Seizure Suppression and Neuroprotection in Soman-Exposed Rats Following Delayed Intramuscular Treatment of Adenosine A_1 Receptor Agonist as an Adjunct to Standard Medical Treatment

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Abstract

Soman produces excitotoxic effects by inhibiting acetylcholinesterase in the cholinergic synapses and neuromuscular junctions and resulting in soman-induced sustained *status epilepticus* (SSE). Our previous work showed delayed intramuscular (i.m.) treatment with A_1 adenosine receptor agonist N-bicyclo-[2.2.1]-hept-2-yl-5'-chloro-5'-deoxyadenosine (ENBA) alone suppressed soman-induced SSE and prevented neuropathology. Using this same rat soman seizure model, we tested if delayed therapy with ENBA (60 mg/kg, i.m.) would terminate seizure, protect neuropathology, and aid in survival when given in conjunction with current standard medical countermeasures (MCMs): atropine sulfate, 2-PAM, and midazolam (MDZ). Either 15- or 30-min following soman-induced SSE onset, male rats received atropine and 2-PAM plus either MDZ or MDZ+ENBA. Electroencephalographic (EEG) activity, physiologic parameters, and motor function were recorded. Either 2- or 14-days following exposure surviving rats were euthanized and perfused for histology. All animals treated with MDZ+ENBA at both time points had 100% EEG seizure termination and reduced total neuropathology compared to animals treated with MDZ (2-day, $p=0.015$ for 15-min, $p=0.002$ for 30-min; 14-day, $p<0.001$ for 15-min, $p=0.006$ for 30-min), showing ENBA enhanced MDZ's anticonvulsant and neuroprotectant efficacy. However, combined MDZ+ENBA treatment, when compared to MDZ treatment groups, had a reduction in the 14-day survival rate regardless of treatment time, indicating possible enhancement of MDZ's neuronal inhibitory effects by ENBA. Based on our findings ENBA shows promise as an anticonvulsant and neuroprotectant in a combined treatment regimen following soman exposure; when given as an adjunct to standard MCMs, the dose of ENBA needs to be adjusted.

Key Words

Adenosine A_1 receptor agonist; Anticonvulsant; Delayed therapy; Neuroprotection; Organophosphorus compound; Soman

List of non-standard abbreviations

A_1AR, Adenosine A_1 receptor; AChE, Acetylcholinesterase; ADO, Adenosine; AMN, Atropine methyl nitrate; A/N, anticonvulsive and neuroprotective; $ATSO_4$, Atropine sulfate; CES, Carboxylesterase; ~~CNS, Central nervous system;~~ EEG, Electroencephalogram, ENBA, N-bicyclo-(2.2.1)-hept-2-yl-5'-chloro-5'-deoxyadenosine; FOB, Functional observational battery; GD, Soman; HI-6, Asoxime chloride; MCM, Medical countermeasure; MDZ, Midazolam; NA, Nerve agent; 2-PAM, 2-Pyridine aldoxime chloride; SSE, Sustained *status epilepticus*.

Highlights

- A_1 adenosine receptor agonist ENBA displays anticonvulsive/neuroprotective effects.
- ENBA as delayed adjunct to medical therapy was evaluated following soman in rats.
- ENBA enhanced the anticonvulsant/neuroprotective effect of atropine and midazolam.
- Results illustrated the roles of adenosine pathway in seizure & brain pathology.

Contribution of Authors

Conceptualization: Tsung-Ming Shih; Data curation: Zora-Maya Keith; Formal Analysis: Zora-Maya Keith; Funding acquisition: Tsung-Ming Shih; Investigation: All authors; Methodology: Tsung-Ming Shih; Project administration: Tsung-Ming Shih; Resources: Cindy Acon-Chen; Supervision: Tsung-Ming Shih; Validation: All authors; Visualization: Zora-May Keith, Crystal Munoz, and Cindy Acon-Chen; Writing – original draft: Zora-Maya Keith; and Writing – review & editing: All authors.

Introduction

Organophosphorus nerve agents (NAs) such as soman (GD) and sarin irreversibly inhibit acetylcholinesterase (AChE), the enzyme responsible for hydrolyzing acetylcholine, an excitatory cholinergic neurotransmitter (reviewed in Taylor, 2011). The buildup of acetylcholine in synapses and neuromuscular junctions leads to excitotoxic activity resulting in mucus secretion, tremors and convulsions, respiratory dysfunction, and sustained *status epilepticus* (SSE). If left untreated, these toxic consequences can lead to severe neurological impairment and/or death (McDonough and Shih, 1997).

Current standard medical countermeasures (MCMs) for NA-exposure include anticholinergics such as atropine sulfate ($ATSO_4$) to block acetylcholine muscarinic receptors, oximes such as 2-pyridine aldoxime (2-PAM) to reactivate NA-bound AChE, and benzodiazepine anticonvulsants such as diazepam or midazolam (MDZ) to control convulsions and seizure activity (Shih et al. 1991; McDonough et al., 2010). These treatments are adequate for controlling peripheral symptoms and reducing lethality. However, unless given promptly following NA exposure, this regimen is insufficient in controlling SSE activity (McDonough et al., 1995; McDonough et al., 2010). Additionally, when NA binds to AChE, this bond becomes irreversible in a process known as aging, and NAs like GD age very rapidly (within a few minutes). Treatments are only effective for a narrow window of time following GD exposure (Brown and Brix, 1998; McDonough et al., 2010). Previous studies found that seizure activity progressing for 20 min or more without intervention, or even with anticonvulsant therapy, yielded substantial neuropathology across multiple brain regions (McDonough et al., 1995). A novel therapeutic modality capable of inhibiting excitotoxic neural activity and offering neuroprotection would potentially improve outcomes in a scenario in which treatment will be delayed.

Dunwiddie and Masino (2001) investigated adenosine (ADO), a homeostatic regulator and purine metabolite produced from the catabolism of adenosine 5'-triphosphate and found that it might be employed as an alternative anticonvulsant to traditional benzodiazepine anticonvulsants. ADO is produced in periods of ischemia or seizures and has demonstrated protective properties on neural and cardiac tissues under stressful conditions through activation of the A_1 ADO receptor (A_1AR) subtype (Schindler et al., 2005; St. Hilaire, 2009). The stimulation of A_1AR inhibits neuronal activity through pre- and post-synaptic mechanisms, preventing excitatory neurotransmitter release and restricting calcium (Ca^{2+}) entry (Dunwiddie and Masino, 2001; Wardas, 2002). The activation of these receptors also reduces the excitability of cardiac tissue, causing bradycardia. Other known effects include hypotension (Schindler et al., 2005) and hypothermia (Thomas and Shih, 2019). In effect, as the body's endogenous anticonvulsant, the A_1AR signaling pathway is a promising therapeutic candidate for treating NA-induced SSE.

Our lab has recognized A_1AR's potential as an anticonvulsive and neuroprotective (A/N) treatment, evaluating candidate A_1AR agonists, such as N^6-cyclopentyladenosine (CPA), 2-chloro-N^6-cyclopentyladenosine (CCPA), and N-bicyclo-(2.2.1)-hept-2-yl-5'-chloro-5'-deoxyadenosine (ENBA, Figure 1) for several years (Thomas and Shih, 2014, 2015; Thomas and Shih, 2019; Thomas, Wegener, & Shih, 2019; Loughery et al., 2021; Meads et al., 2021; Shih 2023). We demonstrated that A_1AR stimulation alone can effectively prevent or terminate NA-induced seizure activity, enhance neuroprotection, and promote survival, even when administered at 15- and 30-min (in case of GD and sarin exposures) or even 60-min (in case of sarin exposure) after ongoing SSE activity (Loughery et al., 2021). Additionally, among these agonists, ENBA offers better advantages with rapid seizure suppression and shorter recovery

time from the effects of A_1AR agonism (Thomas et al., 2019; Loughery et al., 2021; Meads et al., 2021).

The current study extends upon our previous findings (Loughery et al., 2021) of standalone ENBA therapy. Here we evaluate whether adding ENBA (at 60 mg/kg) as an adjunct to current MCMs, $ATSO_4$+2-PAM+MDZ, would enhance the A/N effects under the SSE condition of severe GD intoxication in rats when administered via intramuscular route at delayed treatment time points (e.g., 15- and 30-min after SSE seizure onset) for a duration of 2- and 14-days**.**

Materials and Methods

Subjects

Male Sprague-Dawley rats, weighing 250-300 grams before surgery, were purchased from Charles River Laboratories (Raleigh, NC). Animals were pair-housed during acclimation to the facility, then individually housed after surgery. The environment was a temperature-controlled (21 ± 2 ºC) and humidity-controlled (50 ± 20%) suite with a 12-h light/dark cycle, with light on at 6:00 AM. Food (Laboratory Rodent Diet Chow, LabDiet) and water were available *ad libitum* while animals were in their home cages. Animals were acclimated for 5-7 days prior to surgery.

The experimental protocols were approved by the Institutional Animal Care and Use Committee at U.S. 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.

Materials

Saline (0.9% NaCl) injection, USP, was purchased from Quality Biological (Gaithersburg, MD). Atropine methyl nitrate (AMN) and atropine sulfate ($ATSO_4$) were purchased from

Sigma-Aldrich (St. Louis, MO). Asoxime chloride (HI-6) was synthesized by Kalexyn Medicinal Chemistry (Kalamazoo, MI). Pralidoxime chloride (2-PAM) was purchased from Dishman Pharmaceuticals (Middlesex, NJ). Midazolam (MDZ) solution (in 0.8% NaCl, 0.01% edetate disodium with 1% benzyl alcohol) was purchased from Hospira, Inc (Lake Forrest, IL). N-Bicyclo-(2.2.1)-hept-2-yl-5'-chloro-5'-deoxyadenosine (ENBA) was purchased from R&D Systems (Minneapolis, MN). Soman (GD; pinacolyl methylphosphonofluoridate; > 95% purity by ^{31}P NMR) was obtained from the U.S. Army Combat Capabilities Development Command Chemical Biological Center (Aberdeen Proving Ground, MD). Laboratory Rodent Diet Chow (catalog #5001) was purchased from LabDiet (St. Louis, MO). DietGel Boost (catalog #72-04-5022), DietGel Recovery (catalog #72-06-5022), and HydroGel (catalog #70-01-5022) were purchased from ClearH_2O (Portland, ME). Bacon softies (catalog #F3580) and Fruit and Veggie medley (catalog #F7227) were purchased from Bio-Serv (Flemington, NJ).

Temperature transponders were purchased from Biomedic Data Systems Inc. (Seaford, DE). The MouseOX Plus Pulse Oximeter was obtained from STARR Life Sciences Corporation (Oakmont, PA). Heating Chambers (Model: DW-1, Dimensions: 18" H x 27.625" W x 14.75" L) were purchased from Thermocare (Paso Robles, CA). Electroencephalographic (EEG) activity was recorded using CED 1902 amplifiers and Spike2 software purchased from Cambridge Electronic Design, Ltd. (Cambridge, UK).

ENBA was prepared in multisolvent solution (48.5% distilled H2O, 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol). At the concentration (50 mg/ml) required for the selected dose of administration, ENBA did not readily dissolve and required constant sonication and heating at 37-45 °C until several minutes prior to the time of treatment delivery. GD, HI-6, 2-PAM, AMN, and $ATSO_4$ were dissolved in normal saline solution prior to injection. The

153 volumes of injection were 0.26 ml/kg for GD, 0.25 ml/kg for $ATSO_4$ and 2-PAM; 0.36 ml/kg for
154 MDZ; 0.5 ml/kg for HI-6 and AMN; and 1.2 ml/kg for ENBA.

155 **Experimental Procedures**

156 Rats underwent surgery 5-7 days before experimentation. On the day of surgery, animals were
157 given a subcutaneous (s.c.) injection of meloxicam (1 mg/kg) prior to being anesthetized with
158 isoflurane. A cortical electrode headpiece was implanted to measure brain
159 electroencephalographic (EEG) activity (Thomas et al., 2019; Loughery et al., 2021) and a body
160 temperature transponder was inserted s.c. between shoulder blades. Once animals were able to
161 right themselves following surgery, they were given an injection of sustained-release
162 buprenorphine (0.60 mg/kg, s.c.) to minimize discomfort. Rats were monitored for 3 days
163 following surgery.

164 On experimental day, rats were weighed and placed in monitoring chambers (45.01 cm H x
165 30.99 cm W x 23.11 cm L) and baseline EEG activity was recorded for a minimum of 30 min
166 prior to beginning the experiment (Figure 2). Baseline physiological assessment was scored and
167 recorded at this time, including body temperature, righting reflex, motor activity score, and heart
168 rate. Animals were then treated with HI-6 (125 mg/kg, i.p.) 30 min before an injection of saline
169 (0.5 ml/kg, s.c.), to serve as a control exposure, or a seizure inducing dose of GD (90 µg/kg, s.c.,
170 0.8 x LD_{50}). One min later, rats received an injection of atropine methyl nitrate (AMN, 2-4
171 mg/kg, i.m.) regardless of exposure. The administration of HI-6 as a pretreatment and AMN at 1
172 min post-exposure served to treat peripheral cholinergic symptoms and promote survival so
173 adequate statistical analysis of neuroprotection could be performed. As neither drug crosses the
174 blood brain barrier, they have no effect on the central nervous system (CNS) and do not interfere
175 with the presentation of EEG seizure activity. Based on our previous GD-exposed rat models

(Loughery et al., 2021; Meads et al., 2021), additional doses of AMN (2 mg/kg, i.m.; up to 8 mg/kg) were given as needed after the "+1 min AMN" (Figure 2) and before the medical treatments. All animals were equally considered for AMN boosters based on the observation of the peripheral signs of GD exposure: mucus secretions, salivation, or labored breathing. The additional booster doses of AMN were given to control these peripheral effects of GD, and to clear the airway and aid in survival, without affecting CNS outcomes. It has been shown that even centrally acting $ATSO_4$, at 12 mg/kg, does not affect seizure activity following GD exposure (Shih 1991). To avoid confounding survival data, AMN boosters were not administered after treatments.

