Combination of antiseizure medications phenobarbital, ketamine, and midazolam reduces soman-induced epileptogenesis and brain pathology in rats

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Abstract

Objective: Cholinergic-induced status epilepticus (SE) is associated with a loss of synaptic gamma-aminobutyric acid A receptors (GABA_A) and an increase in N-methyl-D-aspartate receptors (NMDAR) and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) that may contribute to pharmacoresistance when treatment with benzodiazepine antiseizure medication is delayed. The barbiturate phenobarbital enhances inhibitory neurotransmission by binding to a specific site in the GABA_A to increase the open state of the channel, decrease neuronal excitability, and reduce glutamate-induced currents through AMPA/kainate receptors. We hypothesized that phenobarbital as an adjunct to midazolam would augment the amelioration of soman-induced SE and associated neuropathological changes and that further protection would be provided by the addition of an NMDAR antagonist.

Methods: We investigated the efficacy of combining antiseizure medications to include a benzodiazepine and a barbiturate allosteric GABA_A modulator (midazolam and phenobarbital, respectively) to correct loss of inhibition, and ketamine to reduce excitation caused by increased synaptic localization of NMDAR and AMPAR, which are NMDA-dependent. Rats implanted with transmitters to record electroencephalographic (EEG) activity were exposed to soman and treated with atropine sulfate and HI-6 one min after exposure and with antiseizure medication(s) 40 minutes after seizure onset.

Results: The triple therapy combination of phenobarbital, midazolam, and ketamine administered at 40 minutes after seizure onset effectively prevented soman-induced epileptogenesis and reduced neurodegeneration. In addition, dual therapy with phenobarbital and midazolam or ketamine was more effective than...
INTRODUCTION

Organophosphorus nerve agents (OPNAs) bind to and inactivate the enzyme acetylcholinesterase, thereby increasing acetylcholine in cholinergic synapses and neuromuscular junctions, leading to overt toxic signs. In cases of severe OPNA poisoning, seizure may be triggered as a result of the hyperactivation of glutamatergic synapses. Currently approved treatments in the United States include the oxime 2-pralidoxime chloride (2-PAM Cl) for the reactivation of acetylcholinesterase, the muscarinic anticholinergic drug atropine for the amelioration of peripheral effects of OPNA exposure, and the benzodiazepine diazepam as the first-line antiseizure medication. With the FDA approval of injectable midazolam to treat seizure, midazolam is anticipated to replace diazepam once an autoinjector form is approved (reviewed in Ref.1). However, in a scenario of mass casualties and/or attacks to unprepared populations, treatment with an antiseizure medication may be delayed, giving rise to a prolonged self-sustaining seizure (ie, status epilepticus, SE) that becomes resistant to benzodiazepine treatment.2–4 Since the time spent in seizure correlates to neuronal cell damage,5 promptly controlling seizure activity is crucial. Yet, optimal treatments for cholinergic-induced refractory SE are lacking. Novel therapeutic approaches to control SE following exposure to OPNAs must take into consideration the molecular maladaptive changes that underlie its pharmacoresistance.

One of the consequences of prolonged SE is the internalization of γ-aminobutyric acid type A receptors (GABA_A,R), which may explain the loss of efficacy of benzodiazepines.6 Concomitantly, an increase in the number of N-methyl-D-aspartate receptors (NMDAR) at neuronal synapses drives glutamatergic excitation and promotes excitotoxicity.7 In line with this theory, targeting NMDAR with antagonists, such as ketamine, is a highly efficient way of controlling SE. Preclinical studies demonstrated the synergistic properties of combining diazepam and ketamine8 or midazolam and ketamine6 to decrease seizure duration following cholinergic-induced SE. In the rodent models of soman-induced SE, a treatment composed of midazolam and ketamine reduced seizure severity, ameliorated impairment in the Morris water maze, and reduced neuronal loss.10–13 However, the protection afforded by the combination of ketamine and midazolam was incomplete, suggesting a need for an additional adjunct medical countermeasure.

monotherapy (midazolam or phenobarbital) in reducing cholinergic-induced toxicity.

