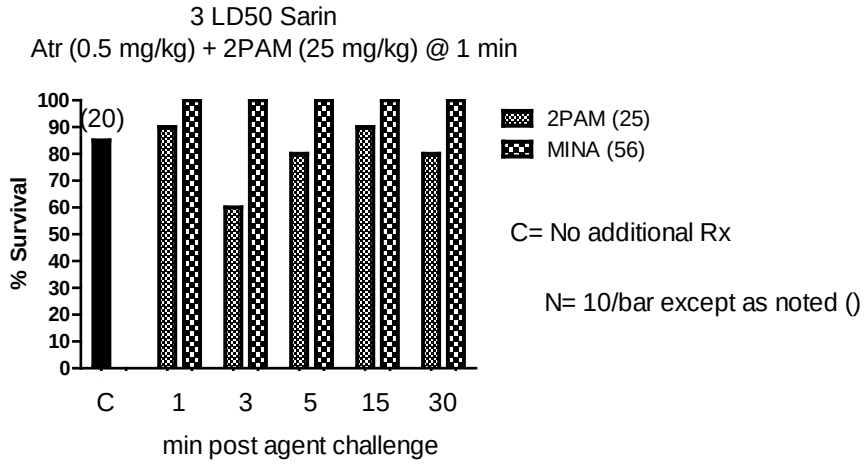
***In Vivo* Brain Partitioning Studies:** The area under the curve (AUC) data by group (MINA or sarin+MINA) and MINA dose did not have equal variances, which violates one of the assumptions for using a parametric analysis, e.g., ANOVA. Therefore, a Shapiro-Wilk test to determine if the data met the assumption of a normal distribution was performed. The raw AUC data was not normally distributed ($p<.0001$), but a log transformation of the AUC data did have a normal distribution ($p=.3383$) and also equal variances of the groups and MINA doses. Therefore, the comparison of groups and MINA doses was conducted on the log-transformed AUC data with a 2-factor ANOVA with interaction of group and MINA dose. No significant interaction of group and MINA dose was observed ($p=.3771$). However, AUCs for the MINA alone group were slightly higher across all MINA doses than the AUCs for the sarin+MINA group (borderline significance $p=.0517$). A highly significant difference ($p<.0001$) in AUCs among MINA doses was found. A Tukey's multiple comparison test of all pairs of MINA doses showed that AUC for 22 mg/kg MINA was significant different from the AUCs for 56 mg/kg ($p=.0002$) and 139 mg/kg ($p<.0001$) MINA, and AUC for 56 mg/kg MINA was significantly different from the AUC for 139 mg/kg MINA ($p=.0002$).

Results

Survival Studies: Figure 2 summarizes the lethality (or survival) results at 2 (Panel A) and 24 (Panel B) hours following lethal sarin exposure. Control animals challenged with 3 x LD_{50}s of sarin and treated with atropine + 2-PAM one min later exhibited a 15% (3/20) lethality rate at 2 hours (Panel A, dark bar on left) and a 60% (12/20) lethality rate at 24 hours (Panel B, dark bar on left). Administering one supplemental 2-PAM treatment at 1, 3, 5, 15 or 30 min after sarin challenge did not significantly improve survival rates at 2 or at 24 hours compared to the control group. In contrast, animals receiving one supplemental treatment with MINA at 1, 3, 5, 15, or 30 min, following the treatment at 1 min with atropine + 2-PAM, displayed significantly ($p<0.05$) less lethality than control animals 24 hours after sarin challenge. Of the 50 animals treated with MINA across all of the delayed treatment times, there was only 1 death within 24 hours in the group where MINA was given 1 min after sarin exposure (Panel B). In contrast, 22 of 50 (44%) animals treated with additional 2-PAM died within 24 hours.