To evaluate the combined neuroprotective efficacy of ENBA with current medical treatments for NAs, all rats received $ATSO_4$ and 2-PAM (0.45 mg/kg and 25 mg/kg, respectively). In addition, rats received either MDZ (1.8 mg/kg) (collectively termed MDZ for this report) or MDZ (1.8 mg/kg) + ENBA (60 mg/kg) (collectively termed MDZ+ENBA for this report) at one of two treatment times (either 15 or 30 min) following SSE seizure onset (or average SSE onset in saline-exposure rats). The doses of $ATSO_4$ and MDZ were human-equivalent doses based on body surface, while dose of 2-PAM and ENBA were the same doses we used in our previous study (Loughery et al., 2021). The total number of animals for each treatment group on exposure day is listed in Table 1.

EEG activity of each rat was monitored for a period of 5 h following saline or GD exposure, marking the end of the first experiment day. During the 5 h observation period, measurements of body temperature, righting reflex, convulsion score (Table 2), and heart rate were taken following saline or GD exposure at 4, 8, 15, 30, 45, and 60 min and, thereafter, at 60-min

198 increments until the end of the initial experiment day. Surviving animals were given follow-up
199 re-assessments at several timepoints based on endpoint assignment of either 2-days or 14-days.

200 **EEG Recording**

201 EEG data was recorded using Spike2 software (version 7, Cambridge Electronic Design).
202 Visual determination of seizure onset and seizure termination required the consensus of two
203 trained individuals following pre-determined criteria. Seizure onset was defined as rhythmic
204 spiking of at least twice the amplitude of baseline recordings lasting at least 10 s (McCarren et
205 al., 2018). Seizure termination was defined as a) spike amplitude below 100 μV, b) spike
206 frequency of at least 10 s apart, and c) absence of spiking for at least 5 min (Loughery et al.,
207 2021). Re-onset was defined as any instance of repetitive spikes or sharp waves exceeding an
208 amplitude of twice that of baseline.

209 **Neuropathological Assessments**

210 Following the final testing session at either 2- or 14-days post-exposure, animals were deeply
211 anesthetized with sodium pentobarbital ($\geq$75 mg/kg), euthanized via exsanguination, and
212 perfused with saline solution and fixed with 10% formalin. The brain, heart, and both kidneys of
213 each animal were submitted to an in-house necropsy lab for analysis. Six brain regions (cerebral
214 cortex, amygdala, piriform cortex, dorsal and ventral hippocampus, and thalamus) associated
215 with severe neurological deficits after NA exposure were each evaluated by a trained pathologist,
216 who was blind to treatment paradigm, and scored using an established standard rubric
217 (McDonough et al., 1995; Loughery et al., 2021): 0 = no lesion; 1 = minimal (1-10%); 2 = mild
218 (11-25%); 3 = moderate (26-45%); 4 = severe (>45%). A total neuropathology score was then
219 calculated for each rat from 0 (normal) to 24 (severe damage). The heart and kidneys were not
220 analyzed as part of this study.

Data Analysis

A "treatment group" was defined as the combination of exposure (saline or GD), treatment (MDZ or MDZ+ENBA) and treatment time-point (15- or 30-min). Due to anticipated death in GD-exposed groups and the lethality-dependent fluctuations in statistical power between comparisons, the number of rats in GD-exposed groups are larger than in saline-exposed groups (Table 1). For comparisons, non-parametric tests (such as the Mann-Whitney U test) were utilized since assumptions are violated to make comparisons among treatment groups and times.

GraphPad Prism (version 9) and QuoStatus (custom Python-based statistical software, University of Utah) were utilized for inferential analysis. The EEG spectral analysis was completed on QuoStatus to assess for change in spike rate and gamma power relative to baseline for each treatment group followed by two-way ANOVAs and Sidak's multiple comparison to evaluate differences between each group. Gamma power was selected as the QuoStatus program filters motion artifacts and other noise sources in the recording to increase the signal-to-noise ratio near this power band (Lehmkuhle et al., 2009).

Animal physiological data from experimental day and neuropathology scores at study endpoints were assessed using Mann-Whitney tests (individual ranks computed for each comparison) and the Holm-Sidak's correction. Physiological data from days post-exposure was compared to baseline measurements (per group) using mixed effect two-way ANOVA with Sidak's multiple comparisons test (with individual variances computed for each comparison). Kaplan-Meier survival curves were generated; Mantel-Cox test was then used to analyze the survival curves. Statistical significance was defined as $p \leq 0.05$ for all tests.

Results

A. Brain Seizure Termination, EEG Power, and Spike Frequency

When evaluating SSE activity in GD-exposed animals, the average latency to SSE was 8.8 ± 5.9 min for all 93 rats. GD/MDZ groups at both treatment timepoints were unable to terminate SSE (0/15 and 0/14 seizure termination for the 15- and 30-min treatment timepoints, respectively; Table 3). On the other hand, the GD/MDZ+ENBA groups experienced 100% seizure termination (31/31 for 15 min and 33/33 for 30 min timepoints). Additionally, we examined the capacity of terminating GD-induced seizure via EEG power analysis. In GD-exposed animals, there was a sharp increase in frequency and power in the gamma range (20-60 Hz). For the GD/MDZ groups at both treatment timepoints the mean power and spike frequency (Figure 3) saw a steady increase after GD administration up until 1 h post-exposure, at which point there was a slight decrease in both parameters before stabilizing. On the other hand, the GD/MDZ+ENBA groups displayed a significant depression both in EEG power and spike frequency compared to their comparative non-ENBA treated groups that began approximately 1 h after GD administration (15-min, $p<0.0001$; 30-min, $p<0.0001$; respectively) and continued through 4 h following exposure ($p<0.0001$, $p<0.0001$; 30-min, $p<0.0001$; respectively). The control of EEG spiking in the gamma power band was maintained only by the MDZ+ENBA treatments, as these groups remained below baseline values throughout the rest of the recording.

B. Motor Convulsive Activity

The GD/MDZ groups did not terminate convulsions during the 5 h observation period, regardless of treatment timepoint (Figure 4). The GD/MDZ+ENBA groups displayed lower convulsion scores compared to GD/MDZ groups by the 30-min timepoint for the 15-min treated groups ($p=0.0027$) and 45-min timepoint for the 30-min treated groups ($p=0.0023$). This trend was maintained through the end of the experiment day (15-min group, $p<0.0001$; 30-min group, $p<0.0001$).

By the 2-day endpoint, the GD/MDZ groups returned to baseline scores (15 min, 0.7 ± 1.3, p=0.4982; 30 min, 0.5 ± 1.2, p=0.8195, Supplementary Tables 5 and 6). This improvement in convulsive score was also shown by the GD/MDZ animals in the 14-day endpoint by Day 3 post-exposure (15-min, 0.0 ± 0.0; 30 min, p=0.8276). Convulsive activity scores in the GD/MDZ+ENBA groups returned to baseline range by the 2-day endpoint (15-min, 0.8 ± 1.4, p=0.3946; 30-min, 1.5 ± 1.6, p=0.1564). Animals in the 14-day endpoint also returned to baseline scores by Day 7 in the 15-min treatment group (0.3 ± 1.0, p=0.7601) and Day 3 in the 30-min treatment group (0.6 ± 1.2, p=0.1313).

B. Righting Reflex

All saline-exposed MDZ-treated (Sal/MDZ) animals (15- and 30-min treatment times) maintained their righting ability for the duration of their respective endpoint (Supplementary Tables 7 and 8).

Righting impairment in the Sal/MDZ-ENBA groups began to differ from the MDZ-only groups by the 30- and 45-min timepoints for the 15- and 30-min treatments, respectively (p=0.0001; p<0.0001; Figures 5 and 6). These animals were no longer impaired, compared to their baseline score, by the 2-day endpoint (15 min, 0.9±0.8, p=0.0517; 30 min, 0.7 ± 1.0, p=0.2077). The animals in the 14-day groups recovered their righting reflex by Day 1 post-exposure (15 min, 0.0 ± 0.0; 30 min, 0.0 ± 0.0).

The GD/MDZ groups began showing righting impairment as early as 4 min after GD exposure, reaching the highest possible score at 1 h following exposure (Figures 5 and 6). Animals in this group exhibited recovery of their righting reflex by their 2-day endpoint (15-min, 0.0 ± 0.0; 30-min, 0.2 ± 0.4, p=0.8195). Animals in the 14-day group recovered their righting ability by Day 1 post-exposure (15-min, 0.0 ± 0.0; 30-min, 0.6 ± 0.9, p=0.2890).

290 Similar to the GD/MDZ groups, the GD/MDZ+ENBA groups began showing righting
291 impairment at 4-min post-exposure, reaching the highest possible score by 45 min post-exposure
292 (Figure 5 and 6). This loss of righting reflex was maintained through the end of the 5 h
293 observation period, with a significantly higher score in the 15-min treated animals compared to
294 their MDZ-only treated counterparts at the 4 and 5 h timepoints ($p=0.0053$, $p=0.0013$
295 respectively). Animals in the GD/MDZ+ENBA 15-min treatment group did not recover their
296 righting reflex by the 2-day endpoint (15-min, 1.1 ± 1.0, $p<0.0001$) while animals in the 30-min
297 treatment group did recover by their endpoint (0.5 ± 0.8, $p=0.4578$). The 14-day endpoint
298 animals had recovered their righting ability by Day 7 post-exposure (15-min, 0.2 ± 0.7,
299 $p=0.7601$; 30-min, 0.0 ± 0.0; supplementary tables).

300 **C. Body Temperature**

301 Saline-exposed groups had baseline body temperatures of ~38 °C (Figures 7 and 8,
302 Supplementary Tables 9 and 10). The Sal/MDZ+ENBA groups showed steady declines in
303 temperature (following treatment) compared to the Sal/MDZ groups by the 45- and 2-h
304 timepoints for the 15-min and 30-min treatments ($p=0.0002$, $p=0.0218$, respectively).
305 MDZ+ENBA treated animals remained hypothermic compared to their baseline temperatures by
306 the 2-day endpoint (15 min, 29.51 ± 5.9 °C, $p=0.0098$; 30 min, 28.7 ± 6.6 °C, $p=0.0057$;
307 Supplementary Tables 9 and 10). The 14-day animals returned to their baseline temperatures by
308 Day 3 (37.5 ± 0.4 °C, $p=0.7323$) and Day 7 (37.9 +/- 0.04 °C, $p=0.3891$) post-exposure for the
309 15- and 30-min treatment groups, respectively.

310 All GD-exposed groups had baseline body temperatures of ~38 °C (Figures 7 and 8). The
311 temperatures of GD/MDZ+ENBA treated groups decreased steadily over the course of the
312 observation period compared to the GD/MDZ groups, beginning at 1 h and 2 h post-exposure for

313 the 15- and 30-min treatment groups (15 min, 33.1 ± 1.3 °C, p=0.0058; 30 min, 33.0 ± 1.0 °C,
314 p<0.0001).

315 GD/MDZ treated animals no longer exhibited significantly lower body temperature compared
316 to their baseline measurements their lowest body temperatures at the 2-day endpoint (15-min,
317 37.5 ± 1.9 °C, p=3974; 30-min, 36.4 ± 1.7 °C, p=0.3968). Animals in the 14-day endpoint had
318 significantly lower body temperatures Day 1 post-exposure (15-min, 36.8 ± 0.9 °C, p=0.0102;
319 30-min, 35.3 ± 1.7 °C, p=0.104), and recovered to baseline temperatures by Day 3 post-exposure
320 (15-min, 38.1 ± 1.2 °C, p>0.9999; 30-min, 37.2 ± 0.3 °C, p=2959). GD/MDZ+ENBA animals
321 did not recover their body temperature by the 2-day endpoint (15-min, 26.5 ± 3.8 °C, p<0.0001;
322 30-min, 26.4 ± 1.0 °C, p<0.0001). Animals in the 14-day endpoint did not recover body
323 temperature until Day 7 post-exposure (15-min, 36.4 ± 4.3 °C, p=0.7473; 30-min, 37.8 ± 0.3 °C,
324 p=0.4679).

325 **D. Heart Rate**

326 The Sal/MDZ+ENBA groups had lower heart rate by the 1-h timepoint than their Sal/MDZ
327 counterparts by the 1 h timepoint (15-min, p<0.0001; 30-min, p<0.0001) that continued through
328 the end of the 5 h observation period (15-min, p<0.0001; p<0.0001; Figures 9 and 10). Animals
329 in the Sal/MDZ group had recorded heart rates that were not significantly different from their
330 baseline at the 2-day endpoint (15-min, p=0.9709; 30-min, p>0.9999) or at the Day 3 timepoint
331 for animals in the 14-day endpoint (15-min, p=0.9394; 30-min, p=0.4365; Supplementary Tables
332 11 and 12). The Sal/MDZ+ENBA animals continued to exhibit depressed heart rates through
333 their 2-day endpoint (15-min, p=0.0003; 30-min, p<0.0001). Animals treated 15-min after SSE
334 onset in the 14-day endpoint began displaying heart rate similar to their baseline by Day 7 post-

exposure (min, p=0.4699). Animals treated 30-min after SSE onset continued to have lower heart rate through the endpoint (p=0.0006).

The heart rates for GD/MDZ+ENBA groups were significantly lower than their GD/MDZ counterparts by the 1-h timepoint (15-min, p<0.0001; 30-min, p<0.0001) and did not recover before the end of the 5 h observation period (15-min, p<0.0001; 30-min, p<0.0001). The heart rates for the GD/MDZ animals remained at baseline in the 2-day endpoint animals (15-min, p>0.9999; 30-min, p=0.1446). In the 14-day animals, the MDZ treated animals were only significantly different from baseline at Day 3 post exposure in the 15-min group (p=0.0440) and at Day 14 in the 30-min treated animals (p=0.0330). The GD/MDZ+ENBA 2-day animals remained below baseline heart rates at their endpoint (15-min, p<0.0001; 30-min, p<0.0001). The 14-day endpoint animals showed recovery to baseline by Day 7 post-exposure (15-min, p=0.9978; 30-min, p=0.9523).