Significance: Benzodiazepine efficacy is drastically reduced with time after seizure onset and inversely related to seizure duration. To overcome pharmacoresistance in severe benzodiazepine-refractory cholinergic-induced SE, simultaneous drug combination to include drugs that target both the loss of inhibition (eg, midazolam, phenobarbital) and the increased excitatory response (eg, ketamine) is more effective than benzodiazepine or barbiturate monotherapy.

KEYWORDS
barbiturate, benzodiazepine, ketamine, organophosphorus nerve agent, refractory status epilepticus

Key Points
• Pharmacoresistance is an unmet therapeutic challenge against cholinergic-induced status epilepticus (SE).
• Efficacy of phenobarbital alone or in combination with midazolam and ketamine was evaluated against soman-induced SE.
• Phenobarbital, midazolam, and ketamine triple therapy reduced time in seizure and prevented epileptogenesis in soman-exposed rats.
• Triple antiseizure medication following soman-induced SE also reduced neurodegeneration and neuronal loss in seizure-sensitive brain regions.
• Antiseizure medication combination to increase GABA_A,R function and reduce glutamate activity may be an effective approach against benzodiazepine-refractory SE.
Modulators of GABA receptors are administered to control seizure activity. The barbiturate phenobarbital, a second-line drug used to treat SE, enhances inhibitory neurotransmission by binding to a specific site in the GABA\(_A\)R to increase the open state of the channel and, thus, decrease neuronal excitability. Additionally, phenobarbital may reduce glutamate-induced currents through AMPA/kainate receptors. The barbiturate pentobarbital (75 mg/kg) had moderate efficacy at reducing soman-induced seizure in rats, with treatment at 5 minutes after seizure onset being more efficacious than a 40-min delay. In midazolam-treated rats exposed to soman, phenobarbital dose-dependently reduced seizure activity, albeit with a delay in seizure termination and without a long-lasting effect. Similar dose-response effects of phenobarbital as an adjunct to midazolam were observed following exposure to the pesticide diisopropyl fluorophosphate (DFP). The latter studies, however, did not investigate the effect of phenobarbital adjunct treatment on epileptogenesis and neuronal cell death at an endpoint longer than 24 or 72 hours. The antiseizure effects of phenobarbital occur in other seizure models, including maximal electroshock, picrotoxin, and electrical hippocampal stimulation. Less is known about the effects of a subanesthetic dose of midazolam in combination with other antiseizure medications.

When treatment of cholinergic-induced seizure is delayed to greater than 30 minutes, blocking one pathway (eg, benzodiazepine monotherapy) is insufficient, and polytherapy is needed to simultaneously activate the remaining GABA\(_A\)R and block NMDAR activation. Currently, we report that the combination of the antiseizure medication phenobarbital with midazolam and ketamine effectively halts acute seizure activity, reduces the incidence of epileptogenesis, and reduces neuronal cell degeneration following SE induced by soman exposure.

2 | METHODS

2.1 | Animals

Male Sprague Dawley rats (350-400 g; Charles River) were individually housed following telemetry implantation surgery, with food and water available ad libitum, on a 12-h:12-h light-dark cycle with lights on at 0600 hours. Rats were weighed daily. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Animal Welfare Act of 1966 (PL 89e544), as amended.

2.2 | Implantation of electrodes and electroencephalographic (EEG) analysis

Electroencephalographic (EEG) telemetry transmitters were subcutaneously (SC) implanted in rats. Under anesthesia by isoflurane (3%-5% induction, 1.5%-5% maintenance), rats were secured in a Kopf stereotaxic apparatus (David Kopf Instruments; Tujunga, CA, USA). Four cortical stainless steel screw electrodes were implanted on the skull 2 mm bilaterally to the midline and 1.6 mm anterior and 4 mm posterior to bregma. Stainless steel wires from the F40-EET transmitters (Data Sciences International [DSI], Inc, St. Paul, MN) were implanted subcutaneously, wrapped around the electrodes, and secured in place using dental acrylic. Rats were administered buprenorphine (0.03 mg/kg, sc) immediately after removal from anesthesia with 1 week to recover from surgery prior to soman exposure. For EEG activity recording, an RPC-1 PhysioTel receiver from DSI was placed under each animal’s home cage for continuous data collection (24 h/d) using Dataquest ART Acquisition software (DSI, Inc). EEG data were recorded continuously for at least 24 hours prior to soman exposure to day of euthanasia (14 days after exposure). EEG data were digitized at 250 Hz to evaluate seizure onset, acute seizure duration, and the development of spontaneous recurrent seizure (SRS).

