HHS Public Access

Author manuscript

Toxicol Appl Pharmacol. Author manuscript; available in PMC 2024 April 01.

Published in final edited form as:

Toxicol Appl Pharmacol. 2023 April 01; 464: 116437. doi:10.1016/j.taap.2023.116437.

A Novel Genetically Modified Mouse Seizure Model for Evaluating Anticonvulsive and Neuroprotective Efficacy of an A_1 Adenosine Receptor Agonist following Soman Intoxication

Tsung-Ming Shih[**]

Neuroscience Department, Medical Toxicology Research Division, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400

Abstract

Recently a novel humanized mouse strain has been successfully generated, in which serum carboxylesterase (CES) knock out (KO) mice (Es1−/−) were further genetically modified by knocking in (KI), or adding, the gene that encodes the human form of acetylcholinesterase (AChE). The resulting human AChE KI and serum CES KO (or KIKO) mouse strain should not only exhibit organophosphorus nerve agent (NA) intoxication in a manner more similar to humans, but also display AChE-specific treatment responses more closely mimicking those of humans to facilitate data translation to pre-clinic trials. In this study, we utilized the KIKO mouse to develop a seizure model for NA medical countermeasure investigation, and then applied it to evaluate the anticonvulsant and neuroprotectant (A/N) efficacy of a specific A_1 adenosine receptor (A_1AR) agonist, N-bicyclo-(2.2.1)hept-2-yl-5'-chloro-5'deoxyadenosine (ENBA), which has been shown in a rat seizure model to be a potent A/N compound. Male mice surgically implanted with cortical electroencephalographic (EEG) electrodes a week earlier were pretreated with HI-6 and challenged with various doses (26 to 47 μg/kg, SC) of soman (GD) to determine a minimum effective dose (MED) that induced sustained *status epilepticus* (SSE) activity in 100% of animals while causing minimum lethality at 24 hours. The GD dose selected was then used to investigate the MED doses of ENBA when given either immediately following SSE initiation (similar to wartime military first aid application) or at 15 min after ongoing SSE seizure activity (applicable to civilian chemical attack emergency triage). The selected GD dose of 33 μg/kg (1.4 x LD_{50}) generated SSE in 100% of KIKO mice and produced only 30% mortality. ENBA at a dose as little as 10 mg/kg, IP, caused isoelectric EEG activity within minutes after administration in naïve un-exposed KIKO mice. The MED doses of ENBA to terminate GD-induced SSE activity were determined to be 10 and 15 mg/kg when treatment was given at the time of SSE onset and when seizure activity was ongoing for 15 min, respectively. These doses were much lower than in the

[**]Corresponding author at: 8350 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400, USA. Tel.: 410-436-3414; fax: 410-436-2690; tsungming.a.shih.civ@health.mil.

Declaration of Interest

The author reports no conflicts of interest or competing financial interests. During the last five years, the author has not appeared in any legal or regulatory proceedings related to the contents of the paper. The author alone is responsible for the content and writing of the paper.

non-genetically modified rat model, which required an ENBA dose of 60 mg/kg to terminate SSE in 100% GD-exposed rats. At MED doses, all mice survived for 24 hours, and no neuropathology was observed when the SSE was stopped. The findings confirmed that ENBA is a potent A/N for both immediate and delayed (i.e., dual purposed) therapy to victims of NA exposure and serves as a promising neuroprotective antidotal and adjunctive medical countermeasure candidate for pre-clinical research and development for human application.

Keywords

A_1 adenosine receptor agonist; Anticonvulsant; Electroencephalographic activity; Genetic mouse strain; Nerve agent; Neuropathology; Neuroprotection; Organophosphorus compound; Seizure activity; Soman; *Status epilepticus*

Introduction

Organophosphorus compounds (OPs) including chemical warfare nerve agents (NAs), such as soman (GD), sarin (GB) and VX, and pesticides are acetylcholinesterase (AChE) inhibitors. Inhibition of AChE by OPs can lead to a progression of toxic signs including hypersecretion, fasciculation, convulsion, seizure, respiratory distress, and death (Taylor 2011). Emergency treatment of acute poisoning with OP AChE inhibitors consists of combined therapy with an anticholinergic drug such as atropine sulfate, an oxime AChE reactivator such as pralidoxime chloride (2-PAM), and a benzodiazepine anticonvulsant such as diazepam or midazolam (Moore et al., 1995). Atropine antagonizes the effects of excess acetylcholine (ACh) at postsynaptic cholinergic 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 activity. In the case of military operations under wartime conditions, pyridostigmine bromide (PB), a reversible AChE inhibitor, may be prophylactically administered to warfighters to shield a fraction of peripheral AChE in anticipation of imminent NA attacks.

While current medical countermeasures (MCMs) described above reduce the toxicity and enhance the likelihood of survival after exposure to OP NAs, they are unable to terminate sustained *status epilepticus* (SSE), which subsequently caused severe neuropathology and debilitating behavioral deficits. Neuronal inhibitory drugs that block ACh receptors, increase the effect of γ-amino-butyric acid ($GABA_A$) receptors, or antagonize N-methyl-D-aspartic acid (NMDA) glutamatergic receptors are efficacious when administered immediately or quickly after NA exposure (McDonough and Shih, 1997; Shih et al., 1991a, 2003). However, after a period of SSE, the CNS often becomes refractory to these treatment strategies, and seizures cannot be readily terminated (Shih and McDonough, 1997; McDonough et al., 2010). Because current neurotransmission inhibitory- and oxime-based strategies are unable to control seizure activity when treatment is delayed, a new modality of ideal MCMs capable of terminating on-going SSE activity needs to be investigated and developed.

Toward that objective, our team has been investigating the adenosine (ADO) signaling pathway as a more effective inhibitory mechanism to prevent or terminate NA-induced seizure and subsequent neuropathology with promising results in rat models (Thomas et

al., 2019). Our *in vivo* ADO research initially utilized the A_1 ADO receptor (A_1AR) agonist N6-cyclopentaladenosine (CPA) to prevent seizure and neuropathology after GB and GD exposure (Thomas and Shih, 2014). While efficacious, CPA's anti-seizure dose produced marked side effects such as sedation, hypothermia, and hypotension, which may complicate future clinical drug transition. Consequently, we assessed two new A_1AR agonists, N-bicyclo-(2.2.1)hept-2-yl-5'-chloro-5'-deoxyadenosine (ENBA) and 2-Chloro-N6-cyclopentyladenosine (CCPA), with greater affinity for the A_1AR (Thomas et al., 2019). They have proven to be efficacious against NA-induced seizure with reduced side effects. When given one minute after GD-induced seizures, all A_1AR agonists proved to be highly efficacious in preventing seizure occurrence. ADO's seizure prevention translated to complete neuroprotection as assessed by histology. While CPA's side effects persisted to the 24-hour time point, rats receiving CCPA and ENBA had recovered consciousness, normal body temperatures, and baseline heart rates at 24 hours (Thomas et al., 2019).

In addition to preventing NA seizure onset, A_1AR agonists are capable of terminating SSE activity and limiting neuropathology when administered 15, 30, or 60 min after seizure onset (analogous to when treatment would occur after a mass NA casualty event). The most remarkable finding was that in GB-exposed rats, ENBA was able to limit the pathology to a score of 11.6 (out of 24 maximum) with a 60-min delay in treatment. Minimal pathology was observed with a 15- or 30-min treatment delay, indicating a strong neuroprotective capability for ENBA. A similar finding was observed in GD-exposed rats (Loughery et al., 2021).

Rat and other rodent models of NA exposure have traditionally been used for the identification of novel therapies that counteract the toxic effects of NA-induced SSE (Shih et al., 2003). However, the presence of plasma/serum carboxylesterase (CES) activity in such animals may confound results, because humans and nonhuman primates do not express serum CES (Li et al., 2005; Duysen et al., 2011). NA binds irreversibly to CES and reduces the systemic concentration of the OP agent that is freely available to inhibit AChE (Maxwell et al., 1987; Wiener and Hoffman 2004; Duysen et al., 2011), thereby providing some protection against lethal doses of certain OP compounds (i.e., scavenging effects), including NAs (Maxwell 1992; Sterri and Fonnum, 2015). This species difference in the expression of plasma CES has been eliminated by the development of a conditional CES gene knockout (Es1−/−) mouse strain that specifically lacks the activity of the enzyme in plasma, while maintaining its activity in other tissues (Duysen et al., 2011). Es1−/− mice, similarly to humans, lack plasma/serum CES (Duysen et al., 2011) and have a lower NA median lethal dose compared to wild-type mice (Duysen et al., 2011; Dunn et al., 2019; Kundrick et al., 2020) and, thus, may be a better model for human OP toxicity investigation.

Although AChE performs the same function in all animals, minor amino acid differences exist across species. These differences cause AChE to react differently to small molecules intended to restore its native activity after inhibition by OP NAs (Worek et al., 2002, 2008, 2010; Aurbek et al., 2006; Luo et al., 2007, 2008; Herkert et al., 2009; Cadieux et al., 2010). Most recently, a novel humanized mouse strain has been successfully generated by U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) scientists (Cerasoli et al., 2019; DeBus et al., 2019), in which serum CES knocked out (KO) mice (Es1−/−)

were cross-bred with a mouse strain (AChE knockin [KI]) in which the gene expressing AChE has been altered to express the amino acid sequence of the human form of the same protein. The resulting AChE KI/Es1 KO (or abbreviated as KIKO) mouse strain incorporates these modifications into a single model that addresses a major advance for the NA medical defense research laboratories. The lack of functional CES in these mice mirrors humans and nonhuman primates, neither of which express serum CES. Most other commonly used small animal models (rats, guinea pigs, other strains of mice) express CES, which directly contributes to those animals' resistance to OP toxicity (Maxwell et al., 1987; Jimmerson et al., 1989a, b; Due et al., 1993; Duysen et al., 2012; Sterri and Fonnum, 2015).

Thus, the production of human AChE in place of mouse AChE combined with the lack of CES in KIKO mice (Cerasoli et al., 2019) establishes an unique animal model (i.e., KIKO mouse strain) that should not only exhibit OP intoxication in a manner more similar to humans, but also display AChE-specific treatment responses more closely mimicking those of humans (DeBus et al., 2019; Reinhardt 2020; Marrero-Rosado et al., 2021). In this study, a KIKO mouse seizure model was developed for OP NA medical countermeasure (MCM) investigation, in particular with regards to anticonvulsant and neuroprotectant (A/N) drug research and development (R&D). It was then utilized as a NA seizure model to evaluate the A/N effects of the A_1AR agonist ENBA (see Figure 1a for chemical structure) following GD (see Figure 1b for chemical structure) exposure at the time of SSE onset and at a delayed time point (15 min after SSE onset) to simulate wartime military chemical warfare and civilian terrorist attack medical rescue operations, respectively, with the hope to facilitate the advanced translation of these results into clinical human application. Additionally, the data obtained will be used to compare with those effects obtained with rat studies (Thomas and Shih, 2014; Thomas et al., 2019; Loughery et al., 2021).

Methods and Materials

Subjects

Male KIKO (C57BL/6-Ces1c$^{tm1.1Loc}$AChE$^{tm1.1Loc}$/J) mice were obtained from the breeding colony at the USAMRICD (Aberdeen Proving Ground, MD) at 10 – 20 weeks of age (body weight: 25 – 35 g). They were housed in individual cages in temperature (21 ± 2°C)- and humidity (50 ± 20%)-controlled quarters that were maintained on a 12-h light-dark cycle (with lights on at 06:00 AM) and received *ad libitu*m access to food (Laboratory Rodent Diet Chow, LabDiet) and water except during experimental periods. Animals were acclimated for 3–10 days prior to surgery.

Materials

Saline (0.9% NaCl) injection, USP, was purchased from Quality Biological (Gaithersburg, MD). Atropine methyl nitrate (AMN) was purchased from Sigma-Aldrich (St. Louis, MO). Asoxime chloride (HI-6; 1-[[2-[(E)-hydroxyiminomethyl]pyridin-1-ium-1-yl]methoxymethyl] pyridin-1-ium-4-carboxamide dichloride) was synthesized by Kalexyn Medicinal Chemistry (Kalamazoo, MI). Soman (GD, pinacolyl methylphosphonofluoridate) was obtained from the U.S. Army Combat Capabilities Development Command Chemical Biological Center (Aberdeen Proving Ground, MD). N- Bicyclo(2.2.1)hept-2-

yl-5'-chloro-5'-deoxyadenosine (ENBA) was purchased from R&D Systems (Minneapolis, MN). IPTT-300 Temperature transponders were purchased from Biomedic Data Systems Inc. (Seaford, DE). The MouseOX Plus Pulse Oximeter was purchased from STARR Life Science Corporation (Oakmont, PA). ENBA was prepared in Multisol vehicle (48.5% distilled H_2O, 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol) on the day of treatment. Diluted preparations were sonicated until the drug was completely dissolved. GD, HI-6, and AMN were diluted in normal saline (0.9% NaCl). GD was injected subcutaneously (SC), and HI-6, AMN, and ENBA were injected intraperitoneally (IP). Injection volumes were 2.2 ml/kg for GD and saline, 4.2 ml/kg for HI-6, 5.3 ml/kg for AMN, and 2.0 – 10.0 ml/kg for ENBA.

Experimental Procedures

Approximately one week prior to experimentation, KIKO mice were surgically prepared with the implantation of a temperature transponder for recording body temperature and cortical wire electrodes for recording brain electroencephalographic (EEG) activity and detecting seizure onset and termination. All surgical procedures used in these experiments were conducted as previously described (Thomas et al., 2019; Loughery et al., 2021; Meads et al., 2021).

On the experiment day, mice were randomly assigned to various experimental groups and placed in individual chambers for EEG recording (dimensions in cm: 23 L × 31 W × 45 H); their EEG activity was recorded using CED 1902 amplifiers and Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, UK). Baseline brain EEG activity was collected for a minimum of 30 minutes. During this time, baseline physiological readings (such as heart rate and body temperature) and neurobehavioral scores (such as general state, motor signs, righting reflex) were recorded. Heart rate was noninvasively monitored using the MouseOX Plus Pulse Oximeter.

Mice were then administered HI-6 (125 mg/kg, IP) at 30 minutes before exposure to GD or saline. One minute later, animals received an IP injection of 2 mg/kg atropine methyl nitrate (AMN) (Shih 1990; Shih et al., 1991b). The administration of HI-6 prior to and AMN immediately after GD exposure, both possessing quaternary ammonium structures, does not affect brain EEG seizure onset or severity; they are used to minimize peripheral cholinergic symptoms and promote survival. Additional doses of AMN (up to 8 mg/kg/mouse) were administered upon observation of peripheral symptoms (i.e., rhinorrhea, mucus secretions, salivation). This is in line with our previous HI-6-pretreated GD seizure rat models (Loughery et al., 2021; Meads et al., 2021).

Two types of experiments were conducted: (I) to determine a minimum dose of GD that would induce 100% EEG seizure activity in exposed KIKO mice while resulting in minimum lethality (<50%) at 24 hours for establishment of a seizure study model, and (II) to determine the minimum effective dose (MED) of ENBA that will stop EEG seizure activity in 100% of animals when treated either at the time of or 15 minutes after EEG seizure onset in this KIKO mouse seizure model.