E. Body Weight

The Sal/MDZ groups in the 2-day endpoint did not experience significant weight loss from baseline (15-min, p=0.4532; 30-min, p=0.9778; Supplementary Tables 13 and 14). 14-day endpoint animals surpassed baseline weights by Day 7 post-exposure (15-min, p=0.0096; 30-min, p=0.0028) through the 14-day endpoint (15-min, p=0.0342; 30-min, p=0.0009). The Sal/MDZ+ENBA groups in the 2-day endpoint did not lose a significant amount of weight before their endpoint (15-min, p=0.0512; 30-min, p=0.3764). The 14-day day experienced significant weight loss by Day 1 post-exposure (15-min, p=0.0045; 30-min, p=0.0011) which continued through Day 3 post-exposure to day 7 post-exposure. These animals were able to surpass their baseline weight levels by the 14-day endpoint (15-min, p<0.0001; 30-min, p=0.0003).

The GD/MDZ groups remained lower than baseline weight for Days 1 through 3 post-exposures. The 2-day endpoint animals were significantly lower than their endpoint (15-min, $p<0.0001$; 30-min, $p<0.0001$). The 14-day animals did not recover to baseline weights until Day 7 post-exposure (15-min, $p>0.9999$) and Day 14 post-exposure (30-min, $p=0.9979$). The GD/MDZ+ENBA 2-day groups were not significantly lower than their baseline when they were treated 15-min after SSE onset ($p=0.0503$) but they were when they were treated 30-min after SSE onset ($p=0.0027$). The 14-day 15-min animals remained below baseline through their endpoint (14-day, $p=0.0129$). Animals treated 30-min after SSE onset remained significantly lower than their baseline through Day 3 post-exposure ($p=0.0003$). By Day 7, and through their endpoint, animals remained comparable to their baseline weight ($p=0.1784$, $p=0.2952$).

F. Survival

Sal/MDZ groups (15- and 30-min) exhibited 100% survival (Table 4, Figures 11 and 12). For Saline/MDZ+ENBA groups at the 2-day endpoint, the 15-min treatment experienced 100% survival (8/8), while the 30-min treatment displayed an 88% survival rate (7/8, $p=0.3762$). However, at the 14-day endpoint both treatment time groups saw the loss of 1 animal each, with 89% survival (8/9, $p=0.5637$) for the 15-min treatment group and 90% (9/10, $p=0.4000$) for the 30-min treatment group (Table 4, Figures 11 and 12).

Following GD-exposure, all MDZ-treated animals, regardless of treatment timepoint, survived to their 2- to 14-day endpoints. However, when ENBA was combined with MDZ in the treatment (GD/MDZ+ENBA) these animals experienced a reduction in survival rate (73% or 8/11 [$p=0.1445$] and 86% or 6/7 [$p=0.3545$] survival for 15-and 30-min treatments, respectively) at the 2-day endpoint, and exhibited a further decrease in survival rate (40% or 8/20 [$p=0.0196$] and

24% or 5/26 [p=0.0006] survival for 15-and 30-min treatments, respectively; Table 4) at the 14-day endpoint compared to GD/MDZ treated groups.

G. Neuropathology

As anticipated, all saline-exposed animals displayed no neuropathology throughout all brain regions and treatment groups (Supplementary Tables 15, 16, 17, and 18), with the exception of one animal in the 14-day groups (Sal/MDZ+ENBA-15 min), which had a minimal total pathology score of 1. There were no notes from the pathologists, so it is possible that the damage was caused by surgery or brain extraction post-perfusion.

At 2-Day Endpoint:

The GD/MDZ groups, 15- and 30-min, had neuropathology scores in all individual brain regions with no significant differences between treatment times (Supplemental Tables 15 and 16). The GD/MDZ+ENBA groups also had no significant differences between treatment times. The GD/MDZ+ENBA 15-min treated animals exhibited lower scores in the thalamus GD/MDZ group (p=0.030). The MDZ+ENBA 30-min group had lower scores in the cerebral cortex and thalamus compared to the MDZ-only treated 30-min group (p=0.013, p=0.013, respectively). Regardless of treatment time, the MDZ+ENBA groups had similar total neuropathology scores (p=0.945); the total scores for these groups were significantly lower than those of their respective MDZ counterparts (15-min, p=0.015; 30-min, p=0.002, Figure 13).

At 14-Day Endpoint:

The GD/MDZ groups had damage in all individual brain regions regardless of treatment time, with a significant difference between the cerebral cortex (p=0.0376, Supplemental Tables 17 and 18). The GD/MDZ+ENBA groups' individual brain regions had no significant differences between treatment times. The GD/MDZ+ENBA 15-min group had lower neuropathology than

403 the GD/MDZ 15-min group in the cerebral cortex (p=0.005), amygdala (p=0.006), thalamus
404 (p<0.001), dorsal hippocampus (p=0.007), and ventral hippocampus (p=0.011). The GD/MDZ
405 30-min treatment group had significantly lower scores than the 15-min treatment group
406 (p=0.041). Regardless of treatment time, the MDZ+ENBA groups had similar total
407 neuropathology scores (p=0.695). The total neuropathology scores for these groups were
408 significantly lower than those of their respective MDZ counterparts (15-min, p<0.001; 30-min,
409 p=0.006, Figure 14).

410 ## Discussion

411 Over the past several years our laboratory has explored A_1AR agonists' potential as an
412 anticonvulsive and neuroprotective (A/N) treatment for organophosphorus NA intoxication. We
413 have evaluated candidate A_1AR agonists, such as N^6-cyclopentyladenosine (CPA), 2-chloro-N^6-
414 cyclopentyladenosine (CCPA), and N- Bicyclo-(2.2.1)-hept-2-yl-5'-chloro-5'-deoxyadenosine
415 (ENBA) as treatments for soman (GD) and sarin exposure (Thomas and Shih, 2014, 2015;
416 Thomas and Shih, 2019; Thomas, Wegener, and Shih, 2019; Loughery et al., 2021; Meads et al.,
417 2021; Shih 2023). Among these agonists, ENBA offers better advantages with rapid seizure
418 suppression and shorter recovery time from the side effects of A_1AR agonism (Thomas et al.,
419 2019; Loughery et al., 2021; Meads et al., 2021).

420 Our earlier findings (Loughery et al., 2021) showed that A_1AR stimulation alone can
421 effectively prevent or terminate NA-induced seizure activity, enhance neuroprotection, and
422 promote survival with both immediate and delayed i.m. administration after SSE activity. With
423 our current study, we sought to expand upon these previous findings and examine whether
424 adding ENBA (at 60 mg/kg) as an adjunct to standard medical treatments ($ATSO_4$+2-
425 PAM+MDZ+ENBA, expressed as MDZ+ENBA) may enhance the A/N effects of standard

426 medical therapy ($ATSO_4$+2-PAM+MDZ, expressed as MDZ) after GD-induced SSE when given
427 at delayed timepoints while seizure activities were on-going (e.g., 15- and 30-min after seizure
428 onset); animals were assessed for 2- or 14-days post-GD exposure prior to euthanasia and
429 neuropathology assessment.
430 When evaluating SSE activity termination, GD/MDZ groups at both treatment timepoints were
431 unable to terminate SSE (0/15 and 0/14 seizure termination for the 15- and 30-min treatment
432 timepoints, respectively). Additionally, we examined the capacity of terminating GD-induced
433 seizure via EEG power analysis. EEG mean power and spike frequency in these GD/MDZ
434 groups saw a steady increase after GD administration up until 1 h post-exposure; at which point,
435 there was a slight decrease in both parameters before stabilizing but remaining well above
436 baseline EEG. On the other hand, the GD/MDZ+ENBA groups experienced 100% seizure
437 termination (31/31 for 15-min and 33/33 for 30-min timepoints) and displayed a significant
438 depression in both EEG power and spike frequency that began approximately 1 h after GD
439 administration and continued through 4 h following exposure. This control of EEG spiking in the
440 gamma power band was maintained only by the MDZ+ENBA treatment, and these groups
441 remained below baseline values throughout the rest of the recording. This finding concurs with
442 the report of Loughery et al. (2021) where most GD/Sal control animals did not terminate SSE
443 and experienced an increase EEG mean power and spike frequency, while ENBA treatment
444 alone terminated sustained SSE and significantly reduced both mean change in gamma power
445 and spiking frequency.
446 In previous investigations, we observed a causal relationship between seizure termination and
447 neuroprotective efficacy of anticonvulsant drugs after NA intoxication (McDonough et al., 1995;
448 Shih et al., 2003). In this study, as expected, the GD/MDZ groups regardless of treatment time

(15- or 30-min after SSE) displayed high neuropathology scores of 16.9 - 21.4 (out of 24 max damage score) at the end of 2- or 14-days, as these groups did not experience seizure termination. Conversely, the animals receiving MDZ+ENBA had 100% seizure suppression, and their neuropathology scores were markedly reduced to scores of 6.8 – 8.4 (out of 24 max damage score) at the end of 2- or 14-days. These results are comparable to previous studies (Loughery et al., 2021; Meads et al., 2021) where animals exposed to GD and treated with ENBA alone experienced significantly reduced neuropathology compared to animals exposed to GD and treated with saline (control) group. Additionally, there was no significant difference in the neuroprotective effects of the combined treatment between the two treatment times at either endpoint, indicating under GD exposure the neuroprotective effect of ENBA can be extended to 30 min after SSE onset, as was observed previously (Loughery et al., 2021).

In addition to the A/N effects of ENBA, A_1AR agonists induce other physiological effects, such as hypothermia, bradycardia, and sedation. These side effects previously dissuaded researchers from exploring the therapeutic potential of A_1AR agonists, such as CPA (Van Helden et al., 1998; Bueters et al., 2002, 2003; Joosen et al., 2004). However, we found that in the case of NA exposure, these effects may be beneficial in preventing neuroinflammation and neurodegeneration, as well as enhancing behavioral recovery and quality of life after severe CNS insults (Meads et al., 2021; Shih, 2023). Furthermore, therapeutic hypothermia was found to offer neuroprotective benefits (Cilio and Ferriero, 2010; Niquet et al., 2015; Thomas and Shih, 2019) and is employed as a medical tool (Sawyer 2011; Karnatovskaia et al., 2014). In our case, we observed that the hypothermic effect of ENBA may reduce cholinergic agent-induced (such as NAs) lethality and improve quality of life (Munoz et al., 2021, 2024). It is equally as important to note that these side effects are temporary. They are only present during the critical

period of excitatory neuronal crisis, and eventually subside, with animals returning to normal baseline activity within 24 - 48 h (Loughery et al., 2021). Animals that received MDZ+ENBA treatment in this study began regaining mobility around 72 h post-exposure and were fully alert, responsive, and mobile by the 14-day study endpoint.

Surprisingly, unlike previous studies, we saw increased lethality in the GD/MDZ+ENBA treatment groups as compared to the GD/MDZ groups. Following GD exposure, all MDZ-treated animals, regardless of treatment timepoint, survived to their 2- or 14-day endpoints. However, when ENBA (60 mg/kg) was combined with MDZ in the treatment (i.e., GD/MDZ+ENBA) these animals experienced a reduction in survival rate (73% and 86% survival for 15- and 30-min treatments, respectively) at the 2-day endpoint, and exhibited a further decrease in survival rate (40% and 15% survival for 15-and 30-min treatments, respectively) at the 14-day endpoint. These results differ from our previous study (Loughery et al., 2021) where giving ENBA alone did not appear to impact survival, and GD/ENBA-treated animals at 7 days did not differ significantly from the GD/Sal controls. We suspect that the discrepancy could be due to toxic drug interactions between ENBA and MDZ, which may have caused the unexpected lethality, since in the current study the dose of MDZ when administered to our Sal/MDZ or GD/MDZ groups caused minimum or very mild effects on EEG activity and other toxic signs.

One possible explanation of the increased mortality and toxic signs observed in the ENBA+MDZ group is that the ENBA dose applied in current study was too high. We chose to give 60 mg/kg of ENBA in combination with MDZ because this dose was effective as a standalone treatment for NA exposure (Thomas et al., 2019; Loughery et al., 2021). The dose was determined based on previous pilot studies, as well as considerations for its A/N efficacy while increasing survival following GD exposure. In those pilot studies (Thomas et al. 2019), we

aimed to evaluate the neuroprotective potential of A_1AR agonists, including ENBA, CCPA, and CPA, administered alone at various time points following sustained *status epilepticus* (SSE) onset. Our decision to utilize a dose of 60 mg/kg was informed by findings from Thomas et al. (2019), where ENBA dosages ranging from 10 to 62 mg/kg were investigated and the dose of 60 mg/kg was optimal for achieving the desired A/N effects. This dose of ENBA was further assessed and evaluated in Loughery et al. (2021). In the context of intense SSE and delayed treatment following SSE, a higher dose alone may be warranted to effectively mitigate the seizure and neurological effects after GD exposure (Meads et al., 2021; Munoz et al., 2024). Thus, this single dose of ENBA was proposed for this study without dose-response testing when in combination with the standard MCMs (including MDZ).