2.3 | Soman-induced seizure and administration of medical countermeasures

Rats were administered 1.2 LD\(_{50}\) of soman (pinacolyl methylphosphonofluoridate; 118.1 μg/kg; 0.5 ml/kg; SC), obtained from the US Army Combat Capabilities Development Command Chemical Biological Center (Aberdeen Proving Ground, MD, USA), and 1 minute later administered intramuscularly (IM) an admix of atropine sulfate (2 mg/kg) and the oxime HI-6 dichloride salt (93.6 mg/kg). Forty minutes after seizure onset, antiseizure medication treatments were administered intraperitoneally (IP). Soman-exposed animals were assigned to one of five groups: (1) midazolam (3 mg/kg) monotherapy; (2) phenobarbital (30 mg/kg) monotherapy; (3) phenobarbital (30 mg/kg) and ketamine (30 mg/kg); (4) phenobarbital (30 mg/kg) and midazolam (3 mg/kg); and (5) phenobarbital (30 mg/kg), ketamine (30 mg/kg), and midazolam (3 mg/kg). Midazolam and ketamine were...
diluted in sterile water, and phenobarbital was diluted in a vehicle containing 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, and 48.5% distilled water. Seizure onset was on average 7 minutes, so treatment was approximately 47 minutes after exposure. Following soman exposure, EEG was monitored in real time and seizure onset was defined as the appearance of rhythmic high-amplitude spikes (>2 × baseline) that lasted at least 10 s (based on Ref.22). Rats were monitored for 6 hours following exposure, and behavioral seizures were scored using a modified Racine scale composed of five stages: 1, startle; 2, head myoclonus; 3, limb clonus or tonus, body tremor; 4, forelimb clonus with rearing; and 5, rearing and falling with generalized tonic-clonic convulsions.

2.4 EEG seizure analysis

Scoring of EEG for the full duration of the recordings was performed using a high-throughput seizure detection algorithm as previously described. Briefly, seizure activity was determined in 2-s epochs with detection thresholds set by the slope of a linear robust fit applied to a fast Fourier transform and normalized power spectra (0.1-10 Hz) during 24 hours of baseline. During seizure episodes, the power spectra increase in magnitude and dominant frequencies are shifted to the left, increasing the slope of the linear robust fit. When both power spectra and slope reach threshold, the 2-s epoch is marked as a potential seizure. Detection using this method generated a list of candidate seizures that an observer blinded to the treatment groups visually inspected for confirmation of seizures and rejection of false positives arising from the detection of normal behavior (eg, sleep activity) or artifacts in the EEG signal. In the triple therapy group, one rat did not survive to 24 hours, and a second rat with poor EEG signal was excluded from the seizure data analysis.

2.5 Brain tissue collection and immunohistochemistry

Two weeks after soman exposure, rats were injected with sodium pentobarbital (75 mg/kg, IP, Fatal Plus; Patterson Veterinary) and perfused with 0.9% heparinized saline in 0.1 M phosphate buffer (FD Neurotechnologies, Columbia, MD), followed by 4% paraformaldehyde. Brains were removed, postfixed for 6 hours in 4% paraformaldehyde, and cryoprotected in 20% sucrose. Histological sectioning and staining of brain tissue were conducted by FD Neurotechnologies. Coronal 50-μm sections were stained with FD NeuroSilver™ to identify neurodegeneration in brain regions previously shown to be affected following soman exposure (eg, Ref.25–27). Brain regions were qualitatively scored by an observer blinded to treatment, on a scale of 0-4, with 4 being the most severe. Brain regions scored included the thalamus (lateral, medial, reuniens), amygdala (basolateral, lateral, medial, central), hippocampus (CA1, CA2, CA3, dentate gyrus, radiatum, stratum lucidum, oriens layer), and piriform cortex (layers 1, 2, 3), as well as fiber tracts (cingulum, corpus callosum, external capsule, internal capsule, fimbria of hippocampus), at bregma −3.00 mm. Medians of the median score for each subregion were taken to represent the overall brain region.