Figure 2. Efficacy of additional 2-PAM or MINA therapy at different times following lethal sarin intoxication

A. 2-hour survival



B. 24-hour survival

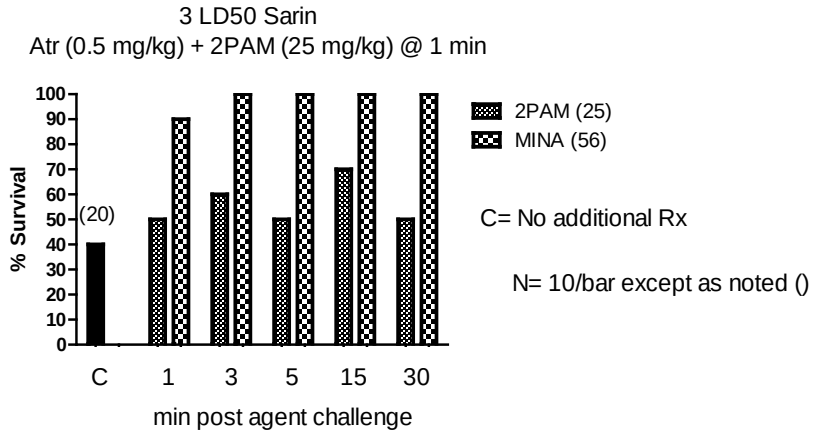


Figure 2. Effect of delayed supplemental oxime treatment on 2-hr (panel A) and 24-hr (panel B) survival following a 3 x LD_{50} s.c. sarin challenge in guinea pigs. Atropine sulfate (0.5 mg/kg) + 2-PAM (25 mg/kg) was administered i.m. 1 min after sarin challenge. Additional 2-PAM (25 mg/kg) or MINA (56 mg/kg) was administered i.m. at 1, 3, 5, 15 or 30 min after sarin challenge, while control (C; dark bar on left) animals received no additional oxime treatment.

Table 1 summarizes morbidity scores at 2 and 24 hours in animals treated with atropine + 2-PAM (control), atropine + 2-PAM + 2-PAM and atropine + 2-PAM + MINA. MINA-treated animals displayed significantly ($p<0.05$) reduced morbidity scores at 2 and 24 hours across all treatment times compared to either the atropine + 2-PAM (controls) or animals receiving supplemental 2-PAM. Most control animals and animals treated with one additional 2-PAM dose exhibited significantly ($p<0.05$) higher morbidity scores at 2 hours after sarin intoxication, and many were still severely intoxicated at 24 hours. No difference in morbidity scores was observed between the atropine + 2-PAM (control) group and the atropine + 2-PAM + 2-PAM group. In contrast, MINA-treated animals displayed lower morbidity scores at 2 hours and were closer to a normal score (0) at 24 hours.

Table 1: Morbidity scores at 2 and 24 hours after sarin challenge and delayed supplemental oxime treatment

Supplemental treatment/Time (min)	**Mean Morbidity Score (N)**					
	Treatment (mg/kg)					
	Atr (0.5) +2-PAM(25) No Supplement		Atr(0.5)+ 2-PAM (25) +2-PAM (25)		Atr (0.5)+ 2-PAM (25) + MINA (56)	
	2 hr	24 hr	2hr	24 hr	2 hr #	24 hr #
1	3.12 (17)	2.75 (8)	3.11 (9)	2.20 (5)	1.10* (10)	0.11* (9)
3			3.00 (6)	2.83 (6)	1.30* (10)	0.10* (10)
5			3.25 (8)	3.00 (5)	0.80* (10)	0.00* (10)
15			3.00 (9)	3.00 (7)	1.10* (10)	0.40* (10)
30			3.00 (8)	3.40 (5)	1.60* (10)	0.30* (10)

(N) = number of surviving animals.

* Significantly different from atropine (Atr) + 2-PAM no supplement (control) group at the corresponding observation time (2 or 24 hrs), $p<.05$.

Significantly different from the Atr + 2-PAM + 2-PAM group at the corresponding observation time (2 or 24 hrs) regardless of treatment time, $p<.05$.