However, when combined with MDZ (particularly because both MDZ and ENBA display similar neuronal inhibitory (e.g., sedation) effects on their own) this dose of ENBA appears to be too potent, producing toxic effects that increase lethality. While MDZ is a benzodiazepine anticonvulsant that causes sedation, hypnosis, and muscular relaxation, it has been associated with decreased tidal volume and respiratory rate (Mihic and Harris, 2011). As previously noted, in addition to its anticonvulsive properties, ENBA also produces physiological inhibitory side effects, such as sedation and slowing down of other metabolic functions. This finding implies that when combining with a benzodiazepine anticonvulsant, the dose of ENBA should be reduced to lessen the side effects of both drugs. To minimize these side effects and improve survival outcomes, a future experiment is necessary to determine a balanced and optimal dose of ENBA that may be given in combination with standard medical treatments for NAs. In view of the results obtained from this study, follow-up dose-response studies focusing on survival rates using this and similar combinations (e.g., lower ENBA doses) will be important to fully

understand the complex role of adenosine signaling in adjunctive therapies. The 100% termination of seizures was impressive in the results with ENBA+MDZ groups and along with marked reduction of neuropathology, re-confirmed the direct connection between seizures and neuropathology (McDonough et al., 1995; Shih et al., 2003). Moving forward, increasing, and not decreasing survival rate is an important indicator and key goal in treating NA intoxication.

It is also interesting to note that, should delayed treatment following NA exposure be necessary, such as in mass casualties scenario, A_1AR agonists (e.g., ENBA) may serve as an alternative to benzodiazepine anticonvulsants (e.g., MDZ, diazepam), since ENBA alone can provide the critical anti-seizure function and eliminate or reduce the subsequent neuropathology and behavioral deficits (Loughery et al., 2021; Meads et al., 2021, Harkins et al., 2023).

In summary, our current study successfully demonstrated the therapeutic efficacy for A_1AR agonism as an A/N medical treatment for GD exposure when given in conjunction with standard medical treatment during a period when SSE is well established. Though future studies are needed to determine an optimal dose of ENBA when administering with MDZ, this combinatorial treatment effectively terminated GD-induced seizure activity and enhanced neuroprotection. The findings of this study together with our earlier study with a singular standalone ENBA treatment (Loughery et al., 2021) suggest that the adenosine signaling pathway, particularly the A_1AR stimulation, is a promising A/N adjunct to standard medical treatment for either immediate therapy or when seizure activity is well established. Additionally, it may serve as an alternative to benzodiazepine anticonvulsant treatment against NAs when the therapy is expected to be delayed pending on arrival of first responders.

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Declaration of Interests Statement

The authors have no conflicts of interest or competing financial interests to declare that are relevant to the content of this article. None of the authors has during the last five years appeared in any legal or regulatory proceedings related to the contents of the paper. The authors alone are responsible for the content and writing of the paper.

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672

673 ## Figure Legends

674 **Figure 1. Chemical structure of ENBA**.

675 **Figure 2. Experimental paradigm**. Rats were treated with HI-6 (125 mg/kg, i.p.) 30 min before
676 an injection of saline (0.5 ml/kg, s.c.), served as control exposure, or a seizure inducing dose of
677 GD (90 µg/kg, s.c.). One min later, rats received an injection of atropine methyl nitrate (AMN,
678 2-4 mg/kg, i.m.). All rats received $ATSO_4$ and 2-PAM (0.45 mg/kg and 25 mg/kg, respectively).
679 In addition, rats received either MDZ (1.8 mg/kg) (collectively termed MDZ treatment group) or
680 MDZ (1.8 mg/kg) + ENBA (60 mg/kg) (collectively termed MDZ+ENBA treatment group) at
681 one of two treatment times (either 15 or 30 min) following SSE seizure onset (or average SSE
682 onset in saline-exposure rats). Rats were euthanized at either 2 days or 14 days after GD or saline
683 exposure for brain neuropathology assessment.

684 **Figure 3. EEG Power and Spike Frequency Analysis following GD Exposure and**
685 **Treatment**. EEG spectral analysis shows change in mean gamma (ɣ) band power (dB) ± SD in
686 (A) 15-min and (B) 30-min treated groups, and mean spiking frequency (Hz) ± SD in (C) 15-min
687 and (D) 30-min treated groups over time (min) for GD-exposed animals over a 4-h experimental
688 window. An assessment of EEG spectral analysis was completed on QuoStatus (Python-based
689 software, University of Utah). Mean change in gamma band power (20-60 Hz) and spike
690 frequency from baseline at hourly intervals relative to baseline for each treatment group was
691 compared. All adenosine A_1 agonist ENBA-treated groups displayed significantly lower power
692 and spike frequency from non-ENBA-treated (i.e., MDZ) groups during the 4-h experimental
693 window (15-min, $p<0.0001$; 30-min, $p<0.0001$; power and spike frequency). (*) indicates
694 datapoints where GD/MDZ+ENBA group is significantly different ($p\leq0.05$) than the GD/MDZ
695 group.

696

Figure 4. Mean Convulsive Score: 15 and 30-min Treatment on Experiment Day, GD Exposure. Displays mean convulsive activity scores ± SD for treatments administered at (A) 15 min or (B) 30 min for the 5-h post-exposure observation period. Convulsive activity scores were recorded at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 60 min intervals for 5 h after GD. The GD/MDZ+ENBA-treated groups displayed lower convulsion scores compared to GD/MDZ groups by the 30-min timepoint ($p=0.0027$) for the 15-min treated groups, and by 45-min timepoint ($p=0.0023$) for the 30-min treated groups. (*) indicates datapoints where GD/MDZ+ENBA group is significantly different ($p\leq0.05$) than the GD/MDZ group.

Figure 5. Mean Righting Score: 15-min Treatment on Experiment Day, Saline and GD Exposure. Displays mean righting reflex ± SD for groups treated at 15 min post seizure onset for the 5-h post-exposure observation period. Righting reflex scores were recorded at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 60 min intervals for 5-h after (A) saline or (B) GD exposure. Righting impairment in the Sal/MDZ-ENBA group began to differ from the MDZ-only group by the 30-min timepoint ($p=0.0001$). Animals in the GD/MDZ+ENBA group had significantly higher scores compared to their MDZ-only treated counterparts at the 4 and 5 h timepoints ($p=0.0053$, $p=0.0013$ respectively). (*) indicates datapoints where MDZ+ENBA group is significantly different ($p\leq0.05$) than the MDZ group**.**

Figure 6. Mean Righting Score: 30-min Treatment on Experiment Day, Saline and GD Exposure. Displays mean righting reflex ± SD for groups treated at 30-min post seizure onset for the 5-h post-exposure observation period. Righting reflex scores were recorded at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 60 min intervals for 5-h after (A) saline or (B) GD exposure. Righting impairment in the Sal/MDZ-ENBA groups began to differ from the MDZ-only groups by the 45-min timepoints ($p<0.0001$). The GD/MDZ and GD/MDZ+ENBA groups

did not significantly differ during the experiment day. (*) indicates datapoints where MDZ+ENBA group is significantly different ($p \leq 0.05$) than the MDZ group**.**

Figure 7. Mean Body Temperature: 15-min Treatment on Experiment Day, Saline and GD Exposure. Displays mean body temperature ± SD for groups treated at 15-min post seizure onset for the 5-h post-exposure observation period. Body temperature was recorded at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 60 min intervals for 5 h after (A) saline or (B) GD exposure. Sal/MDZ+ENBA group showed steady declines in temperature (following treatment) compared to the Sal/MDZ group by the 45-min timepoint ($p=0.0002$). The body temperatures of the GD/MDZ+ENBA animals decreased steadily over the course of the observation period compared to the GD/MDZ animals, beginning at 1 h post exposure ($p=0.0058$). (*) indicates datapoints where MDZ+ENBA group is significantly different ($p \leq 0.05$) than the MDZ group**.**

Figure 8. Mean Body Temperature: 30-min Treatment on Experiment Day, Saline and GD Exposure. Displays mean body temperature ± SD for groups treated at 30-min post seizure onset for the 5-h post-exposure observation period. Body temperature was recorded at 0, 4, 8, 15, 30, 45, and 60 min, and thereafter at 60 min intervals for 5 h after (A) saline or (B) GD exposure. Sal/MDZ+ENBA group showed steady declines in temperature (following treatment) compared to the Sal/MDZ group by the 2 h timepoint ($p=0.0218$). The body temperatures of the GD/MDZ+ENBA animals decreased steadily over the course of the observation period compared to the GD/MDZ animals, beginning at 2 h post exposure ($p<0.0001$). (*) indicates datapoints where MDZ+ENBA group is significantly different ($p \leq 0.05$) than the MDZ group**.**

Figure 9. Mean Percent Change in Heart Rate: 15-min Treatment on Experiment Day, Saline and GD Exposure. Mean percent change in heart rate from baseline by (A) saline and (B) GD exposure, for groups treated 15-min post seizure onset. The percent heart change from

baseline was calculated as: (100 x (24 h heart rate – baseline heart rate))/baseline heart rate for each animal before averaging. Displays mean percent change heart rate ± SD for groups treated at 15-min post seizure onset for the 5-h post-exposure observation period. Heart rates were taken every h for the first 5 h post-exposure. The Sal/MDZ+ENBA group had lower heart rate by the 1-h timepoint than the Sal/MDZ group by the 1 h timepoint ($p<0.0001$) that continued through the end of the 5 h observation period ($p<0.0001$). The heart rates for GD/MDZ+ENBA group was significantly lower than their GD/MDZ counterparts by the 1-h timepoint ($p<0.0001$) and did not recover before the end of the 5 h observation period ($p<0.0001$). (*) indicates datapoints where MDZ+ENBA group is significantly different ($p\leq0.05$) than the MDZ group.

Figure 10. Mean Percent Change in Heart Rate: 30-min Treatment on Experiment Day, Saline and GD Exposure. Mean percent change in heart rate from baseline by (A) saline and (B) GD exposure, for groups treated 30-min post seizure onset. The percent heart change from baseline was calculated as: (100 x (24 h heart rate – baseline heart rate))/baseline heart rate for each animal before averaging. Displays mean percent change heart rate ± SD for groups treated at 30-min post seizure onset for the 5-h post-exposure observation period. Heart rates were taken every h for the first 5 h post-exposure. The Sal/MDZ+ENBA groups had lower heart rate by the 1-h timepoint than their Sal/MDZ counterparts by the 1 h timepoint ($p<0.0001$) that continued through the end of the 5 h observation period ($p<0.0001$). The heart rates for GD/MDZ+ENBA group was significantly lower than their GD/MDZ counterparts by the 1-h timepoint ($p<0.0001$) and did not recover before the end of the 5 h observation period ($p<0.0001$). (*) indicates datapoints where MDZ+ENBA group is significantly different ($p\leq0.05$) than the MDZ group.

Figure 11. Kaplan-Meier Survival Curves: 15-min Treatment, 14-Day Endpoint, Saline and GD Exposure. Kaplan-Meier survival curves for (A) saline or (B) GD exposure, for 14-day

endpoint groups treated 15-min post seizure onset. Displays survival curves for treatments administered at 15 min post seizure for days 1, 3, 7, and 14 post-exposures. Survival was monitored daily. The GD/MDZ+ENBA group did see a significant loss of animals (73% survival) compared to their MDZ-only counterpart (100% survival, p=0.0196).

Figure 12. Kaplan-Meier Survival Curves: 30-min Treatment, 14-Day Endpoint, Saline and GD Exposure. Kaplan-Meier survival curves for (A) saline or (B) GD exposure, for groups treated 30-min post seizure onset. Displays survival curves for treatments administered at 30 min post seizure for days 1, 2, 3, 7, and 14 post-exposures. Survival was monitored daily. The Saline/MDZ+ENBA did not have a significant loss of animals (89% survival) compared to the Saline/MDZ group (100% survival, p=0537). The GD/MDZ+ENBA group did see a significant loss of animals (40% survival) compared to their MDZ-only counterpart (100% survival, p=0.0006).

Figure 13. Mean Total Pathology Scores: 15- and 30-min Treatment, 2-Day Endpoint, GD Exposure. Mean total brain neuropathology scores by (A) 15- and (B) 30-min treatment following GD exposure. Rats were euthanized at 2 days after GD exposure. Six brain regions associated with severe neurological deficits after NA exposure (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 (McDonough et al., 1995): 0 = no lesion; 1 = minimal (1-10%); 2 = mild (11-25%); 3 = moderate (26-45%); 4 = severe (>45%). Displays mean total brain neuropathology scores ± SD. The total scores for the GD/MDZ+ENBA groups were significantly lower than those of the GD/MDZ groups (15-min, p=0.015; 30-min, p=0.002). (*) indicates whether MDZ+ENBA group is significantly different ($p \leq 0.05$) than the MDZ group.

Figure 14. Mean Total Pathology Scores: 15- and 30-min Treatment, 14-Day Endpoint, GD Exposure. Mean total brain neuropathology by (A) 15- and (B) 30-min treatment following GD exposure. Rats were euthanized at 14 days after GD exposure. Six brain regions associated with severe neurological deficits after NA exposure (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 (McDonough et al., 1995): 0 = no lesion; 1 = minimal (1-10%); 2 = mild (11-25%); 3 = moderate (26-45%); 4 = severe (>45%). A total neuropathology score was then calculated for each rat from 0 (normal) to 24 (severe damage). Displays total neuropathology scores ± SD. The total neuropathology scores for the GD/MDZ+ENBA groups were significantly lower than those of their respective MDZ-only counterparts (15-min, $p<0.001$; 30-min, $p=0.006$). (*) indicates whether MDZ+ENBA group is significantly different ($p\leq0.05$) than the MDZ group.

Tables

Table 1. Experimental Groups and Animal Numbers

Exposure	Treatment	Number of Animals (N)	
		15 min after SSE	*30 min after SSE*
Saline	MDZ (control)	6	8
	MDZ+ENBA	17	21
GD (90 ug/kg)	MDZ (control)	15	14
	MDZ+ENBA	31	33

Table 1. All rats received $ATSO_4$ and 2-PAM (0.45 mg/kg and 25 mg/kg, respectively). In addition, rats received either MDZ (1.8 mg/kg) (collectively termed MDZ treatment group) or MDZ + ENBA (60 mg/kg) (collectively termed MDZ+ENBA treatment group) at either 15- or 30-min following SSE seizure onset (or average SSE onset in saline-exposure rats). MDZ is the standard medical treatment for NA intoxication and serves as a control treatment for assessing effect of ENBA as an adjunct therapy. Shown are the initial number of animals for each exposure/treatment group.