I. Development of a KIKO mouse seizure model—In this experiment we took advantage of our rat seizure model for GD that we have been using for the past 3 decades to evaluate potential anticonvulsant drugs (Shih 1990; Shih et al., 1991b) to inform our new mouse model. In our previous experiences in developing a GD seizure model for anticonvulsant drug studies with the rat as a test subject, we observed that 1.0 × LD_{50} of GD did not induce EEG seizure activity in all the animals tested, although most of them did exhibit physical motor convulsive activity. Furthermore, we also observed that giving HI-6 30 min prior to GD challenge resulted in a better survival rate at the 24-hour end point when compared with giving it after GD exposure (Shih 1990). Consequently, in this experiment, HI-6 (125 mg/kg, IP) was given to the KIKO mice 30 min prior to GD challenge, with a starting GD dose of 1.2 x LD_{50}. This was done to ensure that sustained *status epilepticus* (SSE) would occur and that most animals would survive to 24 hours following GD exposure. An appropriate dosage of GD was selected from this experiment and then used in the following experiments to determine anticonvulsant activity of the ENBA in this KIKO mouse seizure model.

Groups of KIKO mice were utilized to identify a GD dose level that induces the maximum rate of seizure onset and maximum rate of survival in 24 hours. A range of GD doses (26, 30, 32, 35, 37, and 47 μg/kg, SC) equivalent to 1.2 x to 2.0 x LD_{50} was tested (Table 1). This dose range selection was based on our earlier study with rat as the test model (Shih 1990; Shih et al., 1991b). Seizure onset was determined by EEG activity and is operationally defined as the appearance of ≥30 sec of continuous high amplitude rhythmic spikes or sharp wave activity in the EEG (McCarren et al., 2018). Mice were left on EEG recording for twenty-four hours after GD exposure. Neurobehavioral responses were assessed using a functional observational battery (FOB) that evaluated the animal's righting reflex, startle reflex, and arousal, as well as the presence of toxic signs like convulsions (Thomas and Shih, 2014; Thomas et al., 2019). Measurements took place at 0, 4, 8, 15, 30, 45, and 60 minutes and thereafter at 1-hour increments for 5 hours following GD or saline exposure. At 24 hours post-exposure, the animals were given a final follow up of neurobehavioral measurement (FOB and toxic signs) before being deeply anesthetized and euthanized. The dose that produced the minimum lethality with maximum rate (i.e., 100%) of seizure occurrence was identified, selected, and utilized in all subsequent experiments involving this KIKO mouse seizure model. Overall, the doses of GD used for the minimum dose determination in this experiment were from 26 (1.2 x LD_{50}) up to 37 μg/kg, with an interval of 2 – 4 μg/kg (i.e., 0.1 x LD_{50}) between doses, which is equivalent to 0.06 – 0.12 μg total dose difference between 2 sequential doses of GD and is narrow enough for this purpose.

II. Determination of an anti-seizure minimum effective dose (MED) of ENBA—Our previous ADO research in rats demonstrated that incidence of seizure termination and time to neuronal suppression are dose dependent, and the time to isoelectric brain activity would plateau with increasing ADO doses (Thomas and Shih, 2014). We started from those effective dose levels for rats to determine a minimum effective dose (MED) of ENBA that would suppress brain SSE activity in 100% of KIKO mice when ENBA was given IP at either the time of seizure onset or 15 min after seizure onset. Groups of mice were

exposed to GD based on the established seizure model (from Experiment I describe above) or saline (sham exposure) and their brain activity was monitored via EEG. Varying ENBA dose levels were tested (Tables 2 – 4). The mouse's heart rate was noninvasively monitored using the MouseOX Plus Pulse Oximeter. The neurobehavioral responses were recorded at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 1-hour increments, for 5 hours after GD or saline exposure. Mice were left on EEG recording for 24 hours after GD or saline exposure. Twenty-four hours after exposure, a final neurobehavioral measurement (FOB and toxic signs) was taken. EEG recordings were analyzed with visual determination to quantify the time to seizure onset and complete neuronal suppression (i.e., isoelectric EEG); this required the consensus of two trained individuals following pre-determined criteria. The seizure termination was defined as the absence of continuous high amplitude rhythmic spike or sharp wave activity. Epileptiform activity had to remain suppressed throughout the entire recording period after drug administration to be considered terminated.

The first dose of ENBA tested after exposure to either GD or saline was 45 mg/kg (based on doses used in previous study with rats; Thomas and Shih, 2014). In GD-exposed groups, ENBA was administered at seizure onset or 15 min after seizure onset. In saline-exposed groups, it was given at an expected seizure onset time plus 15 min, based on the average GD-exposed group's onset time, after saline administration. The criteria for determining the ENBA dose as the MED or whether to step up or step down in dose level are as follows. Briefly, if treatment of 2 or 3 mice induced no seizure termination or isoelectric activity, the dose level was too low, and the dose was increased by one step in a fixed dose range. If treatment of 2 or more mice caused seizure termination or isoelectric activity, the dose was effective, and the next lower dose was then tested. If that lower dose level had already been tested and been shown to provide 100% seizure or normal brain wave suppression, the current dose level was then identified as the MED. Overall, the doses of ENBA used for MED determination in this experiment were from 45 to 5 mg/kg, with an interval of 5 mg/kg between doses, which is equivalent to 0.15 mg total dose difference between 2 sequential doses of ENBA and is narrow enough for this purpose.

Neuropathology

Twenty-four hours after saline or GD exposure and/or ENBA treatment and following final neurobehavioral measurements and body weight were taken, the survivors were deeply anesthetized and perfused. Their brains were extracted and prepared for histology with H&E staining. Six vulnerable brain regions associated with severe neurological deficits after GD exposure (i.e., cerebral cortex, amygdala, piriform cortex, dorsal and ventral hippocampus, and thalamus) were each evaluated by a trained pathologist who was unaware of treatment paradigm and scored using the established standard rubric for each brain region as described in detail elsewhere (McDonough et al. 1995; Meads et al., 2021; Loughery et al., 2021): 0 = No lesion; 1 = Minimal (1 – 10%); 2 = Mild (11 – 25%); 3 = Moderate (26 – 45%); and 4 = Severe (>45%). A total neuropathology score was then calculated and summed for each mouse from 0 (normal) to 24 (the most severe brain damage). Brain pathology for each dose level was compared among those obtained in saline-exposed or GD-exposed and/or ENBA-treated animals.

Data Analysis

Two types of data collection were evaluated based on established criteria: the first, to determine a minimum dose of GD that induced 100% EEG seizure activity in exposed KIKO mice while resulting in minimum lethality (<50%) at 24 hours for establishment of a seizure study model (Experiment I), and the second, to determine the MED of ENBA that would stop EEG seizure activity in 100% of animals when treated at time of *status epilepticus* or 15 minutes after SSE seizure onset in this KIKO mouse seizure model (Experiment II). Additionally, dose-response effects on latency to seizure onset, latency to awake, and duration of ENBA actions were analyzed by one-way ANOVA and followed by Tukey's multiple comparison test (Tables 1–5). Statistical significance is defined as $p \leq 0.05$. Prior to each ANOVA test, we verified normal distribution using a residuals QQ plot and validated equality of variance by Brown-Forsythe test, $p \geq 0.05$.

Results

I. Development of a KIKO mouse seizure model

The purpose of this initial experiment was to determine a minimum dose of GD that would induce 100% EEG seizure activity in exposed KIKO mice while resulting in minimum lethality (<50%) at 24 hours for establishment of a seizure study model. We applied the same treatment paradigm that was used for a rat seizure model (Shih, 1990; Shih et al., 1991). The dose effects of GD on EEG seizure incidents and onset time, 24-hour mortality rates and time, and neuropathology scores are shown on Table 1. GD at a dose of 1.2 x LD_{50} (i.e., 26 μg/kg) was able to generate sustained *status epilepticus* (SSE) in 90% of KIKO mice. Of the 10 mice, one didn't display EEG seizure activity, one had delayed seizure onset at 5.4 hours (326 min), and the remaining 8 mice had seizure onset time ranging from 5.4 to 17.0 min with an average of 12.5±1.5 (N=8) min (if all 9 animals were included 47.3±34.9 min). Doses of GD at or above 1.3 x LD_{50} (30 μg/kg) were required to produce SSE in all animals when HI-6 was administered prior to GD challenge. The time to SSE seizure onset among all 6 GD doses (26 – 37 μg/kg) was not statistically significant (p=0.77, one-way ANOVA). It is interesting to note that the onset of SSE was not straightforward but displayed a variety of wax and wane patterns at the outset of EEG seizure activity (Figure 2). As shown in Figure 2, some of the EEG seizure activity would start and last for a few seconds (< 30 seconds), then stop for few seconds, and then restart. This pattern could repeat several times before eventually becoming *status epileptics*, whereupon continued spiking activity ensued within a minute of two. The seizure activity was still visible at 24 hours after GD exposure, even though the frequency and amplitude were much reduced (Figure 3).

As expected, when the dose of GD increased, the rate of lethality increased (Table 1). The time to death of mice that expired spanned from 1 to 10 hours following GD exposure. There was, however, no GD dose-related effects on time of death. The neuropathology scores for the survivors who were subjected to 24-hour continuous SSE were high with an average of 20 out of 24 points maximum. One mouse challenged with 26 μg/kg GD who didn't display EEG seizure activity and another mouse challenged with 32 μg/kg who developed seizure activity some 10.3 hours after GD exposure had pathology scores of 0 and 1, respectively. When the dose of GD increased from 1.4 x to 1.5 x $LD_{50,}$ a notable reduction in 24-hour

survival rate was observed. A GD dose at 1.4 x LD_{50} yielded 33% mortality and at 1.5 x and 1.6 x LD_{50} yielded double (60%) mortality, while a 2.0 x LD_{50} dose yielded 100% mortality. Therefore, a dose of GD less than 1.5 x LD_{50} (35 μg/kg) was the selected choice for the model to meet the established selection criteria. Since one mouse developed seizure after a delay of 10.3 hours at 32 μg/kg, we chose the slightly higher dose of 33 μg/kg for the GD challenge for our model. Thus, our final KIKO mouse seizure model consisted of KIKO mice being given HI-6 (125 mg/kg, IP) 30 min prior to intoxication with 33 μg/kg, SC, of GD. This is the model we have used in all subsequent experiments.

II. Determination of an anti-seizure minimum effective dose of ENBA

a. ENBA treatment at time of EEG seizure onset.—In the military medical rescue operations of wartime chemical nerve agent exposure, an effective MCM/antidote for immediate care/therapy (e.g., first aid and buddy aid) is critical. The purpose of this experiment was to determine the MED of ENBA that would effectively terminate GD-induced SSE activity in 100% of KIKO mice when given immediately following the onset of seizure activity. We started from 45 mg/kg and went down the dosage scale at 5 mg/kg intervals at each step of testing. Table 2 shows the effects of ENBA at various test doses with regard to the time to EEG seizure termination and isoelectric state, duration of its anti-seizure action, 24-hour lethality, and neuropathology scores in survivors after 33 μg/kg of GD. All 42 mice (100%) exposed to 33 μg/kg GD developed SSE, as was expected from the previous experiment. The average time for GD to induce SSE was different at each ENBA dose groups (Table 2); it ranged from 2.3 to 16.0 min, with an overall mean time to onset of 8.9 ± 0.9 min. ENBA at doses from 45 to 10 mg/kg was capable of terminating SSE in 100% of animals, but at 5 mg/kg, only 2 out of 4 (50%) mice experienced seizure termination. Time to seizure termination following ENBA treatment was rapid in a range from immediate to 24.0 min and showed no dose-related difference. ENBA treatment produced rapid decrements of motor activity and subsequent sedation. Soon after seizure termination, the EEG waves became isoelectric in minutes (ranging from 0.4 to 89.6 min), and the latency to deep sedation was not dose dependent. It took several hours for the majority of animals (>83%) to return to the normal baseline EEG activity (i.e., in an awake state). The time for EEG activity recovery was dose related. On average, the high dose of 45 mg/kg had an effect for 17.4 hours, while the low dose of ENBA, 10 mg/kg, affected the EEG activity for 6.8 hours. The duration of action for ENBA, from its treatment to EEG seizure return, lasted for the entire 24-hour recording period for most doses from 45 to 10 mg/kg, only showing a trend of shorter duration (14.5 hours) at 5 mg/kg. The 24-hour mortality rate was low—about 10 % (4/42) overall—among all 8 ENBA doses tested. For all of the survivors at 24 hours, no brain pathology occurred when their seizure activity was terminated by the treatment of ENBA regardless of doses. The only survivor that did not have seizure termination was treated at an ENBA dose of 5 mg/kg and it had a neuropathology score of 20 (out of 24 points maximum); the other 2 survivors at this same dose that had their seizures terminate had score of 0. Based on these findings, when given at seizure onset, ENBA at a dose of 10 mg/kg, IP, is the minimum effective dose (MED) to terminate 100% GD-induced SSE activity in this KIKO mouse seizure model. In summary, at this dose, the time to seizure termination was on an average of 4.7 min and the latency to isoelectric state was 7.7 min. The latency for EEG to return to its normal state was 6.8

hours, the duration of action of ENBA's suppression of seizure activity was 21.8 hours, and all animals survived without having GD-induced neuropathology (Table 2).

We also tested the actions of ENBA in KIKO mice that received a sham saline exposure to serve as GD exposure controls. The main purpose was to determine the minimum dose of ENBA that would induce an EEG isoelectric state in 100% of control animals. We duplicated the dose selection of ENBA from the GD exposure experiment described above and started from 45 mg/kg and reduced the dosage at 5 mg/kg intervals for each step of testing. Table 3 shows the effects of ENBA alone at various test doses on the time/latency to changing EEG activity from a normal brain wave to isoelectric state, time to awake state, the duration of its sedative action, and 24-hour lethality and neuropathology scores. ENBA treatment produced rapid decrements of motor activity and sedation.