Table 2 summarizes percentage of body weight loss at 24 hours in various treatment groups. Animals receiving one supplemental treatment with MINA at all treatment times showed significantly less body weight loss than either the atropine + 2-PAM (control) group or the animals treated with one additional 2-PAM dose.

Table 2: Body weight loss at 24 hours after sarin challenge and delayed supplemental oxime treatment

Supplemental treatment/ Time (min)	**Mean ± SD % Body Weight Loss @ 24 hr (N)**		
	Treatment (mg/kg)		
	Atr (0.5) +2-PAM(25) No Supplement	Atr(0.5)+ 2-PAM (25) +2-PAM (25)	Atr (0.5)+ 2-PAM (25) + MINA (56) #
1	19.0 ± 1.8(8)	19.4 ± 3.8(5)	3.2* ± 2.7(9)
3		16.0 ± 4.1(6)	6.2* ± 5.8(10)
5		16.8 ± 2.8(5)	3.7* ± 2.3(10)
15		16.1 ± 6.2(7)	5.3* ± 5.0(10)
30		19.0 ± 2.6(5)	5.3* ± 4.3(10)

(N) = number of surviving animals

* Significantly different from the Atr + 2-PAM no supplement (control) group, $p<.05$.

\# Significantly different from the Atr + 2-PAM + 2-PAM group regardless of treatment time, $p<.05$.

AChE reactivation Studies: The capacity of 2-PAM, MINA individually or a combination of 2-PAM and MINA to reactivate sarin-inhibited AChE activity in the tissues of guinea pig is shown in Table 3 and Figure 3. Following sarin exposure, the significant percentage of AChE recovery for 2-PAM (25 mg/kg, i.m.) treatment in the RBC and WB was 47.5% and 57.9 %, respectively, and in the diaphragm, heart, and skeletal muscle was 45.0%, 47.7%, and 27.7%, respectively. Thus, 2-PAM was highly effective in reactivating peripheral AChE. However, no significant AChE reactivation in the CNS was observed. MINA, however, was capable of reactivating AChE activity inhibited by nerve agents in a dose-dependent manner as described elsewhere (Skovira et al, 2010). In this study, MINA at 56.0 mg/kg significantly reactivated sarin-inhibited AChE in all six brain regions and spinal cord (with AChE recovery of 12.0% - 14.8%). MINA-induced recovery of AChE in diaphragm, heart, and skeletal muscle of 16.6%, 12.1%, and

10.9%, respectively, was not statistically significant. In the RBC and WB blood components, MINA produced significant AChE recovery of 11.9% and 8.7%, respectively. When 2-PAM and MINA were used as a combined treatment following sarin intoxication, the percent of AChE recovery of brain AChE activity was not different from that observed for MINA treatment alone. This was expected since 2-PAM rarely passes through the BBB to reactivate brain AChE-inhibited by sarin. In the blood and the 3 peripheral tissues, however, combined treatment with MINA and 2-PAM resulted in additive recovery of AChE activity by 59.1%, 65.7%, 51.6%, 64.9%, and 39.1% in RBC, WB, diaphragm, heart, and skeletal muscle, respectively.

***In Vivo* Brain Partitioning Studies:** A µSPE-LC/MS/MS method was developed and validated for the identification and quantification of the tertiary oxime MINA in aCSF. The relatively simple, high throughput sample preparation and extraction method allows for rapid screening of large sample sets of guinea pig brain microdialysate samples. This method eliminates the need for dilution or combination of time points to achieve a larger and useful sample volume, and also eliminates the need for evaporation and reconstitution steps. A wide dynamic range, which covers the expected microdialysate concentrations, was found to be accurately and reproducibly quantifiable in inter- and intra-day precision and accuracy studies (Table 4). Briefly, three QC concentrations were examined, representing arbitrarily chosen points across the full calibration range. These concentrations were 18.2, 291, and 2330 ng/mL, and are shown in Table 4 as QCL, QCM, and QCH. Five replicates were used for each QC level in both inter- and intra-day experiments. Inter- and intra-day precision and accuracy studies demonstrated accurate and repeatable results, corresponding to accuracies between 98 and 108% and % CVs between 2 and 9%. Acceptable values for accuracy at all three levels were considered to be ± 15% of the known value. Likewise, acceptable precision values at all three levels were considered to be ± 15% CV.