Table 2. Functional Observation Battery (FOB) for Toxic Motor Signs after Exposure and Treatments

	Score			
Observation	0	1	2	3
Righting Reflex	Normal (Immediate righting)	Impaired (delayed righting)	No righting reflex	---
Convulsive Activity	Normal (Absence of activity)	Fasciculation (Twitching, spontaneous)	Tremors (Localized hyperactivity)	Convulsion (Global)

Table 2. Functional observational battery (FOB)-based neurobehavioral scores (such as righting reflex, startle reflex, motor activity, gait, and arousal) were recorded. However, our presentation here focused on righting reflex and convulsive motor activity because these motor activities are the most representative measure of function or incapacitation for rodents under NA exposure and ENBA treatment.

824 **Table 3. Effects of ENBA as an Adjunct Therapy on Soman (GD)-induced Seizure Activity and Total Neuropathology Scores**

Exposure	Treatment			15 min After SSE			30 min After SSE		
Agent (dose)		N	Latency to Seizure Onset (min ± SD)	% Seizure Suppression (Response Ratio)	Latency to Seizure Suppression (min ± SD)	Total Neuropathology (score ± SD)	% Seizure Suppression (Response Ratio)	Latency to Seizure Suppression (min ± SD)	Total Neuropathology (score ± SD)
GD (90 µg/kg)	MDZ	15	11.0±8.5 (29)	0% (0/15)	N/A	19.14±3.86 (14)			
		14					0% (0/14)	N/A	19.64±2.87 (14)
	MDZ+ENBA	31	7.9±0.6 (64)	100% (31/31)	117.4±14.6 (31)	7.38±1.5 (16)			
		33					100% (33/33)	114.1±16.5 (33)	7.55±1.57 (11)

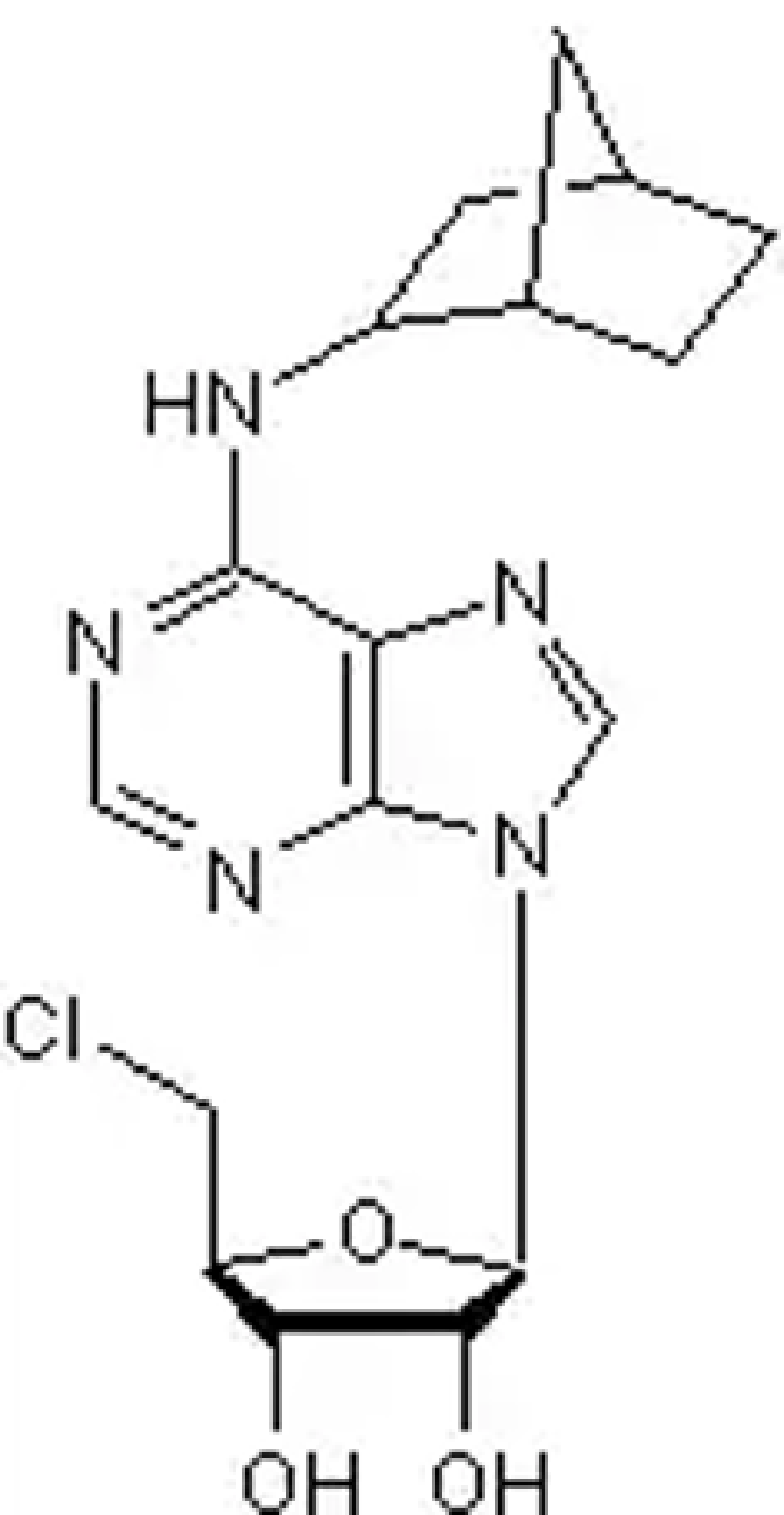
825

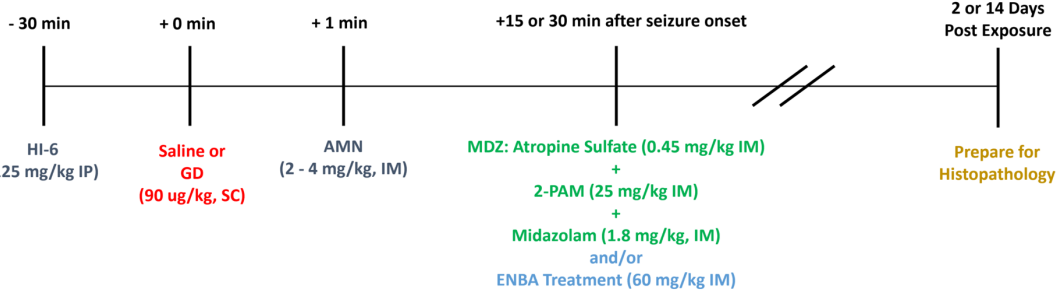
826 **Table 3:** All rats received $ATSO_4$ and 2-PAM (0.45 mg/kg and 25 mg/kg, respectively). In addition, rats received either MDZ (1.8
827 mg/kg) (collectively termed MDZ treatment group) or MDZ+ENBA (60 mg/kg) (collectively termed MDZ+ENBA treatment group)
828 at either 15- or 30-min following SSE seizure onset. Displays seizure terminating summary of MDZ or MDZ+ENBA treatment groups
829 and total neuropathology scores assessed at 14 days after GD exposure.

Table 4. Survival Rate by 15- and 30-min Treatment following Saline and Soman (GD) Exposure at 2- or 14-day Endpoint

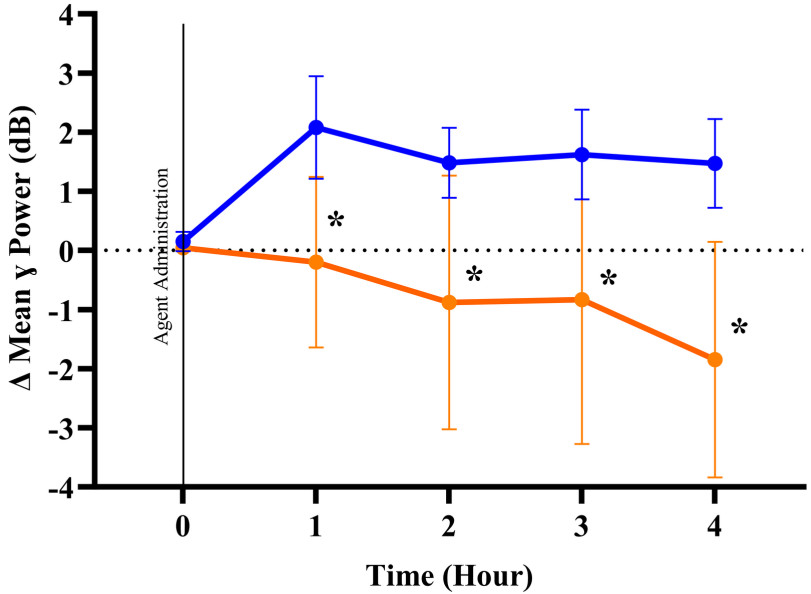
Group			Endpoint	
Exposure	Delayed Treatment after SSE (min)	Treatment	2-Day Survival Rate (%)	14-Day Survival Rate (%)
Saline	**15**	MDZ	3/3 (100)	3/3 (100)
		MDZ+ENBA (60 mg/kg)	8/8 (100)	8/9 (89)
	30	MDZ	4/4 (100)	4/4 (100)
		MDZ+ENBA (60 mg/kg)	7/8 (88)	9/10 (90)
GD (90 ug/kg)	**15**	MDZ	7/7 (100)	7/7 (100)
		MDZ+ENBA (60 mg/kg)	8/11 (73)	8/20 (40)
	30	MDZ	6/6 (100)	8/8 (100)
		MDZ+ENBA (60 mg/kg)	6/7 (86)	5/26 (19)

Table 4. Survival rate (percentage) for groups exposed to saline or GD and treated with MDZ or MDZ+ENBA at either 15-min or 30-min after SSE seizure onset, separated by 2- or 14-day endpoint. Survival was monitored daily. The survival calculation for 14-day endpoint does not include animals from 2-day endpoint and vice versa.

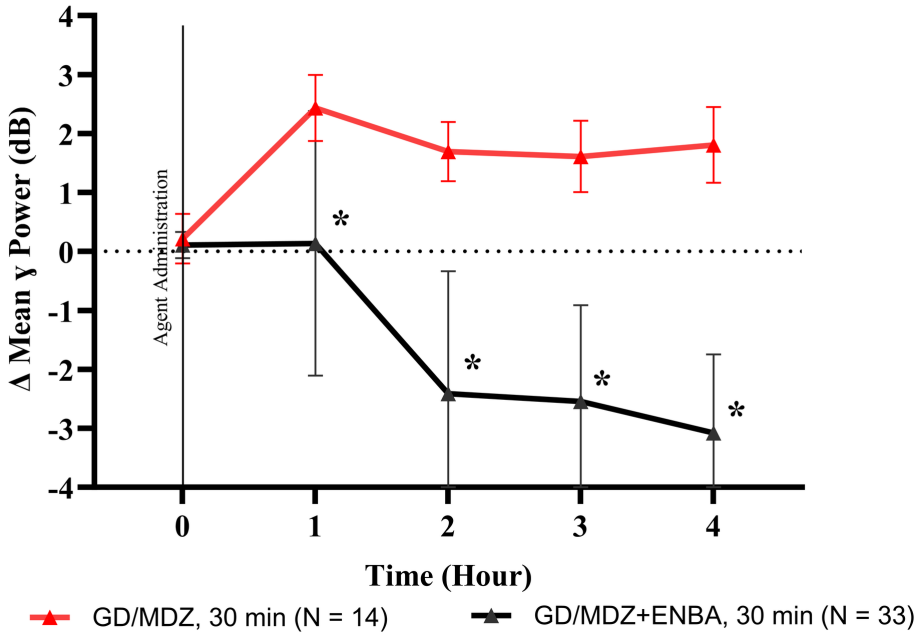

HN
N
N
N
N
Cl
O
OH
OH


- 30 min
+ 0 min
+ 1 min
+15 or 30 min after seizure onset
2 or 14 Days
Post Exposure
HI-6
(125 mg/kg IP)
Saline or
GD
(90 ug/kg, SC)
AMN
(2 - 4 mg/kg, IM)
MDZ: Atropine Sulfate (0.45 mg/kg IM)
+
2-PAM (25 mg/kg IM)
+
Midazolam (1.8 mg/kg, IM)
and/or
ENBA Treatment (60 mg/kg IM)
Prepare for
Histopathology

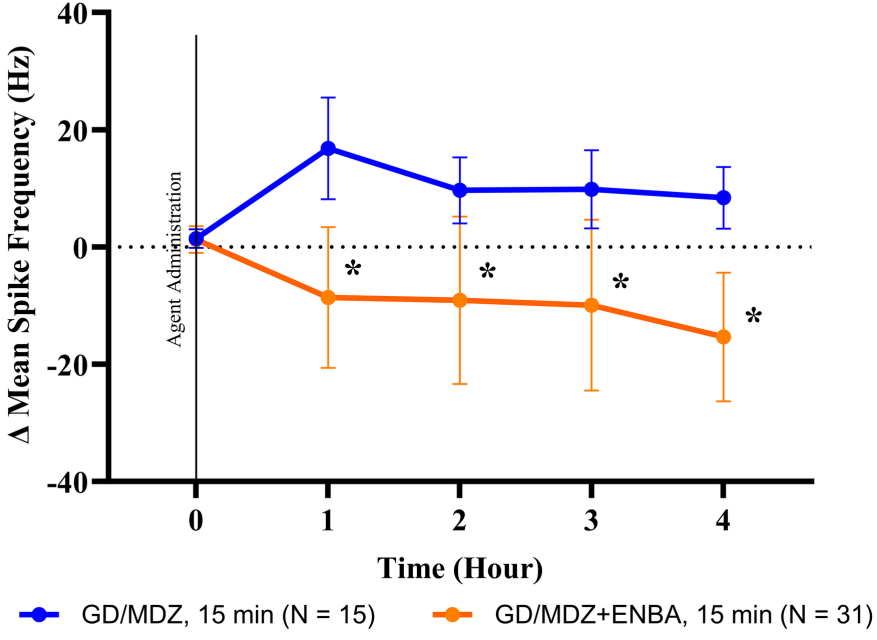
A.