At doses from 45 to 10 mg/kg, it was capable of turning normal EEG activity into an isoelectric state in 100% of animals, but at 5 mg/kg, only in 2 out of 3 (67%) mice did EEG recordings become isoelectric. The time to the isoelectric state of deep sedation following ENBA treatment was rapid. For the majority of the animals (29/34), this occurred within 3 min, showing no dose-related pattern. Figure 4 shows a typical example of the effect of ENBA on the EEG of saline-exposed control mice. For doses from 45 to 30 mg/kg, the isoelectric EEG activity never recovered to baseline activity (or in an awake state) by the 24-hour study end point (Table 3). For doses of ENBA at 25 mg/kg and below, some animals started to display wakefulness and showed recovery of normal EEG activity in a dose-related manner. For example, at the 25 mg/kg dose, one of 5 animals recovered at 19.1 hours after treatment and at 10 mg/kg, 6 of the 8 animals recovered at 9.5 hours after treatment, while at 5 mg/kg, all 2 animals returned to wakefulness in 5.4 hours. A one-way ANOVA was used to analyze the dose effect of 5, 10, and 15 mg/kg on time to awake, but no significant difference was found among them. The duration of action for ENBA was defined as the time from treatment to the return of normal brain waves. This lasted for almost the entire 24-hour recording period for most doses from 45 to 20 mg/kg, showing a trend of shorter duration only at doses of 15 mg/kg and below. There was a significant difference on ENBA's duration of action for 5 doses between 5 – 25 mg/kg ($p \leq 0.05$) by one-way ANOVA, and Tukey's multiple comparison test showed only one significant difference between the 5 and 25 mg/kg doses. Thus, at 5 mg/kg, the entire duration of isoelectric EEG activity lasted for an average of 5.4 hours, which is significantly shorter than for those animals treated with ENBA at doses of 25 mg/kg and above. There was no mortality at 24 hours following any of the 9 doses of ENBA administered; thus, the KIKO mouse is able to tolerate ENBA doses at least up to 45 mg/kg. For all the survivors at 24 hours, no brain pathology was observed, showing no direct damaging effect on neurons by the application of ENBA. Based on these findings, ENBA at dose of 10 mg/kg, IP, was selected as the MED to induce an isoelectric state of deep sedation in the normal KIKO mouse. In summary, at this dose, the average time to isoelectric state was 7.2 min, for EEG to return to the awake state it was 9.5 hours, the duration of action of ENBA's suppression of brain EEG activity was 13.4 hours, and all animals survived without displaying any neuropathology (Table 3). This is the same MED dose that we selected for the KIKO mice that were exposed to a seizure-inducing dose of GD and treated at time of seizure onset.

b. ENBA treatment at 15 minutes after EEG seizure onset.—In the case of a civilian terrorist chemical NA attack, medical rescue operations would likely be carried out by first responders, and a delayed triage medical treatment is expected. Therefore, an efficacious MCM/antidote for delayed care/therapy (i.e., administered by first responders) is mandatory. The purpose of this experiment was to determine the MED of ENBA that would terminate GD-induced SSE activity in 100% of subjects when treatment was delayed for 15 minutes after seizure onset. Table 4 summarizes the effects of ENBA at several test doses, including the time to EEG seizure termination, duration of its anti-seizure action, 24-hour lethality, and neuropathology scores at the 24-hour time point when treatment occurs 15 minutes after SSE seizure onset following GD exposure. We started with an ENBA dose of 10 mg/kg, since that was the MED determined to be effective when treated at the time of seizure onset (from the previous experiment described above). However, at this dose, we observed seizure termination in only 2 of the 4 (50%) KIKO mice challenged with GD (33 μg/kg). We then tested the next higher dose of 15 mg/kg in 8 mice and were able to observe that it terminated seizure activity in all 8 animals at an average of 6.6 ± 3.2 min (Table 4). Figure 5 shows a typical example of EEG tracing of GD-induced SSE onset and termination by a dose of ENBA given at 15 minutes after seizure onset. Further dose testing at 20 mg/kg with 5 mice also displayed 100% (5/5) SSE activity termination. Thus, it solidified and confirmed that 15 mg/kg ENBA is the MED required for 100% seizure termination when SSE activity has been ongoing for 15 minutes. This MED is slightly higher than the MED dose (i.e., 10 mg/kg) required to terminate 100% seizure activity when ENBA is given at the beginning of seizure onset.

The mean time to SSE onset8 for these 3 doses (10, 15, and 20 mg/kg) and 17 mice was 9.1 ± 1.1 min. The duration of action for all 3 doses of ENBA, from its treatment to EEG seizure return, lasted for 24 hours (Table 4). Among these 3 doses tested, there was no dose-response effect on time to seizure termination and ENBA duration of action, and no mortality was observed. All survivors at 24 hours had minimum brain pathology when their seizure activity was terminated by treatment with ENBA, with the exception of one mouse at a dose of 10 mg/kg, whose seizure activity returned at 13 hours after suppression. This mouse had a neuropathological score of 4.0 (out of maximum score of 24). The 2 survivors that didn't experience seizure termination with an ENBA dose of 10 mg/kg had neuropathology scores of 16 and 21 points, respectively. Based on these findings, when treatment is required following a 15-min delay after seizure onset, ENBA at a dose of 15 mg/kg, IP, was selected as the MED to terminate GD-induced SSE in this KIKO mouse seizure model. In summary, at this dose, time to seizure termination was on an average of 6.6 min and latency to isoelectric state was 6.6 min. It took 16.1 hours for EEG to return to its normal state. The duration of action of ENBA's suppression of seizure activity was 24.2 hours, and all animals survived without having GD-induced neuropathology (Table 4).

c. Evaluation of the MED doses of ENBA—Among the three treatment conditions described above there appeared to be some differences in the duration of sedative effects of ENBA at respective MED doses. Table 5 summarizes and compares the effects of ENBA at the MED doses with regard to the following: time to EEG seizure termination, time to isoelectric EEG state, time to awake state, duration of its anti-seizure action, 24-hour

lethality, and neuropathology scores at the 24-hour time point when treatment occurs at SSE onset time and at 15 min after SSE seizure onset following GD exposure and in saline-exposed sham control KIKO mice. For the saline-exposed control group, the time to wakefulness was determined as the median time between the end of the isoelectric state and the onset of awake brain wave. For statistical analysis, a one-way ANOVA and Tukey multiple comparisons test were used for each of the six measured parameters, with only two parameters being significant among these three exposure/treatment groups. For time to wakefulness, there was a significant difference between all the 3 group means ($p<0.01$): specifically, the GD exposure and ENBA 15 mg/kg treatment group took a significantly longer time to wake up than did the GD exposure and ENBA 10 mg/kg treatment group, with $p<0.01$. This difference could possibly be due to the dose of ENBA (15 vs. 10 mg/kg) or timing of therapy (at 15 min after onset vs. at time of onset) or both. For the ENBA duration of action, there was a significant difference between all the 3 means ($p<0.01$): specifically, the saline exposure and ENBA 10 mg/kg treatment was significantly shorter than the GD exposure and either ENBA 10 or 15 mg/kg treatment ($p\leq 0.01$). This difference could possibly be due to the GD intoxication, the toxic effects of which could then enhance the inhibitory action of A_1AR agonists. Surprisingly, the time to seizure termination was not significantly different between the two GD-exposed groups when ENBA was given either at onset of SSE seizure or at 15 min after seizure onset (4.7±3.4 min vs. 6.6±3.2 min), indicating that ENBA's effectiveness was not altered by a delay in its application.

II. Neuropathology

Neuropathological damage was assessed in the three bregmas by a trained veterinary pathologist and scores determined for each of the six brain regions (amygdala, cerebral cortex, piriform cortex, thalamus, dorsal hippocampus, and ventral hippocampus) by the percentage of degeneration and necrosis of neurons in each region using an established rubric: 0 = no lesion; 1 = minimal (1–10%); 2 = mild (11–25%); 3 = moderate (26–45%); and 4 = severe (>45%) (McDonough et al., 1995; Meads et al., 2021; Loughery et al., 2021). Other neuropathologic changes such as neuropil vacuolation and gliosis were noted in the GD-exposed mice but not used to determine the damage severity. A total neuropathology score (0–24) was determined for each mouse by adding the scores from each of the six regions. The higher the total score the more severe the neuropathology (see Tables 1, 2, 3, 4 and 5). Generally, animals with neuropathological damage in one region had damage in all regions, though the hippocampus, was spared in some instances. The degree of damage across regions varied. When exposed to GD, ENBA treatment at either immediately or 15 min after SSE onset showed a total protection in neuropathology score (0.0 ± 0.0) compared to GD-exposed untreated control group (20.8 ± 0.3).

Typical examples of H&E-stained histology of 6 brain regions of male KIKO mice following GD exposure are shown against the GD-exposed and ENBA-treated normal brain regions in Figures 6 and 7 for comparison. Figure 6 displays a comparison of select brain regions (a-b. cerebral cortex, c-d. piriform cortex, and e-f. amygdala) between GD-exposed control mice and GD-exposed + ENBA-treated mice (H&E 10X), while Figure 7 displays select brain regions (a-b. thalamus, c-d. dorsal hippocampus, and e-f. ventral hippocampus) between GD-exposed control mice and GD-exposed + ENBA-treated mice. GD-exposed control

brain sections (a., c., and e.) have multifocally extensive neuronal necrosis and degeneration with neuropil vacuolation and GD-exposed + ENBA-treated brain sections (b., d., and f.) have undamaged neurons with multifocal areas of “dark neurons” (a handling artifact).

Discussion

Current NA medical countermeasures (MCMs), such as atropine sulfate, 2-PAM, and diazepam or midazolam, effectively mitigate peripheral symptoms; however, the brain is often unprotected after prolonged seizure activity. The longer the seizure activity progresses, the less anticonvulsive effectiveness diazepam and midazolam have (Niquet et al., 2016). Failure to terminate NA seizures results in severe brain neuropathology (Shih et al., 2003). Toward developing a critically needed neuroprotectant for NA intoxication, our team has been investigating A_1 adenosine receptor (A_1AR) stimulation as a novel mechanism to shutdown excitotoxicity and to prevent brain damage (Thomas and Shih, 2014, 2019; Beste et al., 2018; Thomas et al., 2019; Loughery et al., 2021; Meads et al., 2021). Adenosine (ADO) is a purine nucleoside that is released during normal metabolic activity and has profound inhibitory effects on neuronal activity and on cholinergic and glutamatergic release via the A_1AR (St Hilaire et al., 2009). We have shown that treatment with A_1AR agonists alone enhanced survival and effectively prevented seizure and neuropathology induced by soman (GD) and sarin (GB) when given acutely and, even more remarkably, could terminate NA-induced sustained seizure and limit brain damage when given 15, 30, or 60 minutes after seizure onset in a rat model (Loughery et al., 2021). Our overall research and development (R&D) goals are to fill the critical gap in current MCM anticonvulsant efficacy and establish a neuroprotective and life-saving comprehensive treatment regimen containing atropine sulfate, 2-PAM, midazolam, and an A_1AR agonist. We aim to accomplish this objective by assessing the clinically relevant regimen of an A_1AR agonist plus standard MCM delivered at both immediate and delayed time points, assessing long-term functional neuroprotection and behavioral recovery, determining if repeated therapy with this combination may eliminate neuropathology, and investigating the sex/gender and age differences in the efficacy of the A1AR agonists. For these studies, we’re using a newly developed humanized esterase KIKO mouse strain as a test model to advance the translation of these results for clinical assessments.

Recently USAMRICD scientists (Cerasoli et al., 2019; DeBus et al., 2019) created a new “humanized” mouse strain (called KIKO strain) that is capable of more accurately predicting both the human response to NA exposures and the effectiveness of MCMs used to treat NA poisoning. Because of this new mouse model, critical drug R&D knowledge could be gained faster and earlier than it is currently, which would enable researchers to focus on developing MCMs most likely to save human lives (Reinhardt 2020). Two specific features of the genetic modification in the KIKO mouse strain make it a powerful animal model for NA MCM investigations. The lack of functional mouse-exclusive serum CES in this strain mirrors humans and nonhuman primates, both of which do not express this enzyme. Most other commonly used small animal research models (i.e., rats, guinea pigs, and other strains of mice) express serum CES, which is known to directly contribute to those animals’ resistance to OP toxicity (Maxwell 1992; Maxwell et al., 1997; Sterri and Fonnum, 2015). Reports also showed that minor amino acid differences across species could cause the AChE

enzyme to react quite differently to small molecules intended to restore the native activity of NA-inhibited AChE. Thus, the production of AChE with the same amino acid sequence as in the human form of the enzyme in these KIKO mice presents a unique opportunity for this mouse strain to act as a model for the study of compounds which interact directly with AChE most representative to human responses. The availability of the KIKO mouse opens up the opportunity of translating results generated from experiments on mice to inform medical breakthroughs in humans (Reinhardt 2020; Marrero-Rosado et al., 2021). Our team has begun the initial step to establish a seizure studying model to test the anticonvulsant and neuroprotective (A/N) efficacy of an A_1AR agonist in the KIKO mouse strain and here is our first report.

We applied the previously established rat GD seizure paradigm (Shih 1990; Shih et al., 1991b) to the KIKO mouse with the following criteria: animal has to generate sustained *status epilepticus* (SSE) activity rapidly and survive for a period of ~24 hours to allow for subsequent brain neuropathology to develop and be detectable by histological staining methods. Consequently, in the preliminary experiment, HI-6 (125 mg/kg, IP) was given to the animals 30 min prior to a subcutaneous GD challenge and followed one min later with an administration of atropine methyl nitrate (AMN) to relieve peripheral side effects, such as rhinorrhea, mucus secretion, and salivation. This exposure and treatment paradigm has been routinely used with rats in our early anticonvulsant screening studies with reproducible results (Shih et al., 1986; 1991a, b; 1996). GD was chosen as the representative NA for the test because of the finding that it's the most difficult NA agent to treat due to rapid aging of its binding complex with AChE. Furthermore, a test compound found to be successful against GD intoxication was usually shown to be equally efficacious toward other NAs as well (McDonough et al., 1999; 2000; Shih et al., 2003). HI-6 and AMN, both charged quaternary compounds, were given to ensure survival long enough for the appropriate measurement of any A/N activity of the tested compounds. Because of rapid aging of the GD-AChE complex, HI-6 was given ahead in anticipation of the GD exposure. HI-6 by itself has been demonstrated to be effective in delaying the time to death without altering the time-course of CNS toxic signs in rats exposed to GD and provides a protective ratio of 2.5 (Shih et al., 1986). The protective effect of HI-6 is attributed to its ability to reactivate inhibited AChE activity in the periphery as well as to other mechanisms of action of H-oximes (Broomfield, 1981).

With this HI-6 pretreatment and AMN treatment regimen, the GD seizure model with KIKO mice was quickly developed. Our first goal was to determine a minimum dose of GD that would induce 100% EEG seizure activity in KIKO mice that resulted in minimum lethality (<50%) at 24 hours for the study model, starting with 1.2 x LD_{50} of GD and moving the exposure doses upward at an 0.1 x LD_{50} interval. At 1.2 x LD_{50} of GD (26 μg/kg) SSE seizure activity was observed in 90% of animals. At 1.4 x LD_{50} (32 μg/kg) all animals (10/10) displayed SSE activity with 33.3% lethality at 24 hours. Any GD dose higher than 1.4 x LD_{50} incurred a higher rate of lethality (≥60%). One mouse in the 1.4 x LD_{50} (32 μg/kg) group developed *status epilepticus* at about 10.3 hours (i.e., an outlier) after GD exposure, as compared with other 9 mice with an average of 12.2±2.0 minutes, and, therefore, the GD dose of 33 μg/kg was selected for the KIKO mouse model. In comparison with our rat seizure model, the dose of GD required was notably lower in the

KIKO mouse than in the rat (Shih 1990): 1.4 x LD_{50} verse 1.8 x LD_{50}, respectively. The difference could be attributed to the knockout of plasma CES in this genetic mouse strain and, thus, reduced the amount of GD required to induce the cholinergic toxic effects. Similar to in rats and guinea pigs (McDonough and Shih, 1993; Koplovitz and Skvorak, 1998; Shih and McDonough, 2000), the onset of SSE in KIKO mice was not straightforward but was displayed in a variety of wax and wane patterns at the outset of EEG seizure activity (Figure 2). The EEG seizure would start and last for a few seconds (< 30 seconds) and stop for several seconds, and then restart. This pattern could repeat several times before eventually becoming *status epilepticus* with high amplitude and spiking activity ensuing and continuing within minutes. Similar to in rats and guinea pigs, the seizure activities were still visible at 24 hours after GD exposure in the survivors, even though the frequency and spike amplitude were much reduced (McDonough and Shih, 1993). All survivors at 24 hours experienced brain pathology with a neuropathological score of 20 out of 24 points maximum (Table 1 and Figures 6 and 7). Neuroprotection by any testing compound could then be easily detected and assessed. Thus, KIKO mice pretreated with HI-6 and subsequently given GD (33 μg/kg, SC) were used as the animal model to evaluate the A/N effects of the A_1AR agonist ENBA in the absence of standard MCMs (atropine sulfate, 2-PAM and midazolam).