In addition, coronal 30-μm sections were stained for the mature neuronal marker NeuN and the inhibitory neuronal marker glutamate decarboxylase 67 (GAD67). A monoclonal mouse anti-NeuN IgG (1:10,000; Millipore, Billerica, MA) and a monoclonal mouse anti-GAD67 IgG (1:2000; Abcam, Cambridge, MA) were used in the immunohistochemistry procedure. Subsequently, the immunoreaction product was visualized according to the avidin-biotin complex method with the Vectastain Elite ABC Kit (Vector Lab., Burlingame, CA). NeuN-positive (NeuN+) and GAD67-positive (GAD67+) cell densities were determined in various brain regions using Image-Pro Plus v7.0 (Media Cybernetics Inc, Rockville, MD, USA; for additional details, see Ref.29). Areas evaluated included the thalamus, basolateral amygdala, piriform cortex, and hippocampus. For neuropathology assessments, controls (no agent) were used for comparison with soman-exposed, treated rats.

2.6 Data analysis

Data analysis was conducted using SPSS v21 (IBM Inc, Armonk, NY, USA). Graphs were compiled using SigmaPlot 14.0 (Systat Software, Inc, San Jose, CA, USA). Repeated-measures ANOVA was used to analyze body temperature, body weight, and motor activity with repeated time points as the within-subjects variable and treatment as the between-subjects variable. If a significant interaction was found, a one-way ANOVA was conducted at each level of the within-subjects variable. For comparison of seizure duration, as well as NeuN+ and GAD67+ cell densities, a general linear model analysis was used, followed by Tukey’s test. A Kaplan-Meier test was performed to compare treatment effects on median time to onset of initial SRS event. To determine the relationship between treatments and percentage of animals that showed SRS by the study endpoint, a logistic regression analysis was performed and the chi-square followed
by Fisher’s exact test was conducted to account for group comparisons. For number of SRS, a one-way ANOVA was used. When homogeneity of variance failed, the Kruskal-Wallis test was conducted, followed by multiple comparisons and adjusted significance to account for multiple comparisons. For comparisons of toxic signs and silver score, a Kruskal-Wallis test was used. Post hoc comparisons were performed using a Mann-Whitney U test. Data are displayed as mean ± standard deviation or median and interquartile range (IQR).

3 | RESULTS

3.1 | Phenobarbital combination therapies reduced acute seizure activity and incidence of epileptogenesis following soman exposure

Subcutaneous exposure to soman resulted in severe and prolonged seizure activity in rats, with average (± SD) seizure onset of 7 minutes (± 4.7 minutes). A general linear model analysis detected a main effect of treatment on acute seizure duration. Therapies that included phenobarbital led to a lower average seizure duration during the first 24 hours following soman exposure compared with midazolam monotherapy (Figure 1A). The average (±SD) seizure duration in the first 24 hours after soman exposure was 542.6 ± 285.7, 235.6 ± 100.1, 161.8 ± 134.2, 156.7 ± 113.2, and 71.2 ± 69.6 minutes for midazolam monotherapy, phenobarbital monotherapy, phenobarbital and ketamine, phenobarbital and diazepam, and phenobarbital, ketamine, and midazolam combination therapy, respectively. Triple therapy also reduced the behavioral seizure, scored using a modified Racine scale, with significantly lower seizure severity score than in rats treated with midazolam or phenobarbital monotherapy or with phenobarbital and midazolam dual therapy by 30 minutes after treatment (Figure 1B). Rats treated with midazolam monotherapy continued to display behavioral seizure for over 5 hours after treatment with significantly higher toxic sign scores compared with triple therapy–treated rats for this duration.