We observed a dose-dependent entry of MINA into the brain extracellular proper as a function of time at three doses (Figure 4). MINA was found in guinea pig brain microdialysate samples, beginning at ~10 minutes post-injection, was representative of all three dosages (22, 56, and 139 mg/kg), and reached the maximal detectable concentrations (C_{max}) of 880 ± 480, 2600 ± 1230, and 3360 ± 1840 ng/mL (error is expressed as ± one standard deviation), respectively, at a time point lying between 20 and 30 minutes after injection. These measurements were made in microdialysates from animals (n = 6) that received MINA with or without sarin exposure.

Table 3. Reactivation of AChE activity by 2-PAM, MINA or a combination of 2-PAM plus MINA in the tissues of guinea pigs following 1xLD_{50} sarin challenge

(A) Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, striatum)									
Agent	Treatment (mg/kg)	N	Brainstem	Cerebellum	Cortex	Hippocampus	Midbrain	Spinal Cord	Striatum
GB	Saline	13	10.76 ± 0.99	6.63 ± 1.00	7.57 ± 0.74	13.22 ± 1.41	9.60 ± 0.88	21.75 ± 2.69	12.41 ± 2.33
	2-PAM (25.0)	8	11.81 ± 0.35	8.00 ± 0.42	13.62 ± 0.59	15.51 ± 1.08	11.25 ± 0.35	19.21 ± 2.02	13.32 ± 1.37
	MINA (56.0)	8	23.87 ± 1.11*	19.88 ± 1.05*	21.37 ± 1.72*	25.85 ± 1.97*	22.82 ± 0.96*	33.72 ± 2.04*	27.24 ± 2.23*
	MINA (56.0) + 2-PAM (25.0)	8	21.16 ± 1.58*	18.79 ± 0.80*	22.95 ± 1.07*	26.47 ± 2.51*	20.31 ± 1.03*	28.57 ± 2.84	26.57 ± 2.13*

(B) Peripheral tissues (diaphragm, heart, skeletal muscle) and Blood (red blood cells and whole blood)							
Agent	Treatment (mg/kg)	N	Diaphragm	Heart	Skeletal Muscle	Red Blood Cells	Whole Blood
GB	Saline	13	21.85 ± 1.38	11.26 ± 0.57	32.08 ± 3.73	7.93 ± 1.08	6.91 ± 0.53
	2-PAM (25.0)	8	66.84 ± 3.18*	58.97 ± 3.63*	59.82 ± 3.53*	55.44 ± 2.60*	64.85 ± 1.46*
	MINA (56.0)	8	38.42 ± 1.89	23.36 ± 1.35	43.02 ± 3.02	19.85 ± 1.80*	15.57 ± 0.70*
	MINA (56.0) + 2-PAM (25.0)	8	73.48 ± 4.18*	76.19 ± 3.97*	71.21 ± 3.66*	67.01 ± 3.18*	72.64 ± 2.27*

Effects of nerve agent sarin (GB) and oxime (2-PAM and MINA) treatments on brain regions (A) and peripheral tissue and blood (B) AChE activity in the guinea pig. Animals were treated with atropine methyl nitrate (2.0 mg/kg, im) 15 min prior to a 1.0 x LD_{50} subcutaneous dose of GB. Oxime treatment was given intramuscularly 15 min after GB challenge. Samples were collected at 45 min after oxime treatment. AChE activity in brain and peripheral tissues was expressed as percentage of saline/saline control AChE activity for each tissue. AChE activity in red blood cell (RBC) and whole blood (WB) was expressed as percentage of individual baseline AChE activity that was obtained 1–3 days prior to the experiment. AChE activity was expressed as mean ± SEM (% of control).