Δ Mean γ Power (dB)
4
3
2
1
0
-1
-2
-3
-4
Agent Administration
*
*
*
*
0
1
2
3
4
Time (Hour)
GD/MDZ, 15 min (N = 15)
GD/MDZ+ENBA, 15 min (N = 31)

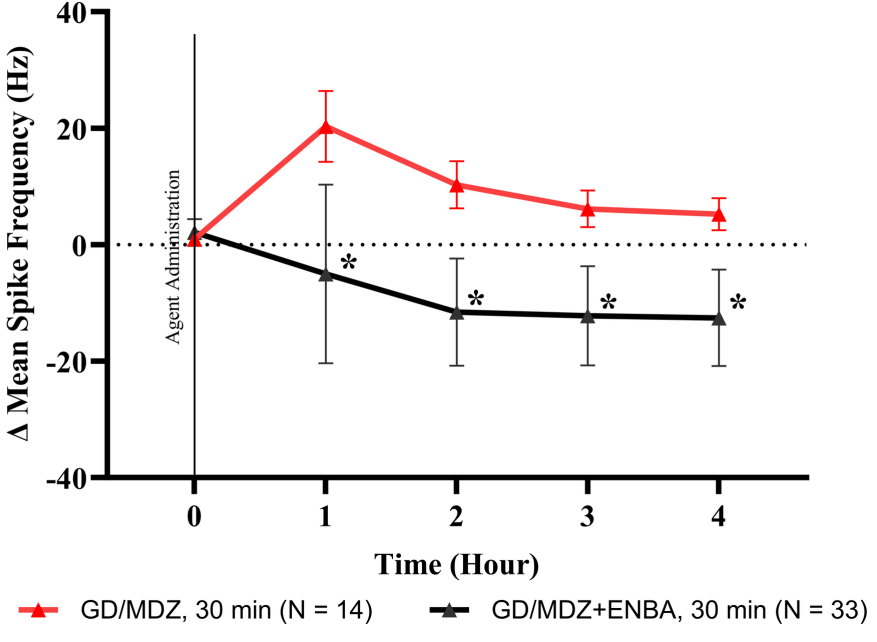
B.


Δ Mean y Power (dB)
4
3
2
1
0
-1
-2
-3
-4
Agent Administration
*
*
*
*
0
1
2
3
4
Time (Hour)
GD/MDZ, 30 min (N = 14)
GD/MDZ+ENBA, 30 min (N = 33)

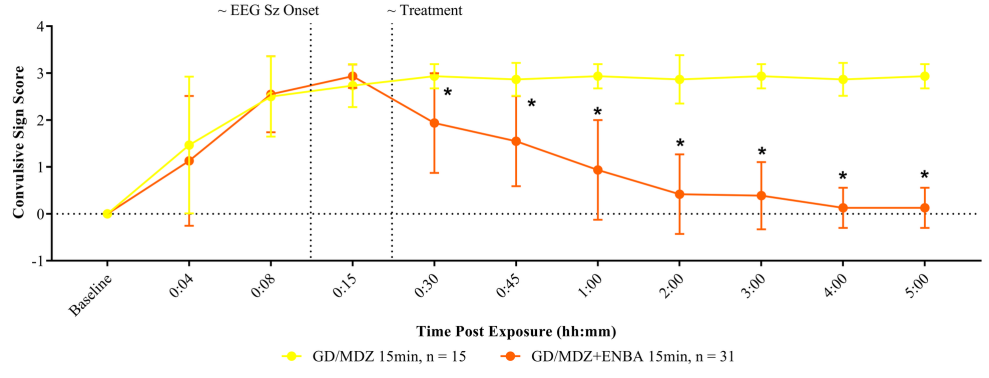
C.


40
20
0
-20
-40
Δ Mean Spike Frequency (Hz)
Agent Administration
*
*
*
*
0
1
2
3
4
Time (Hour)
GD/MDZ, 15 min (N = 15)
GD/MDZ+ENBA, 15 min (N = 31)

D.


40
20
0
-20
-40
Δ Mean Spike Frequency (Hz)
Agent Administration
*
*
*
*
0
1
2
3
4
Time (Hour)
GD/MDZ, 30 min (N = 14)
GD/MDZ+ENBA, 30 min (N = 33)

A.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~ Treatment
Convulsive Sign Score
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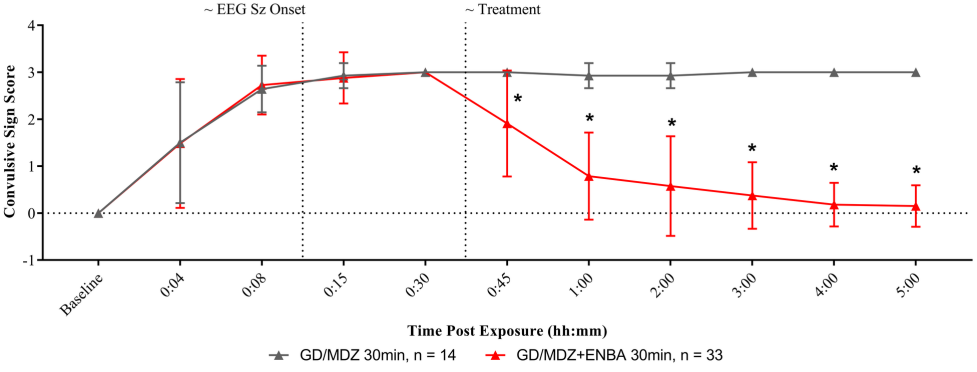
3
2
1
0
-1
*
Baseline
0:04
0:08
0:15
0:30
0:45
1:00
2:00
3:00
4:00
5:00
Time Post Exposure (hh:mm)
GD/MDZ 15min, n = 15
GD/MDZ+ENBA 15min, n = 31

B.


~ EEG Sz Onset
~ Treatment
Convulsive Sign Score
4
3
2
1
0
-1
*
Baseline
0:04
0:08
0:15
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0:45
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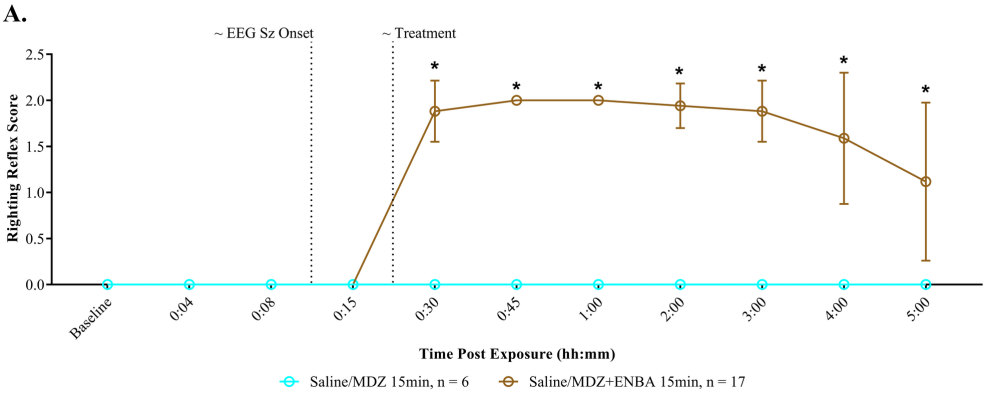
5:00
Time Post Exposure (hh:mm)
GD/MDZ 30min, n = 14
GD/MDZ+ENBA 30min, n = 33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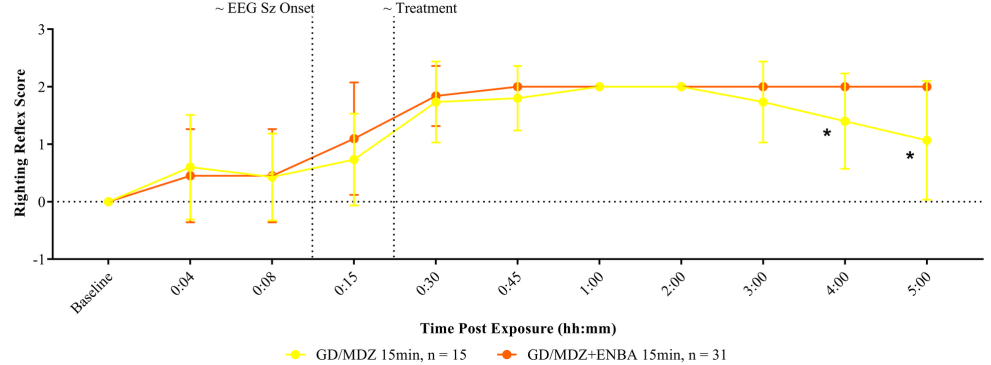
~ Treatment
Righting Reflex Score
2.5
2.0
1.5
1.0
0.5
0.0
*
Baseline
0:04
0:08
0:15
0:30
0:45
1:00
2:00
3:00
4:00
5:00
Time Post Exposure (hh:mm)
Saline/MDZ 15min, n = 6
Saline/MDZ+ENBA 15min, n = 17

B.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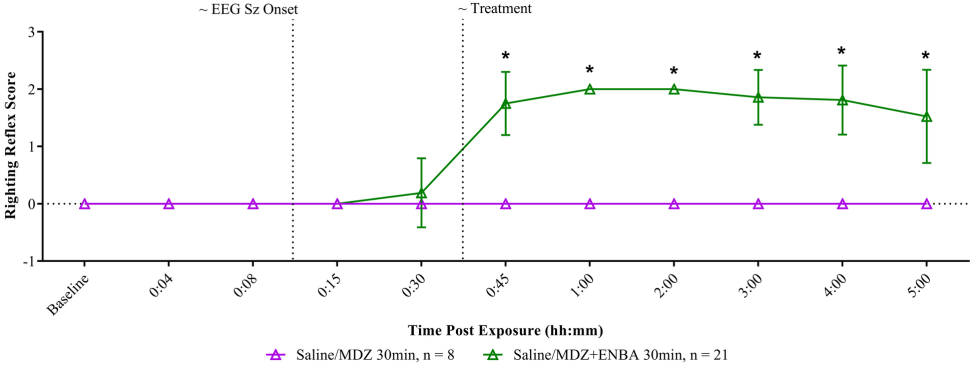
~ Treatment
Righting Reflex Score
3
2
1
0
-1
*
*
Baseline
0:04
0:08
0:15
0:30
0:45
1:00
2:00
3:00
4:00
5:00
Time Post Exposure (hh:mm)
GD/MDZ 15min, n = 15
GD/MDZ+ENBA 15min, n = 31

A.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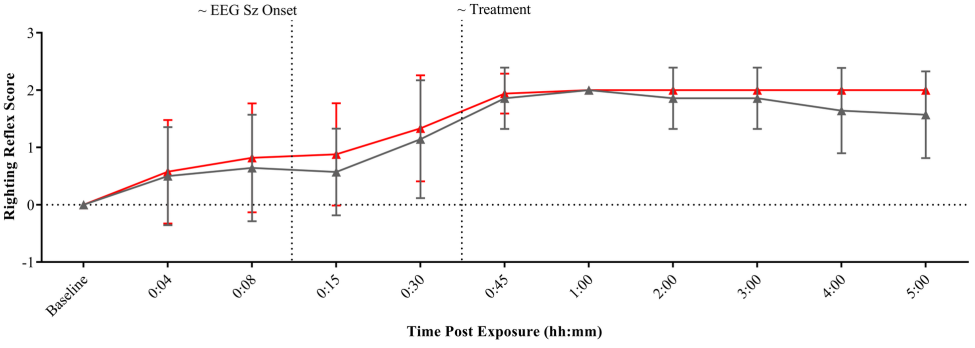
~ Treatment
Righting Reflex Score
3
2
1
0
-1
*
Baseline
0:04
0:08
0:15
0:30
0:45
1:00
2:00
3:00
4:00
5:00
Time Post Exposure (hh:mm)
Saline/MDZ 30min, n = 8
Saline/MDZ+ENBA 30min, n = 21