In the days following soman-induced SE, EEG activity was continuously recorded and subsequently analyzed for the development of SRS. A logistic regression analysis detected a main effect of treatment on the percentage of animals showing SRS by the study endpoint. The group of animals receiving a combination therapy of phenobarbital, ketamine, and midazolam did not

FIGURE 1 Effect of delayed treatment with antiseizure medications on seizure duration and activity following soman (GD) exposure in rats: benefit of simultaneous administration of triple antiseizure medication therapy. Midazolam (MDZ) administered at 40 min after seizure onset induced by GD resulted in prolonged initial seizure activity (status epilepticus). A, Rats that received phenobarbital monotherapy (PHE), dual therapy of phenobarbital with ketamine (PHE + KET) or midazolam (PHE + MDZ) or triple antiseizure medication therapy of midazolam, phenobarbital, and ketamine (MDZ + PHE + KET) had a reduced time in initial seizure compared with MDZ-treated rats. Seizure duration is shown as mean ± SD. **P < .01 and ***P < .001 compared with midazolam. B, Monotherapy with MDZ or PHE was ineffective at terminating behavioral seizure activity, whereas treatment with triple therapy (MDZ + PHE + KET) reduced the Racine score by 30 min after treatment (postexposure time 60 min) compared with midazolam. *P < .05 compared with midazolam. **P < .01 compared with midazolam. #P < .05 compared with midazolam. Racine score is shown as median ± IQR
present SRS, and a chi-square analysis with Fisher’s exact test revealed that this percentage was significantly reduced compared with the percentage of animals developing SRS in the midazolam monotherapy group (Figure 2A). A general linear model analysis detected a main effect of treatment on the average number of SRS events occurring up until study endpoint, with phenobarbital triple therapy showing a significantly fewer average number of SRS (Figure 2B).

### 3.2 Soman-induced transient reduction in body temperature and body weight

A reduction in body temperature was observed in all soman-exposed groups (Figure S1). However, soman-exposed rats treated with phenobarbital and ketamine dual therapy had the shortest decline in temperature, with temperature returning to baseline by 10 hours after soman exposure. Compared with midazolam monotherapy, rats treated with phenobarbital and ketamine dual therapy, midazolam and phenobarbital dual therapy, or phenobarbital, ketamine, and midazolam triple therapy had greater temperature by 10, 16, and 20 hours, respectively, after GD exposure. All soman-exposed groups had significant loss of body weight compared with baseline by 24 hours after exposure, with body weight returning to baseline within a week of exposure. No significant differences in body weight loss were observed among groups.

### 3.3 Combination therapy of midazolam, phenobarbital, and ketamine prevented the development of soman-induced hyperactivity

Gross motor activity measured using DSI telemetry transmitters revealed that hyperactivity occurred during the dark cycle (when rats are most active) by postexposure day 3 in soman-exposed rats treated with midazolam monotherapy at 40 minutes after seizure onset; this hyperactivity continued throughout the study (Figure 3). Activity was also greater in midazolam-treated rats during the light cycle on postexposure day 13, but not at other times as activity is low in general during the light cycle. Development of hyperactivity in soman-exposed rats was reduced in rats that received triple therapy of phenobarbital, ketamine, and midazolam. Comparisons of each group on each day revealed that triple therapy (phenobarbital, ketamine, and midazolam) significantly reduced activity compared with midazolam monotherapy on postexposure days 9 through 13. Those groups treated with dual therapy tended to have reduced activity compared with the midazolam monotherapy group, but it was not significant.
Phenobarbital combination therapies reduced neuropathology following soman-induced SE and delayed treatment with antiseizure medications