*Statistically different ($p<0.05$) from GB/saline group.

Figure 3. AChE reactivation by 2-PAM and MINA following sarin exposure

A

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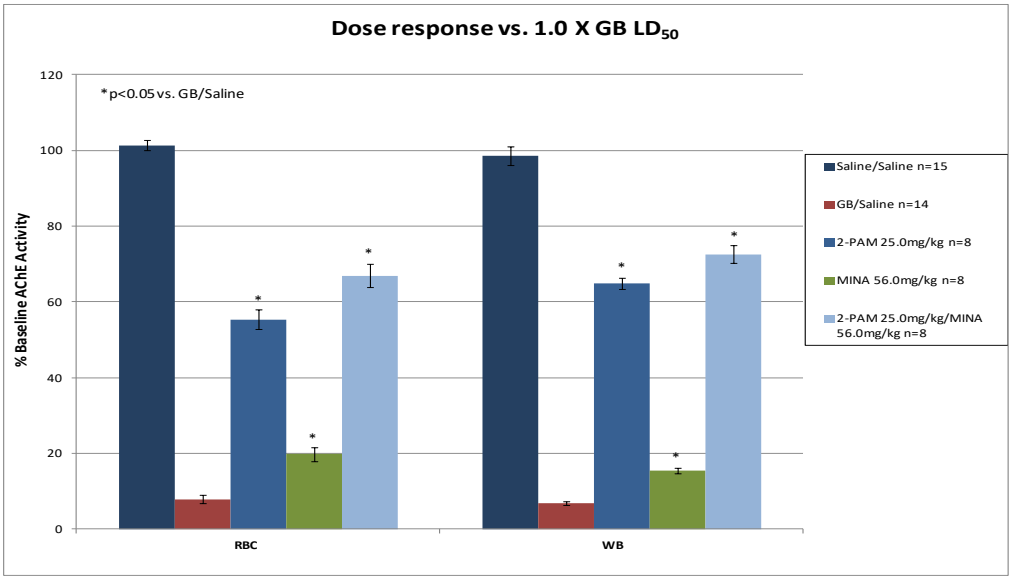

B.