B.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~ Treatment
Righting Reflex Score
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2
1
0
-1
Baseline
0:04
0:08
0:15
0:30
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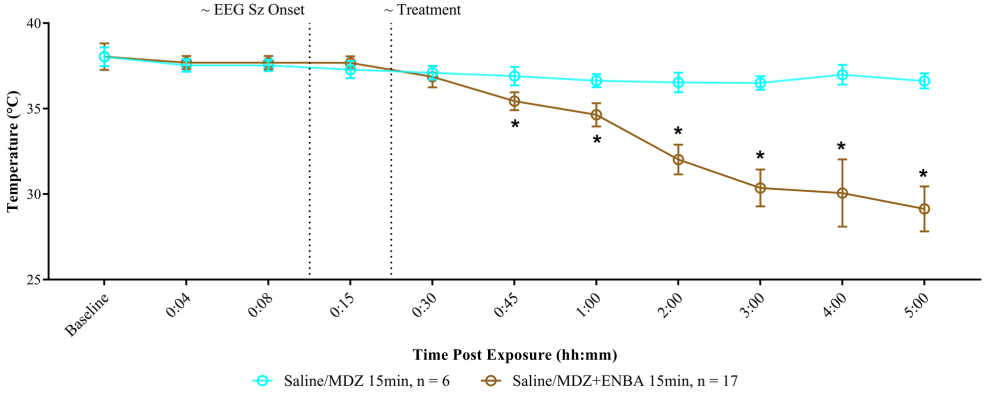
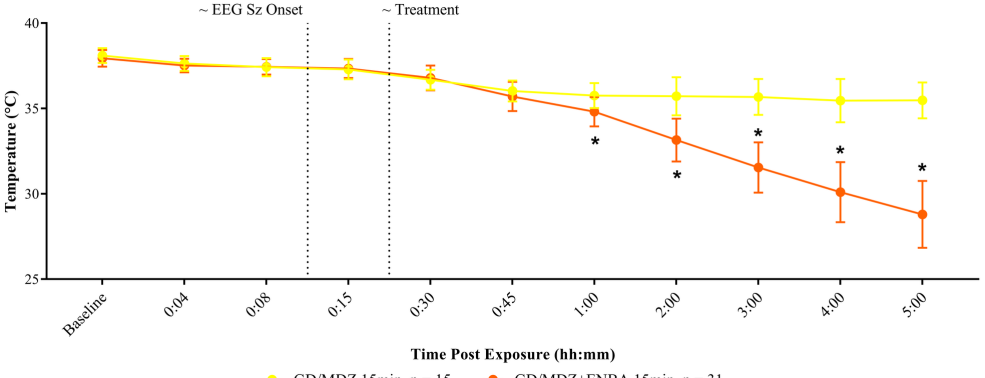
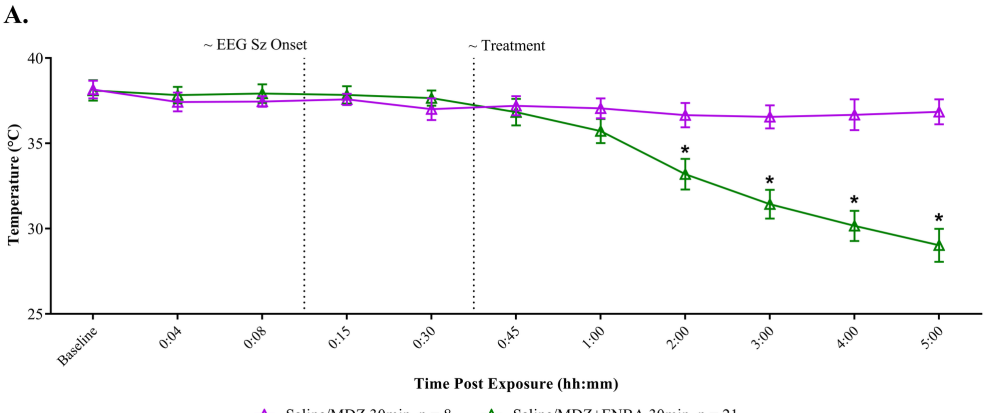
1:00
2:00
3:00
4:00
5:00
Time Post Exposure (hh:mm)
GD/MDZ 30min, n = 14
GD/MDZ+ENBA 30min, n = 31

A.

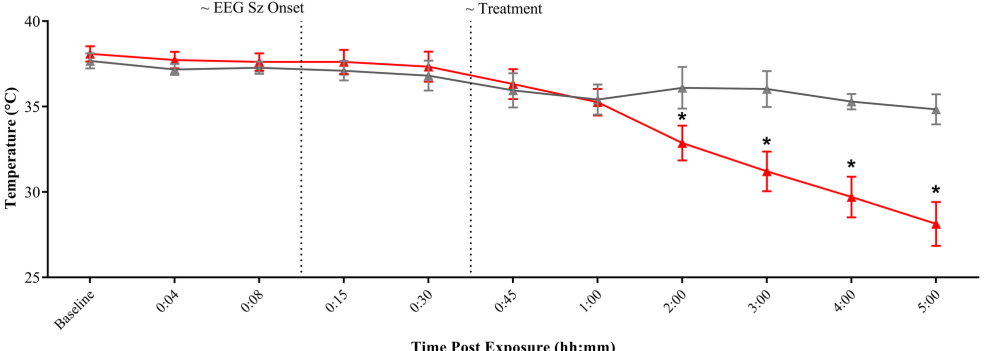

~ EEG Sz Onset
~ Treatment
Temperature (°C)
40
35
30
25
Baseline
0:04
0:08
0:15
0:30
0:45
1:00
2:00
3:00
4:00
5:00
*
Time Post Exposure (hh:mm)
Saline/MDZ 15min, n = 6
Saline/MDZ+ENBA 15min, n = 17

B.

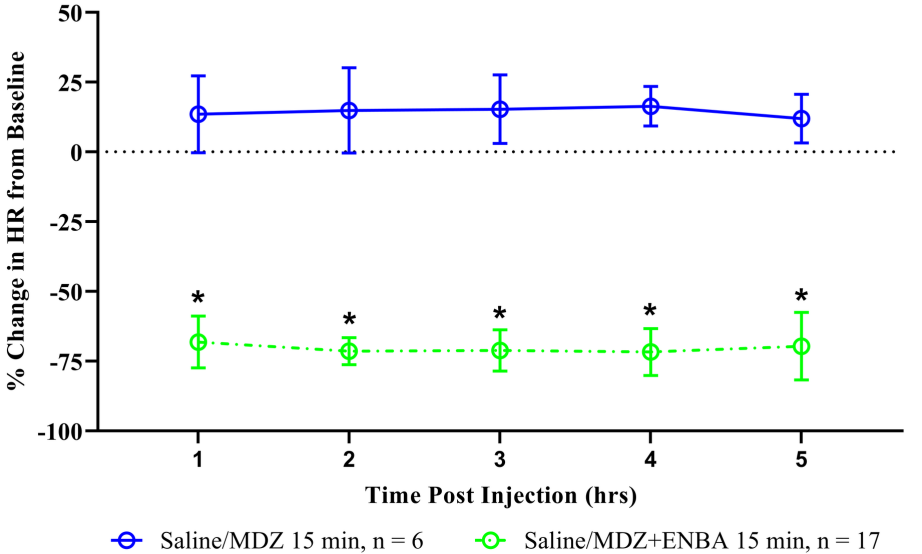

~ EEG Sz Onset
~ Treatment
Temperature (°C)
40
35
30
25
Baseline
0:04
0:08
0:15
0:30
0:45
1:00
2:00
3:00
4:00
5:00
*
Time Post Exposure (hh:mm)
GD/MDZ 15min, n = 15
GD/MDZ+ENBA 15min, n = 31


A.
~ EEG Sz Onset
~ Treatment
40
35
30
25
Temperature (°C)
Baseline
0:04
0:08
0:15
0:30
0:45
1:00
2:00
3:00
4:00
5:00
Time Post Exposure (hh:mm)
Saline/MDZ 30min, n = 8
Saline/MDZ+ENBA 30min, n = 21

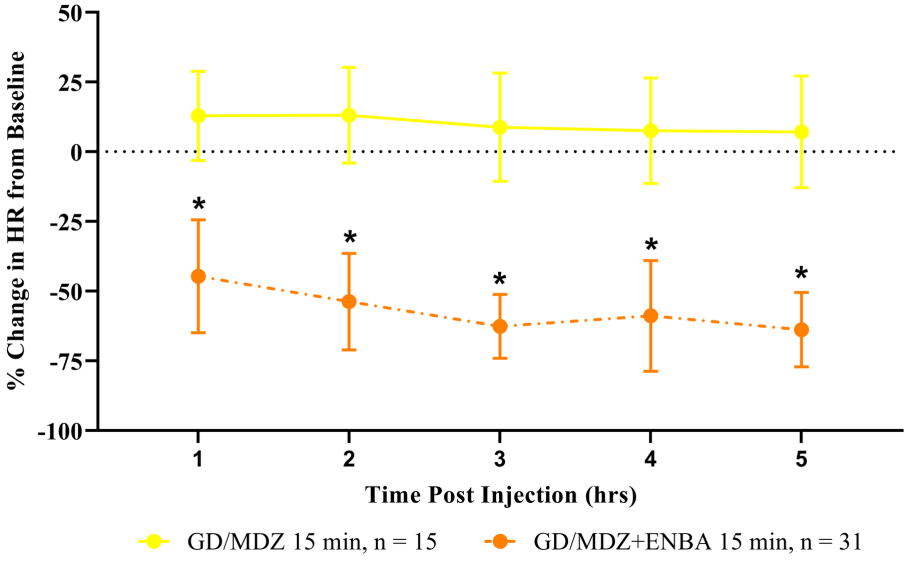
B.


~ EEG Sz Onset
~ Treatment
Temperature (°C)
40
35
30
25
Baseline
0:04
0:08
0:15
0:30
0:45
1:00
2:00
3:00
4:00
5:00
*
Time Post Exposure (hh:mm)
GD/MDZ 30min, n = 14
GD/MDZ+ENBA 30min, n = 33

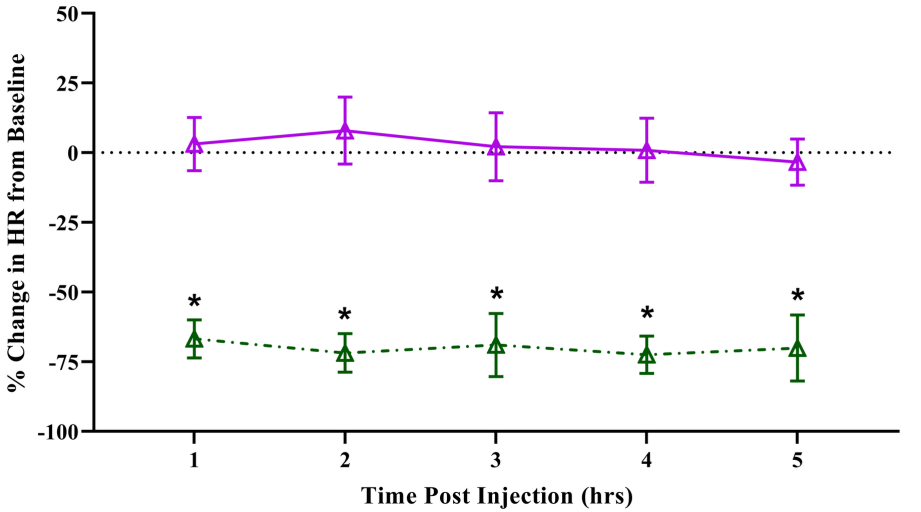
A.


50
25
0
-25
-50
-75
-100
% Change in HR from Baseline
*
*
*
*
*
1
2
3
4
5
Time Post Injection (hrs)
Saline/MDZ 15 min, n = 6
Saline/MDZ+ENBA 15 min, n = 17

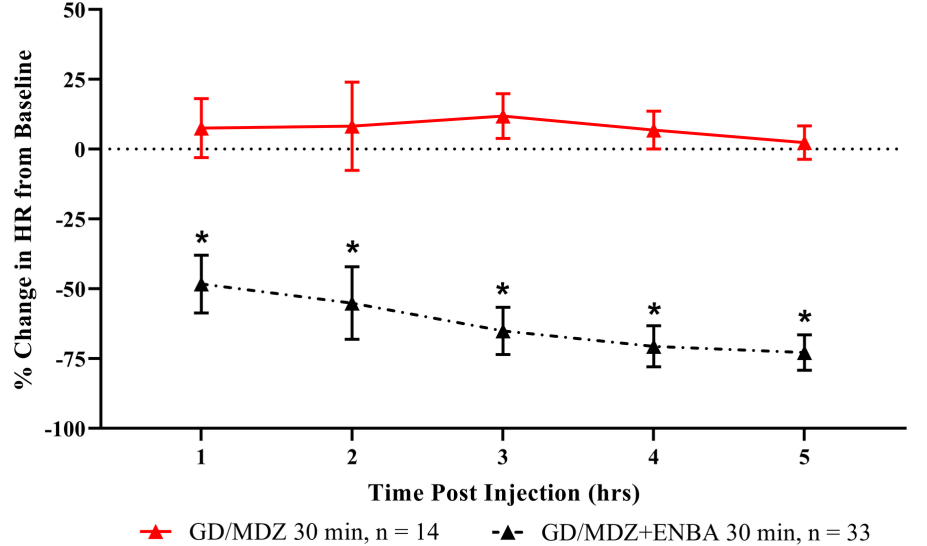
B.


50
25
0
-25
-50
-75
-100
% Change in HR from Baseline
*
*
*
*
*
1
2
3
4
5
Time Post Injection (hrs)
GD/MDZ 15 min, n = 15
GD/MDZ+ENBA 15 min, n = 31

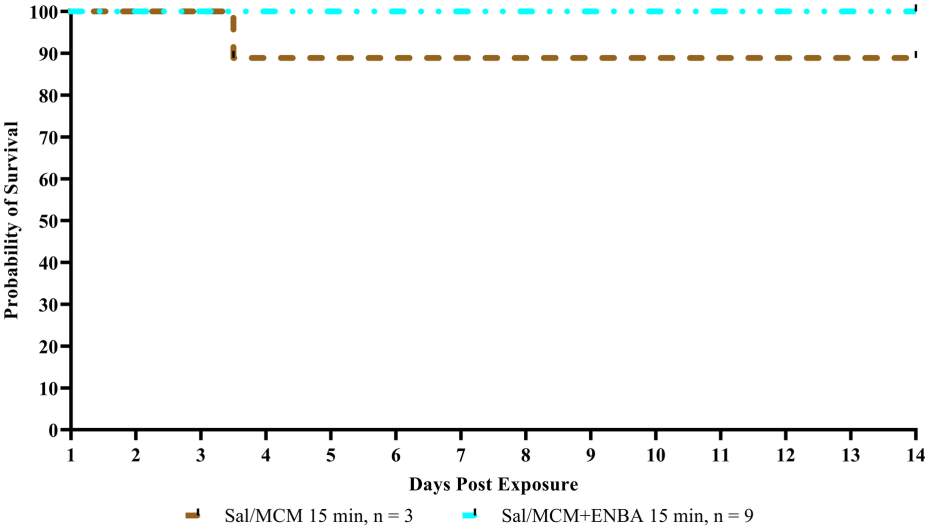
A.