Our second goal was to determine the minimum effective dose (MED) of ENBA that would suppress saline-exposed baseline EEG activity or terminate GD-induced SSE activity in 100% of subjects when treatment at a preselected time, starting with 45 mg/kg and moving the doses downward at a 5 mg/kg (i.e., 0.15 mg total dose per animal) interval. ENBA alone exerted a potent sedative effect and loss of motor activity when given to the saline-exposed control (sham) KIKO mice. Minutes after ENBA administration, the brain EEG activity went into an isoelectric state in all animals tested from 45 to 10 mg/kg (Table 3), but at 5 mg/kg only 2 out of 3 (67%) mice experienced this reaction. The time to an isoelectric state of deep sedation was rapid and occurred within minutes, showing no dose-related pattern. For doses from 45 to 30 mg/kg, the isoelectric EEG activity never recovered to baseline activity (or in an awake state) by the 24-hour study endpoint. For doses of ENBA at 25 mg/kg and below, some animals started to display wakefulness and showed a recovery to normal EEG activity in a dose-related manner. At 5 mg/kg, all 2 animals returned to wakefulness in 5.4 hours. The duration of action for ENBA, from its treatment to EEG seizure return, lasted for almost the entire 24hour recording period for most doses from 45 to 20 mg/kg, showing a trend of shorter duration at doses of 15 mg/kg and below. At 5 mg/kg, the entire duration of isoelectric EEG activity lasted for an average of 5.4 hours, which is significantly shorter than in those animals that were treated with ENBA at doses of 25 mg/kg and above. Twenty-four hours after any of the 9 doses of ENBA, neither mortality nor observable brain pathology was noted. Thus, the KIKO mouse was able to tolerate ENBA doses at least up to 45 mg/kg, IP. Based on these findings, ENBA at dose of 10 mg/kg, IP, was identified as the MED to induce an isoelectric state in the normal KIKO mouse. At this dose, the duration of ENBA's sedative action lasted about 13 – 14 hours.

We investigated the MED of the A/N effects of ENBA under two scenarios using this newly selected KIKO mouse GD seizure model. In one case, we investigated the MED of ENBA when given at the beginning of the SSE activity (similar to wartime military first aid application) and in another case, when given at 15 minutes after ongoing SSE

(applicable to civilian chemical nerve agent attack emergency triage). An ENBA dose of 10 mg/kg successfully terminated early SSE activity, while 15 mg/kg was most effective for the 15-min delayed treatment at stopping EEG seizure activity in 100% of animals. While a higher therapeutic dose of ENBA was needed at the 15-min delayed time point compared to its administration at the onset of SSE activity, the difference in MED was small, from 10 to 15 mg/kg for the 15-min delayed administration. The significance of this difference is currently unknown. It has been shown that a protective post-ictal rise of endogenous adenosine is observed relying on the A_1AR activation and the A_1AR receptor activity is impacted by the epileptic state of SSE, reducing the A_1 specific signal (Boison, 2016; Purnell et al., 2021). It is, therefore, possible that this affected the dose increase by 5 mg/kg (e.g., from 10 to 15 mg/kg) when treatment time was delayed by 15 min after SSE onset. A molecular detection to confirm A_1 adenosine receptor may be warranted.

On the contrary, the time to seizure termination was not significantly different between the two GD-exposed groups when ENBA was given either at onset of SSE seizure or at 15 minutes after seizure onset (4.7±3.4 min vs. 6.6±3.2 min), indicating that the efficiency of ENBA's inhibitory action was not altered by a delay in its administration. This might provide an additional benefit for the victims who had an ongoing SSE activity for a protracted period of time (Loughery et al., 2021). When using benzodiazepine anticonvulsants, such as diazepam, for GD therapy, a time-dependent pharmacoresistance is a major therapeutic deficiency in SSE treatment (Shih et al., 1999; McDonough et al., 2010). As seizures continue, pharmacoresistance develops progressively (Niquet et al., 2016), and the antiseizure potency of benzodiazepines can decrease as much as 20-fold after 30 minutes of seizure activity (Kapur and Macdonald, 1997). This time-dependent pharmacoresistance has also been reported in therapy with phenytoin and barbiturates, but not with N-methyl-D-aspartate (NMDA) receptor blockers (Mazarati et al., 1998; Mazarati and Wasterlain, 1999).

The time-dependent diminished effectiveness of benzodiazepine anticonvulsants against GD-induced seizures had been shown in both rat and guinea pig study models (Shih et al., 1999; McDonough et al., 2010). This situation was observed to be more severe in rats than in guinea pigs. In our earlier anticonvulsant research investigation, we found that diazepam at 9 mg/kg could terminate ongoing seizures in rats when given 5 min after seizure onset; however, when diazepam treatment was delayed to 40 min after seizure onset, even 20 mg/kg of diazepam failed to stop GD-induced seizures (Shih et al., 1999). The results were similar to those reported in other models of *status epilepticus* that have used rats (Walton and Treiman, 1988; Jones et al., 2002). However, in guinea pigs, 10 mg/kg of diazepam was clearly effective in controlling seizure activity in a substantial percentage (57 – 42%) of the guinea pigs, even at the 40- through 80-min treatment delays, respectively (McDonough et al., 2010). Similarly, rats also became more rapidly resistant to the anticonvulsant effects of anticholinergic drugs to terminate NA-induced seizures than did guinea pigs (McDonough and Shih, 1993; McDonough et al., 2000; Shih et al., 2007). This suggests that the duration of seizure activity needed to develop pharmacoresistance may be progressively longer in more developed species, such as humans, providing some beneficial clinical applications.

Clinically, benzodiazepines are reported to lose anticonvulsant effectiveness the greater the delay between seizure onset and benzodiazepine treatment (Towne et al., 1994; Lowenstein

and Alldredge, 1998; Rice and DeLorenzo, 1999). The pharmcoresistant phenomena are most likely due to the trafficking and internalization of benzodiazepine receptors (Niquet et al., 2016). Unlike the benzodiazepines, adenosine signaling pathway receptor agonists appear to show only small or no time-related change in pharmacologic seizure suppressing effectiveness in the KIKO mouse or in the rat. We have observed in the rat GD seizure model that the same 60 mg/kg dose of ENBA was required to produce the anticonvulsant effectiveness whether the treatment was delayed for 15, 30, or up to 60 min after seizure onset (Loughery et al., 2021), whereas in the present study with the KIKO mouse, the increase in MED was from 10 to 15 mg/kg when treatment was delayed for 15 min after seizure onset. However, we have noticed that a huge dosage difference exists in the anticonvulsant MED between the KIKO mouse and the rat. In the KIKO mouse, the MED dose was 15 mg/kg, while in the rat, it was 60 mg/kg (Thomas et al., 2019). The significance of these 2 variations in dosage of ENBA is currently not understood, because the pharmacoresistant phenomena have not been fully characterized for A_1AR agonists in the literature. As was discussed earlier a protective post-ictal rise of endogenous adenosine was observed and the A1AR receptor activity was impacted by the epileptic state of SSE, reducing the A_1 specific signal (Boison, 2016; Purnell et al., 2021). It is, thus, possible that this affected the dose increase by 5 mg/kg (e.g., from 10 to 15) when treatment time was delayed. Moreover, our use of a genetically modified mouse strain that eliminates plasma CES and incorporates the human AChE gene might have modified the pharmacologic responses to this class of compounds.

Due to the time-sensitive deficiency, the benzodiazepine class of anticonvulsants (i.e., diazepam, midazolam) may not be suitable for the sole therapeutic treatment of OP NA exposures, especially when treatment is expected to be delayed, such as in a NA terrorist attack scenario. This fact, coupled with its lower effective dose requirement and quick antiseizure actions at all times of application, makes the A_1AR agonist an extremely attractive choice as a more versatile A/N antidote for seizure suppression following OP NA exposure. With the lower dose of ENBA, the inherent side effects from this A_1AR class of drugs, such as sedation, hypothermia and hypotension, can be expected to be milder and have a relatively rapid recovery time. Overall, in our investigations all A_1AR agonists (i.e., CPA, CCPA, and ENBA) proved to be highly efficacious in stopping ongoing seizure activity and preventing neuropathology follow NA intoxication. Our data suggested that among them ENBA therapy alone appeared to have the best recovery from consciousness, body temperatures, heart rates, and behaviors following GD exposure in rat test model (Thomas et al., 2019; Meads et al, 2021). The effectiveness of ENBA may be attributable to its highly specific A_1AR actions than those of CPA and CCPA. Even though the literature for ENBA is limited, the neuronal inhibitory actions of CPA and CCPAhave been very much researched and reported. The available literature of this class of compounds implies that ENBA might have actions other than direct agonism at adenosine receptors, which might be responsible for its effects noted within this report, such as reduction on cholinergic and glutamatergic neurotransmitter release via the A1AR (St Hilaire et al., 2009; Borea et al., 2018; Thomas and Shih, 2019), apoptosis and inflammatory action (Effendi et al., 2020), hypothermia (Thomas and Shih, 2019), competition against the NA (such as sarin and soman) for reversible binding of the active site of AChE (Beste et al., 2018), effect

on adenosine homeostasis (Purnell et al 2021, Liu et al 2019, Németh et al 2003, Pastor-Anglada and Perez-Torras 2018), and other adenosine receptor independent effects (Purnell et al., 2021; Boison 2016; Masino et al., 2011; Liu et al., 2019; Németh et al., 2003; Chen et al., 2019).

Our successful results so far, using an adenosine-based therapy with A_1AR agonist as an A/N compound against GD exposure, have been focused primarily on male rodent models. In view of the recent publication by Pierling et al. (2021), who provided direct evidence that there is a gender/sex difference in cerebral A_1AR availability in humans, an investigation with female animal model may be necessary for the efficacy of A_1AR agonists. It has been known for sometimes that there are gender/sex and age differences in the toxic effects of OP NAs and their therapeutics. A gender/sex and age comparisons are valuable for therapeutic R&D, as many other researchers have provided evidence in literatures to support gendered/sexed and aged response differences to many antidotal compounds against NAs (Smith et al., 2015; Wright et al., 2015; Borgus et al., 2020). However, such data on gender/sex differences in ENBA's (or any A_1AR agonists) efficacy against OP NAs are currently unavailable. Consequently, it has been in our plan to investigate in more details the impact and importance of the A_1AR agonists in this regard.

In view of the successful verification of the effectiveness of ENBA alone as an A/N treatment against GD in this humanized KIKO mouse model, we are ready to move forward to assess the clinically relevant regimen of an A_1AR agonist plus standard MCM at both immediate and delayed time points of therapy, to evaluate long-term functional neuroprotection and behavioral recovery, to determine if repeated therapy with this combination would totally eliminate neuropathology and lethality, and to investigate the sex/gender and age differences in the efficacy of the A1AR agonists in order to advance the translation of these results to clinical trials.

In summary, pre-clinical research to better anticipate human care for the treatment of OP NA intoxication is essential. We utilized a genetically modified mouse strain that lacks the mouse-exclusive serum CES gene and knocks-in the human AChE gene to develop a KIKO seizure model for the evaluation of the A/N efficacy of an A_1AR agonist, ENBA. In the first phase, male mice receiving 33 μg/kg (i.e., 1.4 x LD_{50}) of GD exhibited SSE 100% of the time with 30% mortality. This dose was selected for our seizure model. For the second phase, we identified 10 and 15 mg/kg ENBA, doses which were much lower than in the rat model of 60 mg/kg, to be the MEDs when given alone at either the beginning of the SSE activity (similar to wartime military first aid application) or 15 minutes after ongoing SSE (applicable to civilian chemical agent attack emergency triage), respectively. Additionally, at the MED dose, SSE was suppressed within minutes at both treatment time points, neuropathology remained absent, physical recovery was markedly enhanced, and survival improved following GD exposure. The findings confirm ENBA is a potent anticonvulsant for both immediate and delayed (i.e., dual purposed) therapy to male KIKO mice of NA intoxication and is a promising A/N candidate for further advanced preclinic antidotal and therapeutic research and development.

Acknowledgments

The author acknowledges the generous supply of KIKO mice from Dr. C. Linn Cadieux and her research team who breed the in-house colony at USAMRICD for this study, the neuropathological evaluation of brain samples and brain images provided by Dr. Kimberly A. Whitten, and the excellent technical supporting work of Ms. Cindy Acon-Chen, Ms. Zora-Maya Keith, and Ms. Crystal Emily Munoz. The research described was supported by interagency agreements (AOD21005-001-00000 and AOD22011-001-00000) between the NIH Office of the Director (OD) and the U.S. Army Medical Research Institute of Chemical Defense under the oversight of the Chemical Countermeasures Research Program (CCRP) within the Office of Biodefense Research (OBRS) at the National Institute of Allergy and Infectious Diseases/National Institutes of Health (NIAID/NIH). The views expressed are solely those of the authors and do not necessarily represent the official views of the CCRP, NIAID, NIH, HHS, USAMRICD or DoD.

The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the United States Army Medical Research Institute of Chemical Defense (USAMRICD), and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. The research described was supported by an interagency agreement (AOD21005-001-00000) between the NIH Office of the Director (OD) and the USAMRICD under the oversight of the Chemical Countermeasures Research Program (CCRP) within the Office of Biodefense Research (OBRS) at the National Institute of Allergy and Infectious Diseases/National Institutes of Health (NIAID/NIH). The views expressed are solely those of the authors and do not necessarily represent the official views of the CCRP, NIAID, NIH, HHS, USAMRICD or DoD.