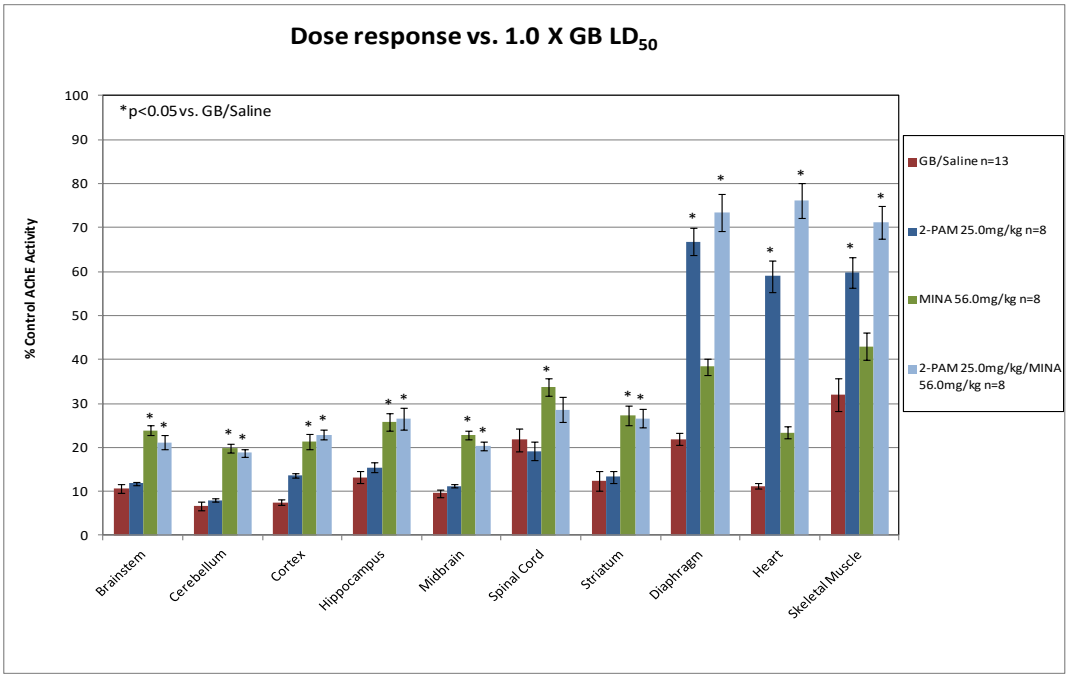

Figure 3: Effects of nerve agent sarin (GB) and oxime (2-PAM and MINA) treatments on brain regions (A) and peripheral tissue and blood (B) AChE activity in the guinea pig. Animals were treated with atropine methyl nitrate (2.0 mg/kg, im) 15 min prior to a 1.0 x LD_{50} subcutaneous dose of GB. Oxime treatment was given intramuscularly 15 min after GB challenge. Samples were collected at 45 min after oxime treatment. AChE activity in brain and peripheral tissues was expressed as percentage of saline/saline control AChE activity for each tissue. AChE activity in red blood cell (RBC) and whole blood (WB) was expressed as percentage of individual baseline AChE activity that was obtained 1–3 days prior to the experiment. AChE activity was expressed as mean ± SEM (% of control).

Table 4A. Interday precision and accuracy

	QCL (18.2 ng/mL)	QCM (291 ng/mL)	QCH (2330 ng/mL)
Interday 1	19.7	302	2280
Interday 2	22.7	290	2640
Interday 3	18.7	288	2320
Interday 4	18.5	308	2260
Interday 5	18.3	282	2320
Mean	19.6	294	2364
Std. Dev.	1.83	10.68	156
%CV	9.3%	3.63%	6.62%
Accuracy	108%	101%	101%

Interday variability was determined by examining three QC samples (18.2, 291 and 2330 ng/ml) over the course of five days. Samples were quantified by back calculating to a calibration curve run in parallel.

Table 4B. Intraday precision and accuracy

	QCL (18.2 ng/mL)	QCM (291 ng/mL)	QCH (2330 ng/mL)
Intraday 1	18.3	282	2320
Intraday 2	20.0	278	2150
Intraday 3	19.2	276	2430
Intraday 4	17.2	295	2450
Intraday 5	17.7	291	2560
Mean	18.5	284	2382
Std. Dev.	0.923	6.75	127
%CV	4.99%	2.37%	5.32%
Accuracy	102%	98%	102%

Intraday variability was determined by examining three QC samples (18.2, 291 and 2330 ng/ml) injected in replicate (n=5) in a single day. Samples were quantified by back calculating to a calibration curve run in parallel.

Figure 4. Time-course and dose-dependent penetration of MINA into the brain with or without sarin exposure