50
25
0
-25
-50
-75
-100
% Change in HR from Baseline
*
*
*
*
*
1
2
3
4
5
Time Post Injection (hrs)
Saline/MDZ 30 min, n = 8
Saline/MDZ+ENBA 30 min, n = 21

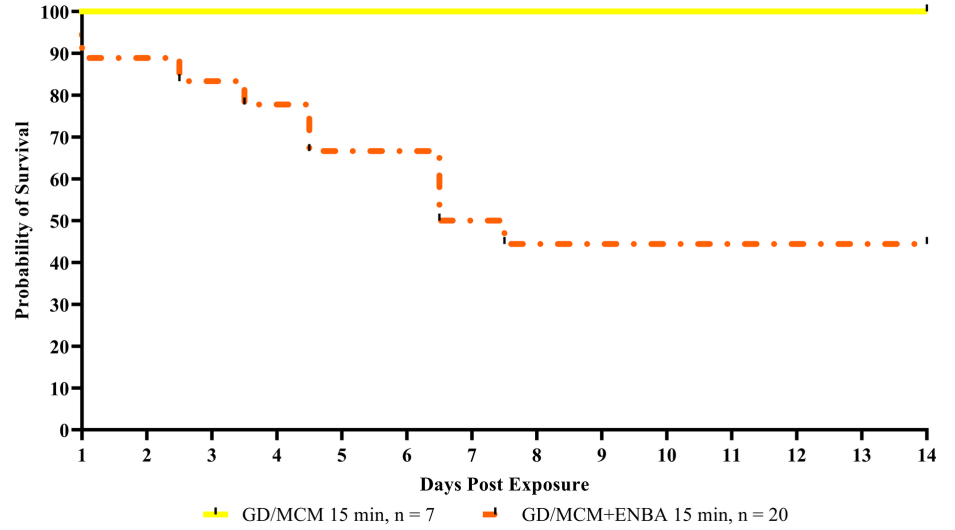
B.


50
25
0
-25
-50
-75
-100
% Change in HR from Baseline
*
*
*
*
*
1
2
3
4
5
Time Post Injection (hrs)
GD/MDZ 30 min, n = 14
GD/MDZ+ENBA 30 min, n = 33

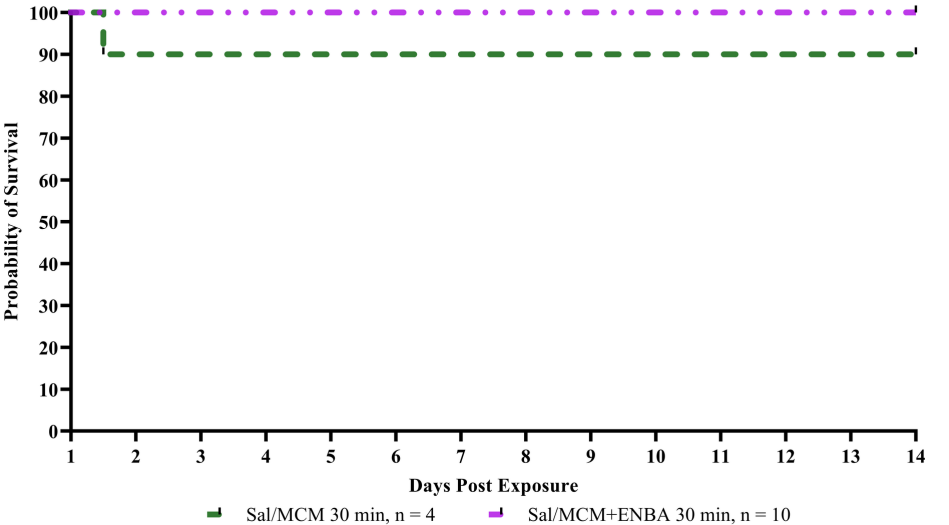
A.


100
90
80
70
60
50
40
30
20
10
0
Probability of Survival
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days Post Exposure
Sal/MCM 15 min, n = 3
Sal/MCM+ENBA 15 min, n = 9

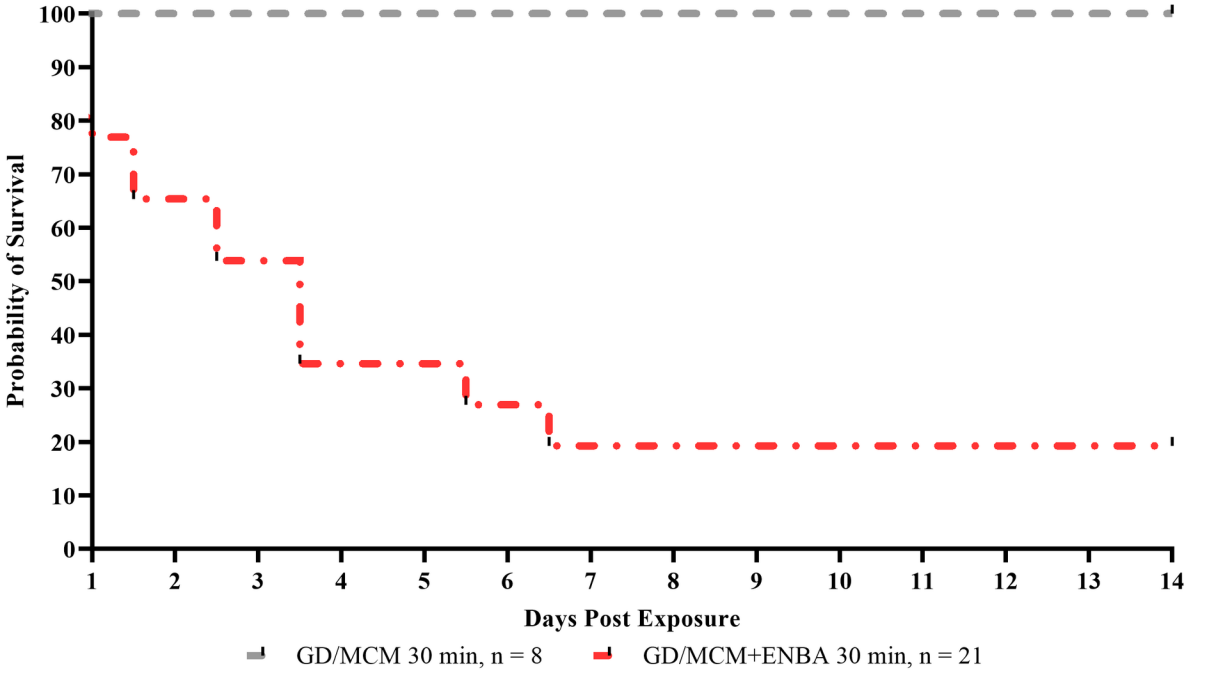
B.

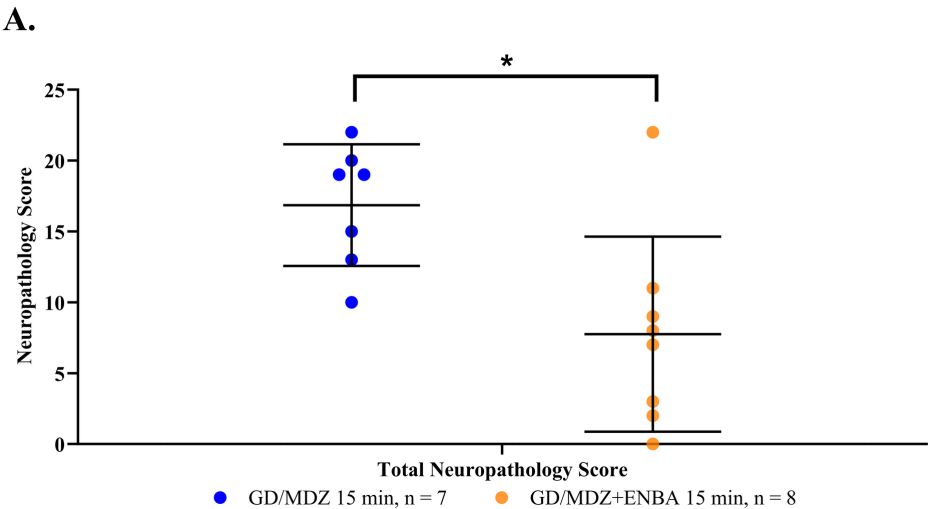

100
90
80
70
60
50
40
30
20
10
0
Probability of Survival
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days Post Exposure
GD/MCM 15 min, n = 7
GD/MCM+ENBA 15 min, n = 20

A.

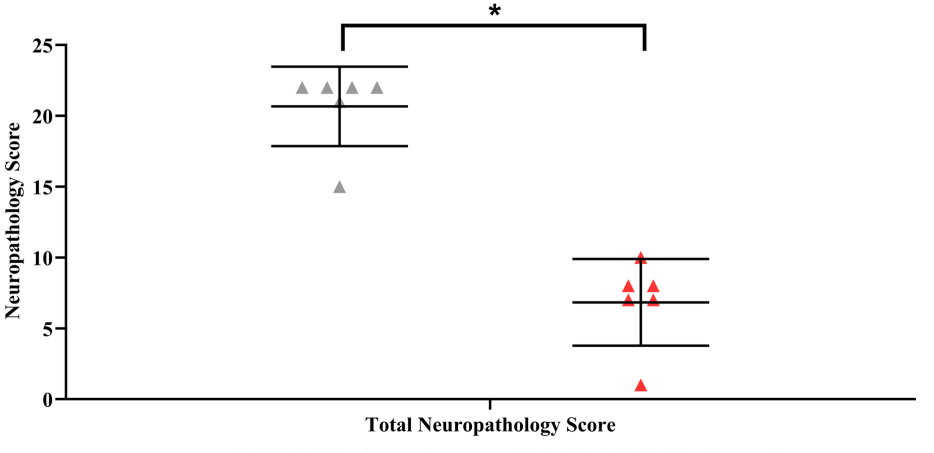

100
90
80
70
60
50
40
30
20
10
0
Probability of Survival
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days Post Exposure
Sal/MCM 30 min, n = 4
Sal/MCM+ENBA 30 min, n = 10

B.

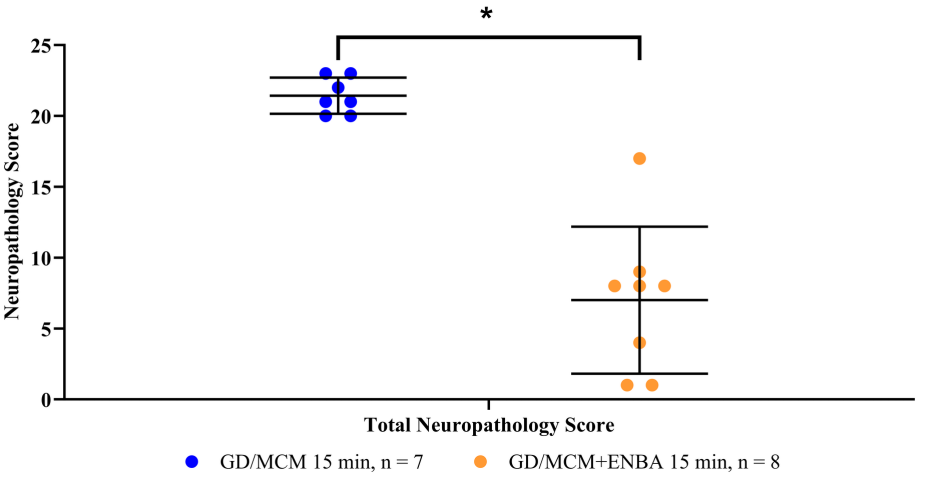

100
90
80
70
60
50
40
30
20
10
0
Probability of Survival
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days Post Exposure
GD/MCM 30 min, n = 8
GD/MCM+ENBA 30 min, n = 21


A.
*
25
20
15
10
5
0
Neuropathology Score
Total Neuropathology Score
GD/MDZ 15 min, n = 7
GD/MDZ+ENBA 15 min, n = 8

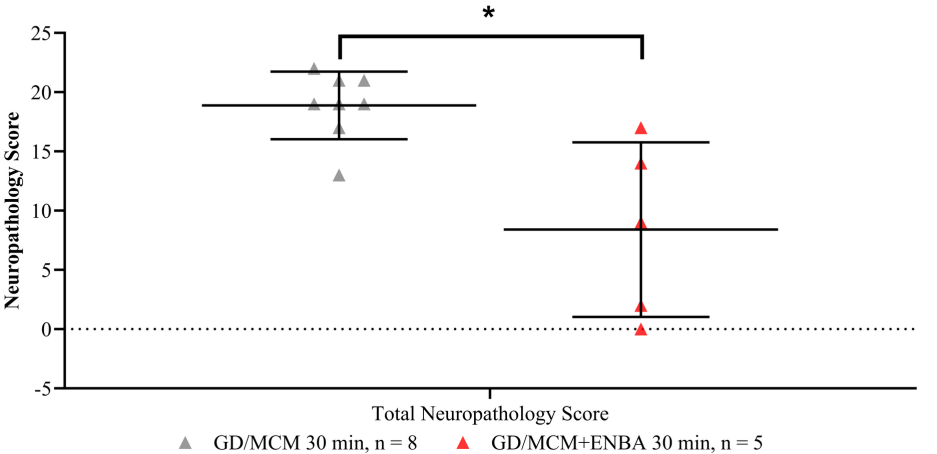
B.


*
25
20
15
10
5
0
Neuropathology Score
Total Neuropathology Score
GD/MDZ 30 min, n = 6
GD/MDZ+ENBA 30 min, n = 6

A.


*
25
20
15
10
5
0
Neuropathology Score
Total Neuropathology Score
GD/MCM 15 min, n = 7
GD/MCM+ENBA 15 min, n = 8

B.


*
25
20
15
10
5
0
-5
Neuropathology Score
Total Neuropathology Score
GD/MCM 30 min, n = 8
GD/MCM+ENBA 30 min, n = 5