Abbreviations:

A_1AR	A_1 adenosine receptor
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADO	Adenosine
AMN	Atropine methyl nitrate
A/N	anticonvulsant and neuroprotective
CES	Carboxylesterase
CPA	N6-cyclopentyladenosine
CCPA	2-Chloro-N6-cyclopentyladenosine
CNS	Central nervous system
EEG	Electroencephalogram
ENBA	N-bicyclo-(2.2.1)hept-2-yl-5’-chloro-5’-deoxyadenosine
FOB	Functional observational battery
GABA	γ-amino-butyric acid
GB	Sarin
GD	Soman
HI-6	Asoxime chloride

IP	Intraperitoneal injection
KIKO	Knock in and knock out
MCM	Medical countermeasure
MED	Minimum effective dose
NA	Nerve agent
NMDA	N-methyl-D-aspartic acid
OP	Organophosphorus compound
PB	Pyridostigmine bromide
R&D	Research and development
SC	Subcutaneous injection
SSE	Sustained *status epilepticus*

References

Aurbek N, Thiermann H, Szinicz L, Eyer P, and Worek F. (2006). Analysis of inhibition, reactivation and aging kinetics of highly toxic organophosphorus compounds with human and pig acetylcholinesterase. Toxicology, 224(1–2): 91–99. [PubMed: 16720069]

Beste A, Taylora DE, Shih T-M, and Thomas TP (2018). Mechanisms of acetylcholinesterase protection against sarin and soman by adenosine A1 receptor agonist N6-cyclopentyladenosine. Comput. Biol. Chem 75: 74–81. 10.1016/j.compbiolchem.2018.04.017. [PubMed: 29747078]

Borgus JR, Puthongkham P, and Venton BJ (2020). Complex sex and estrous cycle differences in spontaneous transient adenosine. J. Neurochem 153(2): 216–229. 10.1111/jnc.14981. [PubMed: 32040198]

Boison D. (2016). Adenosinergic signaling in epilepsy. Neuropharmacology 104: 131–139. 10.1016/j.neuropharm.2015.08.046. [PubMed: 26341819]

Borea PA, Gessi S, Merighi S, Vincenzi F, and Varani K. (2018). Pharmacology of adenosine receptors: The state of the art. Physiological Review 98(3): 1591–1625. 10.1152/physrev.00049.2017.

Broomfield CA (1981). Some proposals for the mechanisms of action of the H-oximes. In: Evaluation of H-Series Oximes: Proceedings of a Symposium, Edited by Broomfield CA, Hackley BE, Hahn FE, Lenz DE, and Maxwell DM USABML-SP-81–001. Aberdeen Proving Ground, MD: U.S. Army Biomedical Laboratory, ADA098806, pp. 35–53.

Cadieux CL, Broomfield CA, Kirkpatrick MG, Kazanski ME, Lenz DE, and Cerasoli DM (2010). Comparison of human and guinea pig acetylcholinesterase sequences and rates of oxime-assisted reactivation. Chem Biol Interact. 187(1–3): 229–233. [PubMed: 20433814]

Carlin JL, Jain S, Gizewski E, Wan TC, Tosh DK, Xiao C, Auchampach JA, Jacobson KA,, Gavrilova O, and Reitman ML (2017). Hypothermia in mouse is caused by adenosine A1 and A3 receptor agonists and AMP via three distinct mechanisms. Neuropharmacology. 114:101–113. 10.1016/j.neuropharm.2016.11.026. [PubMed: 27914963]

Cerasoli D, Matson E, Barry T, DeBus S, Hall N, Hamilton T, Zimmerman D, McDonough J, Shih T-M, Anderson D, and Cadieux CL (2019). A novel genetically modified mouse model for evaluating nerve agent countermeasures. In Proceedings of the 3rd International CBRNE Research & Innovation Conference, Nantes, France, on 20–23 May 2019.

Chen F, He X, Luan G, and Li T. (2019) Role of ONA Methylation and Adenosine in Ketogenic Diet for Pharmacoresistant Epilepsy: Focus on Epileptogenesis and Associated Comorbidities. Front. Neural 10: 119. 10.3389/fneur.2019.00119.

DeBus SJ, Matson EM, Barry TB, and Cadieux CL (2019). Characterization of nerve agent intoxication in a novel genetically modified mouse model. . In Proceedings of the 3rd International CBRNE Research & Innovation Conference, Nantes, France, on 20– 23 May 2019.

Due A, Trap HC, Van Der Wiel HJ, and Benschop HP (1993). Effect of pretreatment with CBDP on the toxicokinetics of soman sterioisomers in rats and guinea pigs. Arch Toxicol, 67(10): 706–711. [PubMed: 8135661]

Dunn EN, Ferrara-Bowens TM, Chachich ME, Honnold CL, Rothwell CC, Hoard-Fruchey HM, Lesyna CA, Johnson EA, Cerasoli DM, McDonough JH, and Cadieux CL (2019). Evaluating mice lacking serum carboxylesterase as a behavioral model for nerve agent intoxication. Toxicol Mech Methods. 28(8): 563–572.

Duysen EG, Cashman JR, Schopfer LM, Nachon F, Masson P, and Lockridge O. (2012). Differential sensitivity of plasma carboxylesterase-null mice to parathion, chlorpyrifos and chlorpyrifos oxon, but not to diazinon, dichlorvos, diisopropylfluorophosphate, cresyl saligenin phosphate, cyclosarin thiocholine, tabun thiocholine, and carbofuran. Chem Biol Interact. 195: 189–198. [PubMed: 22209767]

Duysen EG, Koentgen F, Williams GR, Timperley CM, Schopfer LM, Cerasoli DM and Lockridge O. (2011). Production of ES1 plasma carboxylesterase knockout mice for toxicity studies. Chem. Res. Toxicol 24: 1891–1898. [PubMed: 21875074]

Effendi WI, Nagano T, Kobayashi K, and Nishimura Y. (2020). Focusing on adenosine receptors as a potential targeted therapy in human diseases. Cells 9, 785. https://safe.menlosecurity.com/10.3390/cells9030785. [PubMed: 32213945]

Herkert NM, Lallement G, Clarençon, Thiermann H, and Worek F. (2009). Comparison of the oxime-induced reactivation of rhesus monkey, swine and guinea pig erythrocyte acetylcholinesterase following inhibition by sarin or paraoxon, using a perfusion model for the real-time determination of membrane-bound acetylcholinesterase activity. Toxicology, 258(2–3): 79–83. [PubMed: 19428926]

Jimmerson VR, Shih T-M, Maxwell DM, Kaminskis A, and Mailman RB (1989a). The Effect of 2-(o-Cresyl)-4H-1:3:2benzodioxaphosphorin-2-oxide on Tissue Cholinesterase and Caboxylesterase Activities of the Rat. Fundamental and Applied Toxicology, 13: 568–575. [PubMed: 2612789]

Jimmerson VR, Shih T-M, Maxwell DM, and Mailman RB (1989b). Cresylbenzodioxaphosphorin oxide pretreatment alters soman-induced toxicity and inhibition of tissue cholinesterase activity of the rat. Toxicol Lett, 48(1): 93–103. [PubMed: 2749782]

Jones DM, Esmaeil N, Maren S, and Macdonald RL (2002). Characterization of pharmacoresistance to benzodiazepines in the rat Li-pilocarpine model of status epilepticus. Epilepsy Res. 50: 301–312. [PubMed: 12200221]

Kapur J. and Macdonald RL (1997). Rapid seizure-induced reduction of benzodiazepine and Zn^{2+} sensitivity of hippocampal dentate granule cell GABAA receptors. J. Neurosci 17: 7532–7540. [PubMed: 9295398] [PubMed: 9295398]

Koplovitz I. and Skvorak JP {1998). Electrocorticographic changes during generalized convulsive status epilepticus in soman intoxicated rats. Epilepsy Res. 30: 159–164. [PubMed: 9600547]

Kundrick E, Marrero-Rosado B,Stone M, Schultz C, Walker K, Lee-Stubbs RB, de Araujo Furtado M, and Lumley LA (2020). Delayed midazolam dose effects against soman in male and female plasma carboxylesterase knockout mice. Ann. N.Y. Acad. Sci 1479(1): 94–107. [PubMed: 32027397]

Li B, Sedlacek M, Manoharan I, Boopathy R, Duysen EG, Masson P, and Lockridge O. (2005). Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. Biochem. Pharmacology, 70 (11): 1673–1684.

Liu YJ, Chen J, Li X, Zhou X, Hu YM, Chu SF, Peng Y, and Chen NH (2019). Research progress on adenosine in central nervous system diseases. CNS neuroscience & therapeutics 25(9), 899–910. 10.1111/cns.13190. [PubMed: 31334608]

Loughery T, Whitten K, Acon-Chen C, Shih T-M (2021). Intramuscularly administered A1 adenosine receptor agonists as delayed treatment for organophosphorus nerve agent-induced Status Epilepticus. Toxicology and Applied Pharmacology, 419, (2021) 115515.

Lowenstein DH and Alldredge BK (1998). Status epilepticus. NEJM 338: 970–976. [PubMed: 9521986]

Luo C, Tong M, Chilukuri N, Brecht K, Maxwell DM, and Saxena A. (2007). An in vitro comparative study on the reactivation of nerve agent-inhibited guinea pig and human acetylcholinesterases by oximes. Biochemistry, 46(42): 11771–11779. [PubMed: 17900152]

Luo C, Tong M, Maxwell DM, and Saxena A. (2008). Comparison of oxime reactivation and aging of nerve agent-inhibited monkey and human acetylcholinesterases. Chem Biol Interact, 175(1–3): 261–266. [PubMed: 18555982]

Marrero-Rosado BM, Stone M, de Araujo Furtado M, Schultz CR, Cadieux CL, and Lumley LA (2021). Novel genetically modified mouse model to assess soman-induced toxicity and medical countermeasure efficacy: human acetylcholinesterase knock- in serum carboxylesterase knockout mice. Int. J. Mol. Sci 2021, 22, 1893. 10.3390/ijms22041893. [PubMed: 33672922]

Masino SA, Li T, Theofilas P, Sandau US, Ruskin DN, Fredholm BB, Geiger JD, Aronica E, and Boison D. (2011). A ketogenic diet suppresses seizures in mice through adenosine A1 receptors. J Clin Inv. 121: 2679–2683.

Maxwell DM (1992). The specificity of carboxylesterase protection against the toxicity of organophosphorus compounds. Toxicol. Appl. Pharmacol 114: 306–312. [PubMed: 1609424]

Maxwell DM, Brecht KM, and O'Neill BL (1987). The effect of carboxylesterase inhibition on interspecies differences in soman toxicity. Toxicol. Lett 39: 35–42. [PubMed: 3672554]

Mazarati AM, Baldwin RA, Sankar R, and Wasterlain CG (1998). Time-dependent decrease in the effectiveness of antiepileptic drugs during the course of self-sustaining status epilepticus. Brain Res. 814:179–185. [PubMed: 9838100]. [PubMed: 9838100]

Mazarati AM and Wasterlain CG (1999). N-methyl-D-asparate receptor antagonists abolish the maintenance phase of selfsustaining status epilepticus in rat. Neurosci Lett. 265: 187–190. [PubMed:]. [PubMed: 10327162]

McCarren H, Arbutus J, Ardinger C, Dunn E, Jackson C, and McDonough J. (2018). Dexmedetomidine stops benzodiazepinerefractory nerve agent-induced status epilepticus. Epilepsy Res, 141(1): 1–12. [PubMed: 29414381]

McDonough JH, McMonagle J, Copeland T, Zoeffel D. and Shih T-M (1999). Comparative evaluation of benzodiazepines for control of soman-induced seizures. Arch. Toxicol, 73: 473–478. [PubMed: 10650919]

McDonough JH, Dochterman LW, Smith CD and Shih T-M (1995). Protection against nerve agent-induced neuropathology, but not cardiac pathology, is associated with the anticonvulsant action of the drug treatment. NeuroToxicology 15:123–132.

McDonough JH, McMonagle J. and Shih T-M (2010). Time-dependent reduction in the anticonvulsant effectiveness of diazepam against soman-induced seizures in guinea pigs. Drug Chem. Toxicol, 33(3): 279–283. [PubMed: 20429808]

McDonough JH and Shih T-M (1993). Pharmacological modulation of soman-induced seizures. Neurosci. Biobehav. Rev 17: 203–215. [PubMed: 8515903]

McDonough JH and Shih T-M (1997). Neuropharmacological Mechanisms of Nerve Agent-induced Seizure and Neuropathology. Neuroscience and Biobehavioral Reviews, 21(5), 559–579. [PubMed: 9353792]

McDonough JH, Zoeffel LD, McMonagle J, Copeland TL, Smith CD and Shih T-M (2000). Anticonvulsant treatment of nerve agent seizures: anticholinergics versus diazepam in soman-intoxicated guinea pigs. Epilepsy Res, 38: 1–14. [PubMed: 10604601]

Meads K, Thomas T, Langston J, Myers T, and Shih T-M (2021). Evaluation of adenosine A1 receptor agonists as neuroprotective countermeasures against Soman intoxication in rats. Toxicology and Applied Pharmacology, 416(2021), 1–10.

Moore DH, Clifford CB, Crawford IT, Cole GM, and Baggett JM (1995). Review of nerve agent inhibitors and reactivators of acetylcholinesterase. In Enzymes of Cholinesterase Family, ed. by Quinn DM, Balasubramanian ASDoctor BP and Taylor P, pp. 297–304. Plenum Press, New York.

Németh ZH, Leibovich SJ, Deitch EA, Sperlágh B, Virág L, Vizi ES, Szabó C, and Haskó G. (2003). Adenosine stimulates CREB activation in macrophages via a p38 MAPK-mediated mechanism. Biochemical and biophysical research communications, 312(4), 883–888. 10.1016/j.bbrc.2003.11.006. [PubMed: 14651954]

Niquet J, Baldwin R, Suchomelova L, Lumley L, Naylor D, Eavey R, and Wasterlain CG (2016). Benzodiazepine-refractory status epilepticus: pathophysiology and principles of treatment. Ann. N. Y. Acad. Sci 1378(1): 166–173. [PubMed: 27392038]

Pastor-Anglada M. and Pérez-Torras S. (2018). Who is who in adenosine transport. Frontiers in Pharmacology, 9: 627. 10.3389/fphar.2018.00627. [PubMed: 29962948]

Pierling AL, Elmenhorst E-M, Lange D, Hennecke E, Baur DM, Beer S, Kroll T, Neumaier B, Aeschbach D, Bauer A, Landolt H-P, and Elmenhorst D. (2021). Cerebral A1 adenosine receptor availability in female and male participants and its relationship to sleep. NeuroImage 245: 118695, 10.1016/j.neuroimage.2021.118695.

Purnell B, Murugan M, Jani R, and Boison D. (2021). The Good, the Bad, and the Deadly: Adenosinergic Mechanisms Underlying Sudden Unexpected Death in Epilepsy. Frontiers in Neuroscience. 15: 1–15.

Reinhardt BC (2020). Of mice and human. JSTO in the News, Vol. 10 (No. 5): 7–9, published by Defense Threat Reduction Agency, Fort Belvoir, VA, USA.