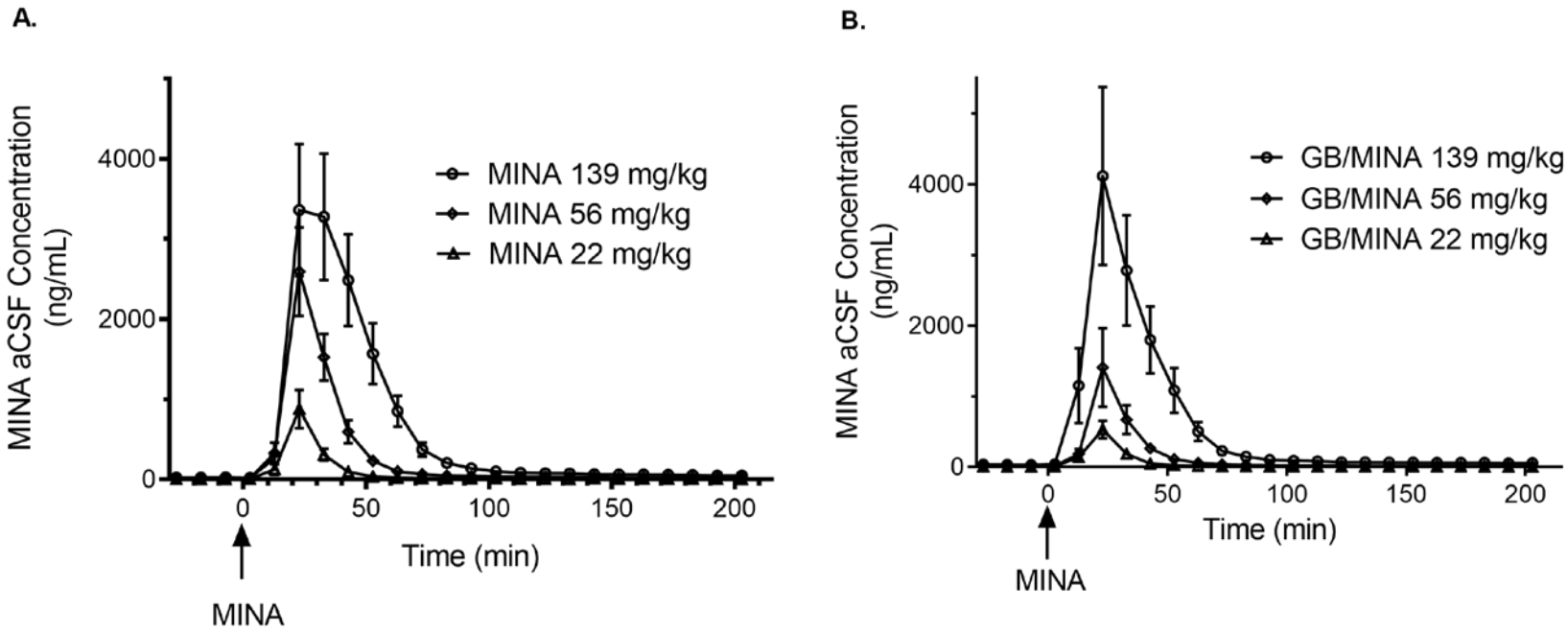

Figure 4: Guinea pigs were treated with MINA (22, 56 or 139 mg/kg, i.m.) alone (Panel A) or immediately following sarin exposure (Panel B). Extracellular samples were collected in the striatum by *in vivo* microdialysis procedure for 4 hours, and concentrations of MINA were analyzed by high performance liquid chromatography-tandem mass spectrometry.

Discussion

The CNS is a critical site of action for the toxic effects of OP compounds, including pesticides and nerve agents (NAs). Inhibition of AChE in the CNS and the subsequent increased amount of acetylcholine at CNS cholinergic synapses disrupt the cholinergic neuronal transmission, initiate convulsions and seizures, paralyze respiratory control centers, and induce neurotoxic mechanisms, all of which contribute to the incapacitating and lethal effects of OPs. While atropine and diazepam or midazolam can counteract some of these CNS effects through muscarinic receptor blockade and seizure control, currently available oximes (2-PAM, HI-6, obidoxime and TMB-4) provide little if any reactivation of inhibited AChE in the brain, largely because they are quaternary ammonium compounds with limited capacity to cross the BBB passively. The inability of currently available oximes to readily cross the BBB and reactivate OP-inhibited AChE in the CNS is a major limitation of current oxime treatment.