Rice AC and DeLorenzo RJ (1999). N-methyl-D-aspartate receptor activation regulates refractoriness of *status epilepticus* to diazepam. Neuroscience 93: 117–123. [PubMed: 10430476]

Shih T-M (1990). Anticonvulsant effects of doazepam & MK-801 in soman poisoning. Epilepsy Res, 7:105–116. [PubMed: 2289469]

Shih T-M, Duniho SM and McDonough JH (2003). Control of nerve agent-induced seizures if critical for neuroprotection and survival. Toxicol Appl Pharmacol. 188: 69–80. [PubMed: 12691725]

Shih T-M, Koviak TA, and Capacio BR (1991a). Anticonvulsants for poisoning by the organophosphorus compound soman: pharmacological mechanisms. Neurosci. Biobehav. Rev, 15: 349–362. [PubMed: 1683477]

Shih T-M and McDonough JH (1997). Neurochemical mechanisms in soman-induced seizures. J. Appl. Toxicol 17: 255–264. [PubMed: 9285539]

Shih T-M and McDonough JH (2000). Efficacy of biperiden and atropine as anticonvulsant treatment for organophosphorus nerve agent intoxication. Arch. Toxicol, 74: 165–172. [PubMed: 10877003]

Shih T-M, McDonough JH and Koplovitz I. (1996). Evaluation of anticonvulsant drugs for soman-induced seizure activity. J. Amer. Coll. Toxicol 15 (Suppl. 2): S43–S60.

Shih T-M, McDonough JH and Koplovitz I. (1999). Anticonvulsants for soman-induced seizure activity. J. Biomed. Sci 6: 86–96. [PubMed: 10087439]

Shih T-M, Whalley CE and Valdes JJ (1991b). A comparison of cholinergic effects of HI-6 and 2-PAM in soman poisoning. Toxicol. Lett 55: 131–147. [PubMed: 1998202]

Shih T-M, Whalley CE, Valdes JJ, Lundy PM and Lockwood PA (1986). Cholinergic effects of HI 6 in soman poisoning. In: Dynamics of Cholinergic Function, Ed. Hanin I, Plenum Press, New York, pp 767 779.

Smith CD, Wright LKM, Garcia GE, Lee RB, and Lumley L/A. (2015). Hormone-dependence of sarin lethality in rats: sex differences and stage of the estrous cycle. Toxicol Appl Pharmacol. 287(3): 253–257. 10.1016/j.taap.2015.06.010. [PubMed: 26079828]

St Hilaire C, Carroll SH, Chen H, and Ravid K. (2009). Mechanisms of induction of adenosine receptor genes and its functional significance. J. Cell Physiol 218: 35–44. [PubMed: 18767039]

Sterri SH and Fonnum F. (2015). The role of carboxylesterases in therapeutic intervention of nerve gases poisoning. In Handbook of Toxicology of Chemical Warfare Agents. Academic Press; pp. 1099–1106.

Taylor P. (2011). “Anticholinesterase Agents.” In Goodman & Gilman’s The Pharmacological Basis of Therapeutics. Brunton LL, Chabner B& Knollman B, Eds. New York: McGraw-Hill, pp. 239–254.

Thomas TP and Shih T-M (2014). The Neuroprotective benefits of central adenosine receptor stimulation in a soman nerve agent rat model. Toxicol. Mech. Methods, 24(6): 385–395. [PubMed: 24785252]

Thomas TP and Shih T-M (2019). The use of adenosine agonists to treat nerve agent-induced seizure and neuropathology. Biomedical Journal of Scientific and Technical Research (BJSTR), 17 (5): 13147–13154 (ISSN: 2574–1241; DOI: 10.26717/BJSTR.2019.17.003074.

Thomas TP, Wegener A, and Shih T-M (2019). In vivo evaluation of a1 adenosine agonists as novel anticonvulsant medical countermeasures to nerve agent intoxication in a rat soman seizure model. Neurotoxicity Research, 36(2), 323–333. [PubMed: 31069755]

Towne AR, Pellock JH, Ko D, and DeLorenzo RJ (1994). Determinants of mortality in *status epilepticus*: a retrospective study of 292 adult patients. Epilepsia 35: 27–34. [PubMed: 8112254]

Walton NY and Treiman DM (1988). Response of status epilep- ticus induced by lithium and pilocarpine to treatment with diazepam. Exp. Neurol 101:267–275. [PubMed: 3396644]

Wiener SW and Hoffman RS (2004). Nerve agents: a comprehensive review. J Intensive Care Med. 19: 22–37. [PubMed: 15035752]

Worek F, Reiter G, Eyer P. and Szinicz L. (2002). Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. Arch. Toxicol 76 (9): 523–529. [PubMed: 12242610]

Worek F, Aurbek N, Wetherell J, Pearce P, Mann T, and Thiermann H. (2008). Inhibition, reactivation and aging kinetics of highly toxic organophosphorus compounds: pig versus minipig acetylcholinesterase. Toxicology, 244(1): 35–41. [PubMed: 18054823]

Worek F, Wille T, Aurbek N, Eyer P, and Thiermann H. (2010). Reactivation of organophosphate-inhibited human, Cynomolgus monkey, swine and guinea pig acetylcholinesterase by MMB-4: a modified kinetic approach. Toxicol Appl Pharmacol, 249(3): 231–237. [PubMed: 20888357]

Wright LKM, Lee RB, Clarkson ED, and Lumley LA (2015). Female rats are less susceptible during puberty to the lethal effects of percutaneous exposure to VX. Toxicology Reports 10.1016/j.toxrep.2015.12.003.

Highlights

- Soman toxicity was tested in a genetically modified esterase (KIKO) mouse strain.
- Minimum dose of GD to induce sustained seizure (SSE) was evaluated in KIKO mice.
- Minimum efficacy doses (MEDs) of ENBA in stopping GD-induced SSE were evaluated.
- At MEDs the anti-seizure effects of ENBA lasted for more than 20 hours.
- Once seizure was terminated by ENBA no neuropathology was evident.

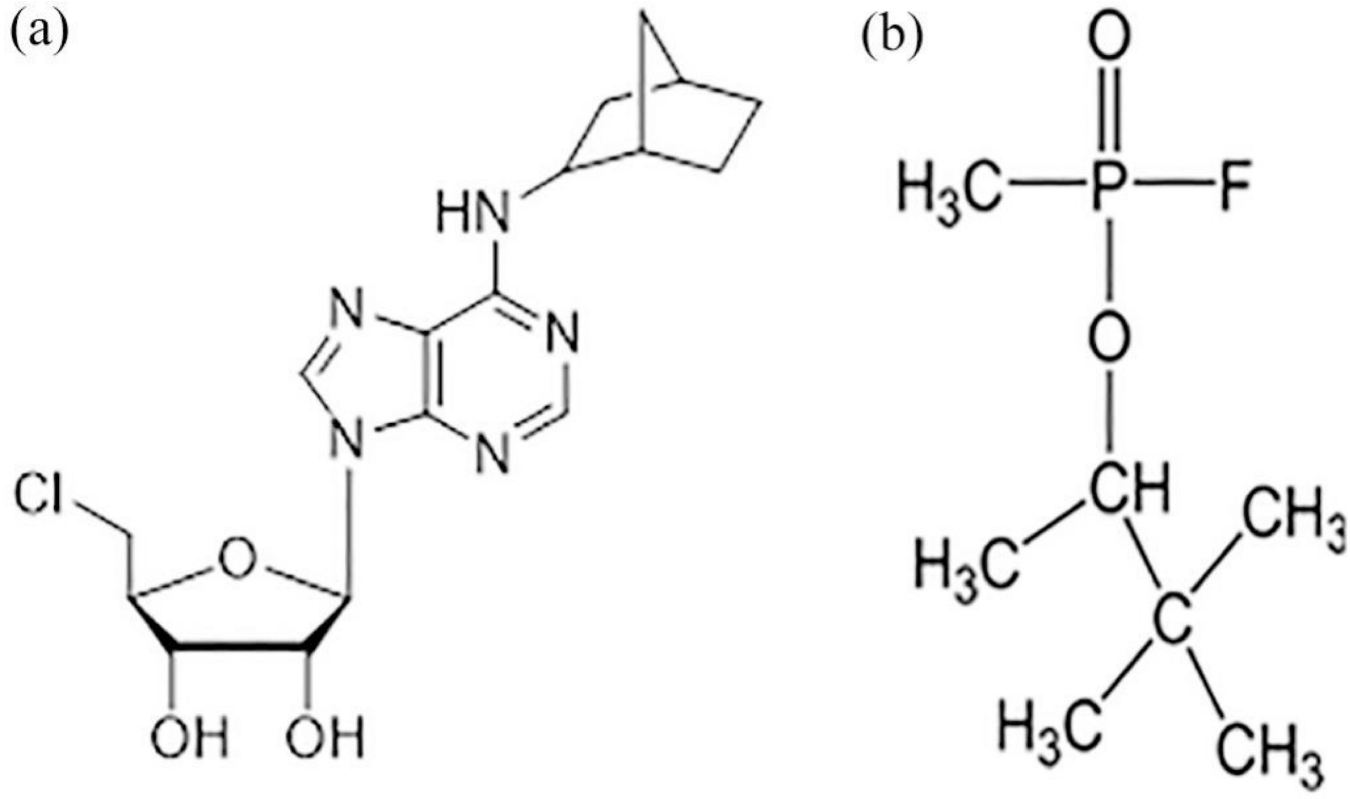

Figure 1.

Figure 1a. Chemical structure of ENBA.

Figure 1b. Chemical structure of soman

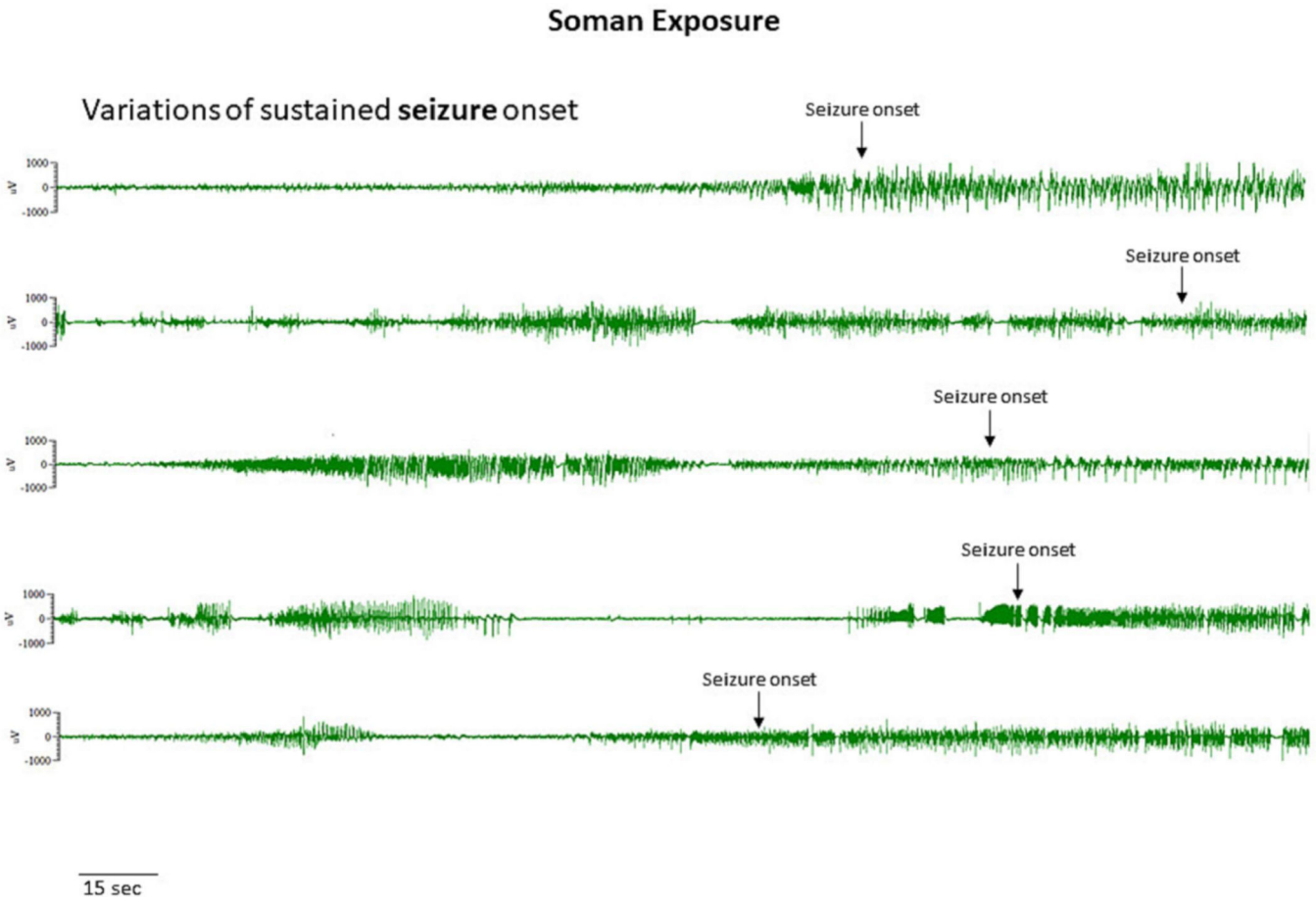

Figure 2.

Variations in sustained EEG seizure (*status epilepticus*) onset following soman exposure. Male KIKO mice were pretreated with HI-6 (125 mg/kg, IP) 30 min prior to challenge with a dose of soman (33 μg/kg, SC) and treated one min later with atropine methyl nitrate (2 mg/kg, IP). Sustained *status epilepticus* (SSE) onset was determined by EEG activity and is operationally defined as the appearance of ≥30 sec of continuous high amplitude rhythmic spikes or sharp wave activity in the EEG.

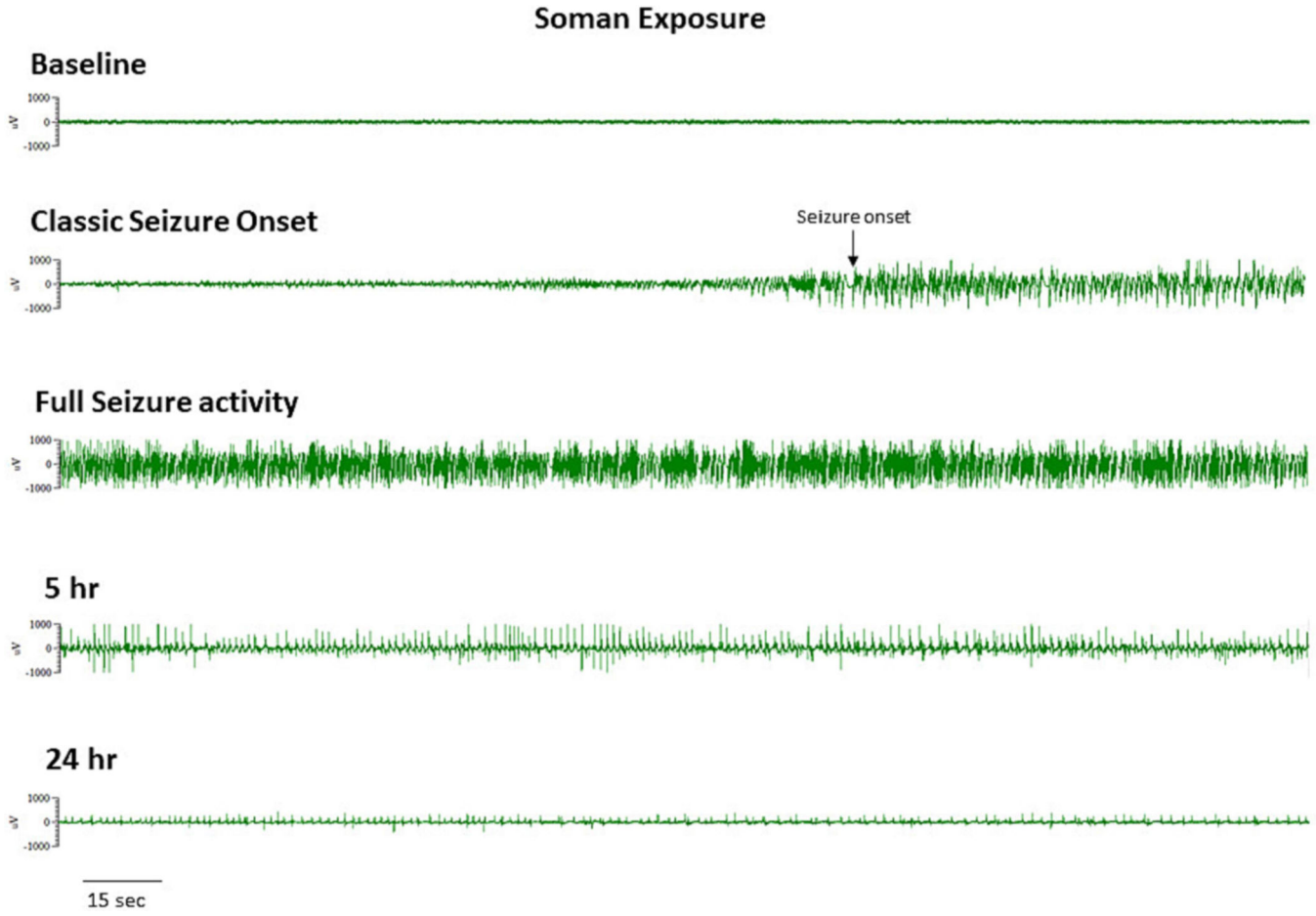

Figure 3.

A typical example of an EEG tracing showing soman-induced seizure onset and sustained *status epilepticus* (SSE) that followed and lasted for 24 hours in a male KIKO mouse.

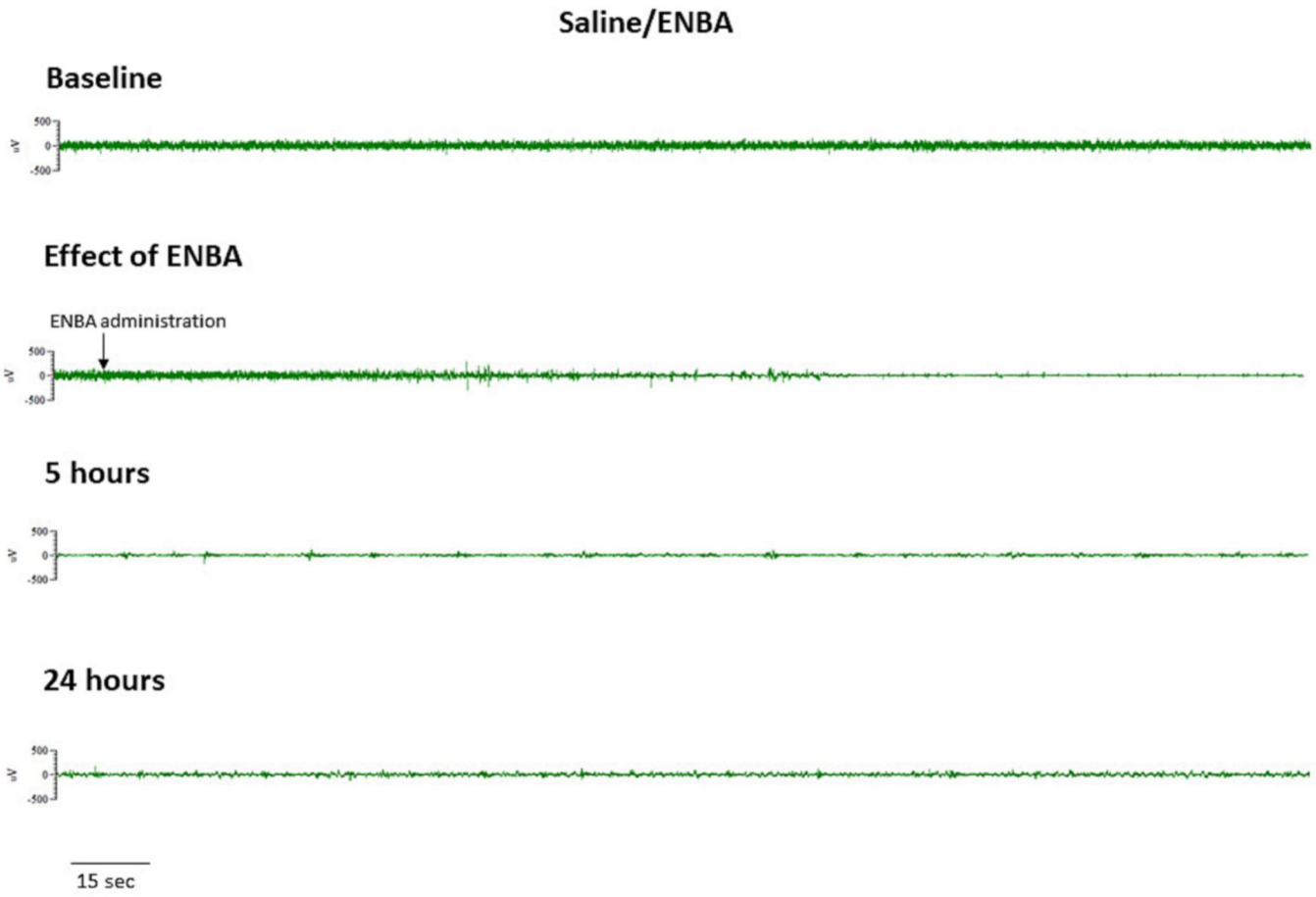

Figure 4.

A typical example of an EEG tracing of saline-exposed control brain wave and effect of ENBA in a male KIKO mouse. The effect of ENBA was extremely rapid, and EEG showed isoelectric-like activity within minutes after its administration and lasted for 24 hours.

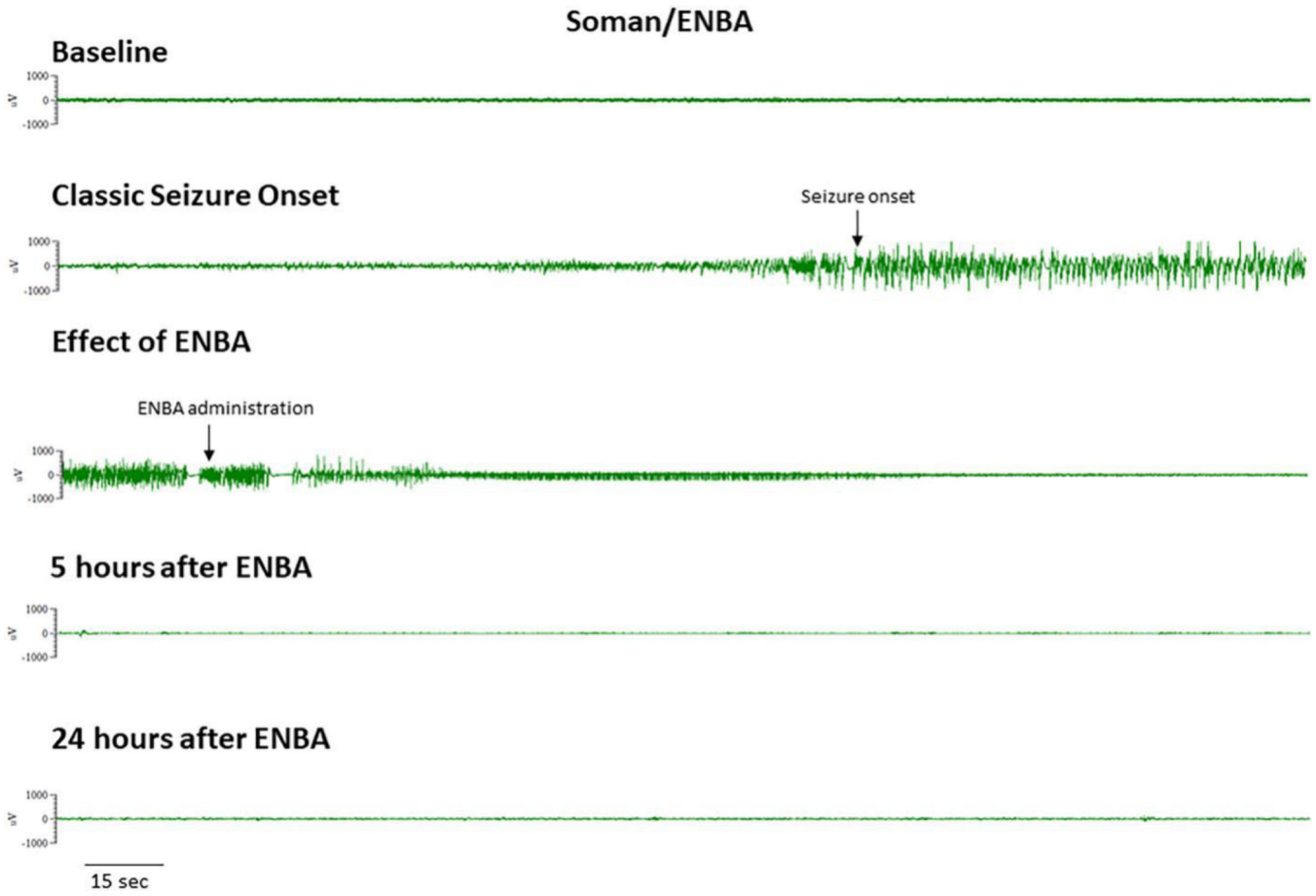

Figure 5.

A typical example of an EEG tracing of soman-induced SSE onset and termination by a dose of ENBA given at 15 minutes after seizure onset in a male KIKO mouse. The effect of ENBA was extremely rapid, and the EEG indicated isoelectric activity that began minutes after the administration of ENBA and lasted for 24 hours.

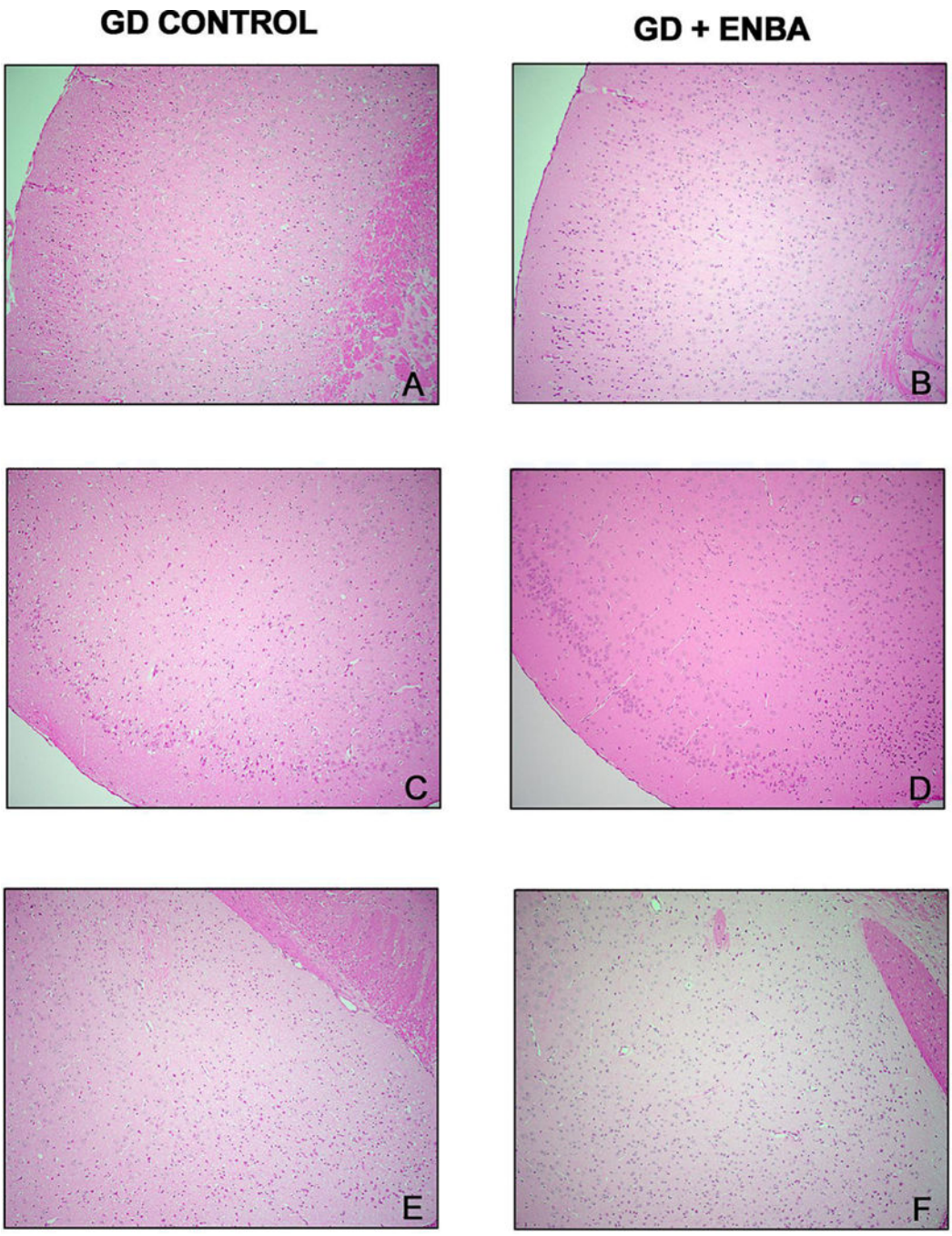

Figure 6.

Comparison of select brain regions (a-b. cerebral cortex, c-d. piriform cortex, and e-f. amygdala) between GD-exposed control mice and GD-exposed + ENBA-treated mice (H&E 10X). GD-exposed control brain sections (a., c., and e.) have multifocally extensive neuronal necrosis and degeneration with neuropil vacuolation. GD-exposed + ENBA-treated brain sections (b., d., and f.) have undamaged neurons with multifocal areas of “dark neurons” (a handling artifact).