In the present study, a three-prong research approach was conducted. A dose-related penetration of the tertiary oxime MINA into the extracellular cerebrospinal fluid in the striatum was observed, using an *in vivo* microdialysis procedure. For this purpose, a micro-elution SPE procedure coupled to LC-MS/MS was developed to determine minute quantities of MINA in guinea pig brain microdialysates. MINA, when administered after sarin had maximally inhibited AChE in the tissues, reactivated sarin-inhibited AChE in both brain regions and peripheral tissues. In blood and peripheral tissues, the recovery of AChE activity was additive when MINA was combined with 2-PAM in the treatment regimen following sarin exposure. A supplemental treatment with MINA at various times up to 30 min following lethal sarin (3 x LD_{50}) exposure and immediate atropine plus 2-PAM treatment resulted in reduced 24-hour lethality and 2- and 24-hour morbidity. In contrast, supplemental treatment with the quaternary oxime 2-PAM at any delayed treatment time did not improve survival or morbidity beyond that provided by early emergency treatment alone.

In the 1950s many reports had shown that MINA, a simple, small aliphatic structure tertiary oxime, was able to reactivate sarin-inhibited AChE in the brain and save lives (Askew, 1956; 1957; Dultz et al., 1957; Rutland, 1958; Myers, 1959; Wills, 1959; Cohen and Wiersinga, 1960). Recently, the neurochemical and antidotal effects of MINA have been extended to other NAs in terms of AChE reactivation, protective efficacy, and anticonvulsant and neuroprotective effects (Shih et al., 2010a, 2010b, Skovira et al., 2010). Due to its capacity to reactivate sarin-inhibited AChE in the CNS, MINA was generally believed to readily pass through the BBB and into the CNS due to its tertiary ammonium structure. Here we used the microdialysis procedure to provide the first direct evidence that MINA indeed partitions into the CNS within 20 min after administration. Maximum concentrations reached after intramuscular administration were 880, 2600, and 3360 ng/mL, at 22.0, 56.0, and 139 mg/kg, respectively. All doses provided maximal concentrations at 35 min post-injection. MINA at 139 mg/kg sustained relatively high concentrations that were close to the maximum observed (between 2,800 and 3050 ng/ml) from 35 to 55 min after administration. Although, the lower two doses also reached maximum concentrations at 35 min, a relatively rapid decline was noted thereafter. The data suggest that the residence time in the brain was related to administered dose.

The recovery by MINA of NA-inhibited AChE activities, whether they are in the brain regions or the peripheral tissues, is in general very mild when compared with the recovery induced by 2-PAM (Shih et al., 2010; Skovira et al., 2010). In the present study, 2-PAM at a dose of 25.0 mg/kg reactivated sarin-inhibited AChE in the peripheral tissues by 28-48%, whereas MINA at 56.0 mg/kg only reactivated AChE in these same tissues by 11-16%. Even at the highest dose tested (139.0 mg/kg), it reactivated AChE in peripheral tissues by a 22% maximum (Skovira et al., 2010). When it reached the CNS, the same degree of AChE reactivation (12-15%) as in the periphery was observed. This degree of AChE reactivation (12-15%) in the CNS is probably the critical mass of the AChE enzyme that protects against the lethal consequences of sarin, since the addition of 2-PAM to MINA raised AChE activity to about 40-65% of control in the peripheral tissues, which is lower than that of the combination of 2-PAM plus 2-PAM therapy in this study. The supplemental addition of 2-PAM did not significantly enhance the survival rate at any time from 5 to 30 min after sarin exposure, but the MINA supplement did enhance the survival rate at any of these time points.

In summary, the major finding in this study was that supplemental treatment with the CNS-penetrating oxime MINA reduced 24-hour lethality and 2- and 24-hour morbidity after lethal sarin intoxication, even if MINA treatment was delayed by up to 30 min. In contrast, supplemental treatment with the quaternary oxime 2-PAM at any delayed treatment time did not improve survival or morbidity beyond that provided by early emergency treatment alone. The data strongly suggest that a centrally penetrating oxime could provide significant benefit as an adjunct treatment for NA intoxication. The increased survival and reduced morbidity resulting from supplemental MINA treatment was most likely due to AChE reactivation by MINA of sarin-inhibited AChE in the brain following its passing through the BBB.

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