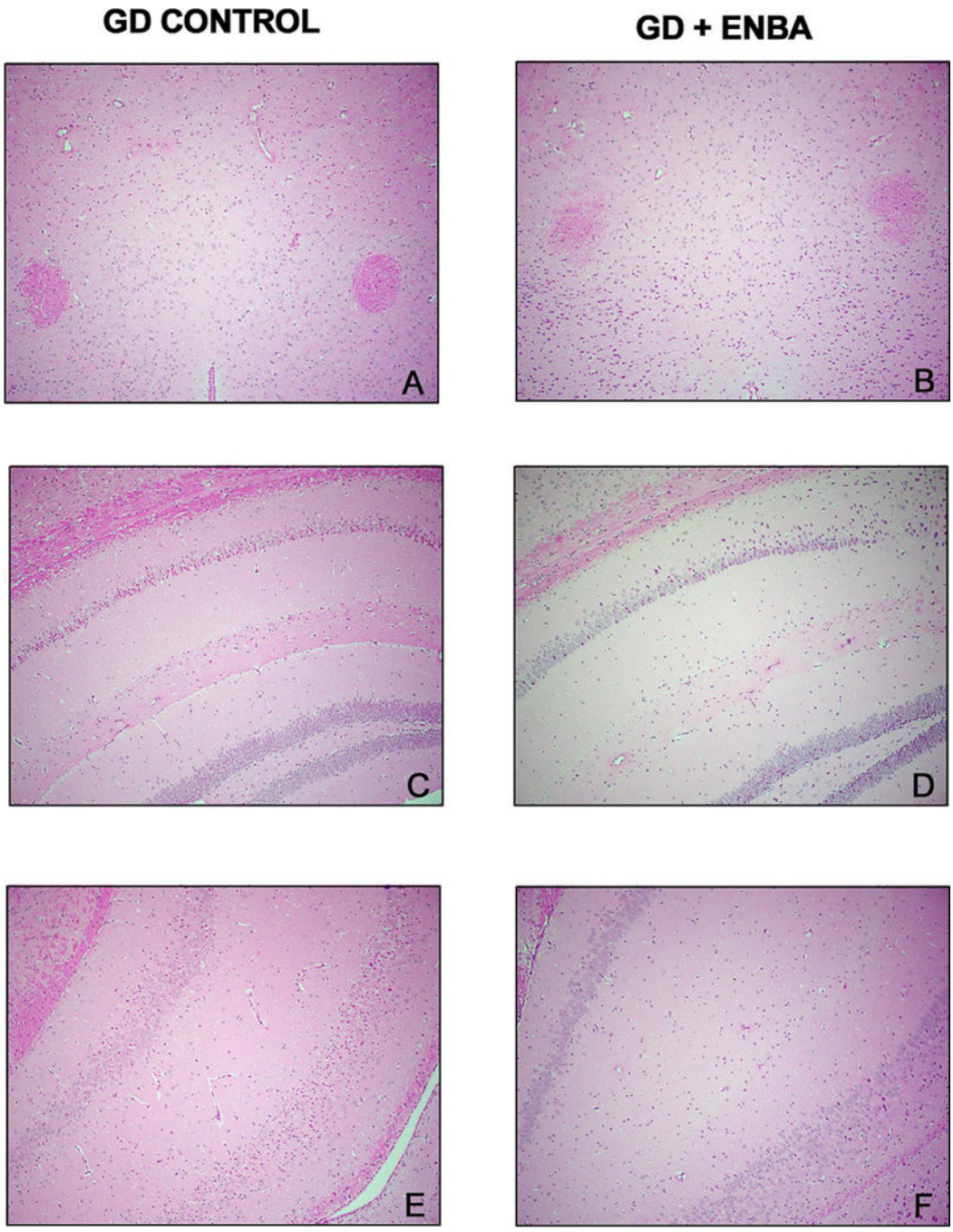

Figure 7.

Comparison of select brain regions (a-b. thalamus, c-d. dorsal hippocampus, and e-f. ventral hippocampus) between GD-exposed control mice and GD-exposed + ENBA-treated mice (H&E 10X). GD-exposed control brain sections (a., c., and e.) have multifocally extensive neuronal necrosis and degeneration with neuropil vacuolation. GD-exposed + ENBA-treated brain sections (b., d., and f.) have undamaged neurons with multifocal areas of “dark neurons” (a handling artifact).

Table 1.

Male KIKO Mouse Soman-Induced Seizure Dose Determination

Dose of Soman		No.	Seizures Activity		24-Hour Lethality		Neuropathology
µg/kg, SC	LD_{50} Fraction	N=	Response Fraction	Time to Onset[#] min ± SEM (N)	Response Fraction	Time to Death hour ± SEM (N)	Mean ± SEM (N)
26	1.2	10	9/10	12.5±1.5 (8)[##]	3/10	7.4±3.3 (3)	20.5 ± 0.4 (6)
30	1.3	10	10/10	16.5±5.3 (10)	2/10	3.8±3.1 (2)	20.6 ± 0.2 (8)
32	1.4	9[*]	9/9	12.2±2.0 (9)	3/9	3.1±1.8 (3)	20.8 ± 0.3 (6)
35	1.5	10	10/10	11.9±2.6 (10)	6/10	7.3±2.0 (6)	18.0 ± 2.4 (4)
37	1.6	10	10/10	9.2±1.6 (10)	6/10	3.1±1.7 (6)	20.0 ± 0.0 (4)
47	2.0	3	3/3	11.2±7.1 (3)	3/3	5.6±4.8 (3)	╲

Table 1. Male KIKO mice were pretreated with HI-6 (125 mg/kg, IP) 30 min prior to challenge with a dose of soman (26 −47 µg/kg, SC) and treated one min later with atropine methyl nitrate (4 mg/kg, IP). Time to EEG seizure onset and death were recorded. Neuropathology scores were assessed at 24 hours following soman exposure using established rubric scores (McDonough et al. 1995; Meads et al., 2021).

[#]No significant difference in time to seizure onset among all doses (p=0.77). Ran on prism using an ordinary one-way ANOVA.

[##]One mouse seized at 5.4 hours after soman exposure and was excluded from this calculation. If included the average was 47.3±34.9 (9) min.

[*]At 32 µg/kg, one mouse seized at 10.3 hours after soman exposure and was excluded from all data calculation.

Toxicol Appl Pharmacol. Author manuscript; available in PMC 2024 April 01.

Table 2.

Minimum effective dose determination of ENBA immediately following soman-induced seizure onset in male KIKO mice

Dose of ENBA	No. of Mice	Seizure Activity			Sedation			ENBA Duration of Action	24-Hour Lethality		Neuropathology
mg/kg, IP	N=	Time to Onset* min ± SEM (N)	Termination Response Fraction	Time to Termination min ± SEM (N)	Latency to Isoelectric State min ± SEM (N)	Return of Baseline-EEG Response Fraction	Latency to Awake hour ± SEM (N)	From Treatment to Seizure Return Time hour ± SEM (N)	Response Fraction	Time from Treatment hour (N)	Mean ± SEM (N)
45	4	16.0±0.7 (4)	4/4	2.8±2.1 (4)	26.2±21.0 (4)	4/4	17.4±0.5 (4)	24.5±0.1 (4)	0/4	\	0±0 (4)
40	6	9.5±1.9 (6)	6/6	2.2±1.2 (6)	4.1± 1.1 (6)	4/6	16.2±0.8 (4)	24.7±0.1 (6)	0/6	\	0±0 (6)
35	6	8.5±2.6 (6)	6/6	1.5±0.8 (6)	2.3±0.7***(5)	5/6	12.8±3.7 (5)	24.7±0.1 (5)	1/6	16.7 (1)	0±0 (6)
30	6	6.8±1.9 (6)	6/6	0.9±0.6 (6)	1.5±0.5 (6)	5/6	15.0±1.1 (5)	24.7±0.1 (5)	1/6	17.3 (1)	0±0 (6)
25	7	7.4±2.5 (7)	7/7	16.1±12.4 (7)	19.2±12.1 (7)	5/7	8.9±2.3 (5)	23.5±2.0 (6)	1/7	22.2 (1)	0±0 (6)
20	2	2.3±0.1 (2)	2/2	0±0**(2)	2.3±0.2 (2)	2/2	8.6±6.5 (2)	24.8±0.0 (2)	0/2	\	0±0 (2)
10	7	9.5±2.1 (7)	7/7	4.7±3.4 (7)	7.7±3.5 (7)	6/7	6.8±2.3 (6)	21.8±3.0 (7)	0/7	\	0±0 (7)
5	4	9.9±2.7 (4)	2/4	0.5±0.1 (2)	0.9±0.4 (2)	1/2	8.1 (1)	14.5± 10.6 (2)	1/4	11.8 (1)	6.7±6.7 (3)
****Animal had outlier time to isoelectric state, 107.8 minutes, was excluded only from this calculation.*											

Table 2. Male KIKO mice were pretreated with HI-6 (125 mg/kg, IP) 30 min prior to challenge with soman (33 µg/kg, SC) and treated one min later with atropine methyl nitrate (4 mg/kg, IP). A dose of ENBA (45 – 5 mg/kg, IP) was administrated at time of EEG seizure onset. Brain EEG seizure activity and death were recorded. Neuropathology scores were assessed at 24 hours following soman exposure.

*Average time to seizure onset for all animals is 8.90 ± 0.87 (42) minutes [mean ± SEM (N)].

**Seizure activity terminated within seconds after ENBA.

***One mouse had an outlier time to isoelectric state and was excluded from Latency to Isoelectric State calculation only. None of the survivors at 24 hours showed any pathology with the exception of one mouse with 5 mg/kg who didn't have seizure termination and had a score of 20.

Table 3.

Minimum effective dose determination of ENBA following saline exposure in KIKO male mice

Dose of ENBA	No. of Mice	Sedation				ENBA Duration of Action (hour)	24-Hour Lethality	Neuropathology
mg/kg, IP	N=	Response Fraction	Latency to Isoelectric State min ± SEM (N)	Awake at 24-hour Response Fraction	Time to Awake hour ± SEM (N)	From Treatment To End of Isoelectric Time hour ± SEM (N)	Response Fraction	Mean ± SEM (N)
45	3	3/3	0.9±0.1 (3)	0/3	\	24.5±0.0 (3)	0/3	0±0 (3)
40	4	4/4	2.8±1.1 (4)	0/4	\	24.7±0.1 (4)	0/4	0±0 (4)
35	4	4/4	1.4±0.1 (4)	0/4	\	24.8±0.0 (4)	0/4	0±0 (4)
30	*3*	3/3	1.2±0.1 (3)	0/3	\	24.6±0.0 (3)	0/3	0±0 (3)
25	*5*	5/5	1.0±0.1 (5)	1/5	19.09 (1)	24.0±1.2 (5)	0/5	0±0 (5)
20	*2*	2/2	1.0±0.4 (2)	1/2	16.73 (1)	20.7±4.0 (2)	0/2	0±0 (2)
15	*3*	3/3	15.0± 13.0 (3)	2/3	10.0±3.3 (2)	15.2±5.1 (3)	0/3	0±0 (3)
10	*8*	8/8	7.2±4.4 (8)	6/8	9.5±2.3(6)	13.4±3.1 (8)	0/8	0±0 (8)
5	*3*	2/3	1.8±0.0 (2)	2/2	5.4±0.1 (2)	5.4±0.0 (2)	0/3	0±0 (2)

Table 3. Male KIKO mice were pretreated with HI-6 (125 mg /kg, IP) 30 min prior to challenge with a dose of normal saline (2.2 ml/kg, SC) and treated one min later with atropine methyl nitrate (2 mg/kg, IP). A dose of ENBA (45 – 5 mg/kg, IP) was administrated at time of expected EEG seizure onset, based on the average time of onset induced by soman. Brain EEG activity and death were recorded. Neuropathology scores were assessed at 24 hours following saline exposure.

*Time to awake state was determined as median time between end of isoelectric state and onset of awake brain wave.

Table 4.

Minimum effective dose determination of ENBA at 15 min following soman-induced seizure onset in male KIKO mice

Dose of ENBA	No. of mice	Seizure Activity					Sedation			ENBA Duration of Action	24-Hour Lethality	Neuropathology
mg/kg, IP	N=	Time to Onset* min ± SEM (N)	Termination Response Fraction	Latency to Termination min ± SEM (N)	Return from Suppression Response Fraction	Latency to Return from Suppression hour(N)	Latency to Isoelectric State min ± SEM (N)	Return of Baseline-EEG Response Fraction	Latency to Awake hour ± SEM (N)	From Treatment Time hour ± SEM (N)	Response Fraction	Mean ± SEM (N)
10	4	6.6±1.3 (4)	2/4	3.4±0.4 (2)	1/2	13.00 (1)	3.4±0.5 (2)	0/4	\	18.8±5.7 (2)	0/4	10.3±5.0 (4)
15	8	10.1±1.3 (8)	8/8	6.6±3.2 (8)	0/8	\	6.6±3.2 (8)	6/8	16.1 ± 0.2 (6)	24.2±0.4 (8)	0/8	0±0 (7)
20	5	9.4±3.0 (5)	5/5	23.3±21.5 (5)	1/5	19.50 (1)	23.3±21.5 (5)	0/5	\	24.0±0.7 (5)	0/5	0±0 (5)

Table 4. Male KIKO mice were pretreated with HI-6 (125 mg/kg, IP) 30 min prior to challenge with a dose of soman (33 μg/kg, SC) and treated one min later with atropine methyl nitrate (4 mg/kg, IP). A dose of ENBA (10 – 20 mg/kg, IP) was administrated at 15 min after soman-induced SSE onset. Brain EEG seizure activity and death were recorded. Neuropathology scores were assessed at 24 hours following soman exposure.

*Average time to seizure onset for all animals is 9.1±1.1 minutes [mean ± SEM (N)].

Table 5.

Comparative efficacy of minimum effective dose (MED) of ENBA treatment at time of seizure onset or 15 min after seizure onset in male KIKO mice

Exposure to	ENBA Treatment Time at Seizure Onset	ENBA MED (mg/kg)	Time to Seizure Termination min ± SEM (N)	Time to Isoelectric State min ± SEM (N)	Time to Awake State[#] hour ± SEM (N)	ENBA Duration of Action hour ± SEM (N)	24-Hour Lethality	Pathology Score
Soman	Yes	10	4.7±3.4 (7)	7.7±3.5 (7)	6.8±2.3 (6)	21.8±3.0 (7)	0/7	0
Soman	+15 min	15	6.6±3.2 (8)	6.6±3.2 (8)	16.1±0.2 (6)[*]	24.2±0.4 (8)	0/8	0
Saline	---	10	---	7.2±4.4 (8)	9.5±2.3 (6)	13.4±3.1 (8)[**]	0/8	0

Table 5. Male KIKO mice were pretreated with HI-6 (125 mg/kg, IP) 30 min prior to challenge with a dose of soman (33 μg/kg, SC) or saline and treated one min later with atropine methyl nitrate (2 or 4 mg/kg, IP). A minimum effective dose (MED) of ENBA (10 or 15 mg/kg, IP) was administrated at either soman-induced SSE seizure onset or 15 min after seizure onset. Brain EEG activity and death were recorded. Neuropathology scores were assessed at 24 hours.

[#]For saline-exposed control group, time to awake state was determined as median time between end of isoelectric state and onset of awake brain wave.

[*]Significantly different from both saline-exposed group and soman-exposed group treated at time of seizure onset ($p \leq 0.01$).

[**]Significant difference from both soman exposure groups. ($p \leq 0.01$).