The triple antiseizure medication combination of phenobarbital, ketamine, and midazolam prevented or lessened damage in several brain regions, as shown with silver stain and with NeuN+ and GAD67+ immunohistochemistry 2 weeks after soman exposure. Median silver stain scores were compared in brain regions typically damaged by prolonged seizure following soman exposure. Triple therapy with phenobarbital, ketamine, and midazolam resulted in significantly less damage in fiber tracts, thalamus, amygdala, hippocampus, and piriform cortex compared with midazolam monotherapy (Figure 4). NeuN immunohistochemistry was used to measure viable neurons in the thalamus, piriform cortex, and amygdala following soman exposure. Significant loss of neurons was observed in the thalamus, amygdala, and piriform cortex in rats treated with midazolam monotherapy compared with no agent control (Figure 5). Rats receiving treatment with triple therapy following soman exposure had reduced neuronal loss compared with those that received midazolam monotherapy and were not different from no agent controls. In addition, soman-exposed rats treated with midazolam monotherapy had significantly fewer number of GAD67+ cells in the amygdala and piriform cortex compared with no agent controls (Figure 6). Treatment with triple therapy of phenobarbital, ketamine, and midazolam reduced the soman-induced loss of GAD67+ cells in the amygdala and piriform cortex. In addition, soman-exposed rats treated with phenobarbital and midazolam dual therapy had reduced loss of GAD67+ neurons in the piriform cortex compared to soman-exposed rats treated with midazolam monotherapy.

4 | DISCUSSION

In the current study, we report on the beneficial effects of combining the antiseizure medication phenobarbital...
with ketamine and midazolam to counter the effects of soman-induced SE in rats. The therapeutic limitations of benzodiazepines highlight the need for improved antiseizure medications to treat pharmacoresistant seizure. Our current findings provide further support that targeting cholinergic-induced maladaptive glutamatergic (increased NMDAR and AMPAR) and GABAergic (reduced GABAAR) components that contribute to pharmacoresistance following OPNA exposure is an effective approach to reducing cholinergic-induced seizure and neuropathology. Our laboratory and others previously reported on the beneficial effect of administering the NMDAR antagonist ketamine in combination with a benzodiazepine in reducing cholinergic-induced seizure severity, epileptogenesis, functional impairment, and brain pathology in rodent models. Previous studies also showed the importance of simultaneous administration of antiseizure medications, as opposed to sequential drug administration, in reducing cholinergic-induced seizure severity. The inclusion of a third antiseizure medication, valproate, in combination with ketamine and midazolam further reduces seizure severity, epileptogenesis, and neuronal loss following soman exposure. Our current findings report on the benefits of phenobarbital when used as an adjunct to midazolam and that inclusion of ketamine provides additional protection against soman-induced SE, similar to our previous observations with combination therapy of valproate with ketamine and midazolam. Phenobarbital monotherapy or dual therapy of phenobarbital (and midazolam or ketamine) improved outcome, but the greatest benefit of treatment was in the group that received the triple therapy of phenobarbital, ketamine, and midazolam.

Although early administration of benzodiazepines is effective at reducing cholinergic-induced seizure, the therapeutic window for terminating seizure is limited to 40 minutes or less after seizure onset, and treatment success with benzodiazepines is inversely correlated with seizure duration. In the weeks after exposure, many rats and mice develop SRS. Since the time spent in seizure correlates with neuronal cell damage, prompt control of seizure activity is critical. Phenobarbital reduced time in the initial seizure when administered as a monotherapy, as a dual therapy (with ketamine or midazolam) or as a triple therapy (with ketamine and midazolam) 40 minutes after soman-induced seizure compared
with midazolam monotherapy. In agreement with the antiseizure effects of phenobarbital against soman exposure, phenobarbital at this dose (30 mg/kg) was also effective against kainate-induced seizure and brain damage.\(^{40}\)

In the current study, behavioral seizure as measured on the Racine scale was primarily reduced in animals that received triple therapy; these animals had significantly less seizure than those treated with monotherapy (midazolam or phenobarbital) or dual therapy (phenobarbital plus midazolam). Treatment efficacy of triple therapy to reduce behavioral seizure occurred within 30 minutes of treatment and persisted for the duration of toxic sign monitoring. In addition, treatment with triple therapy (phenobarbital with midazolam and ketamine), but not phenobarbital monotherapy or dual therapy, reduced the number of SRS compared with midazolam monotherapy. No animals that received triple therapy following soman exposure developed SRS compared with 75% of animals that received midazolam monotherapy. In mice exposed to a seizure-inducing dose of soman, a low dose of phenobarbital monotherapy (20 mg/kg) resulted in a delay in onset to SRS but did not reduce neuropathology.\(^{41}\)

Higher doses of phenobarbital as evaluated against DFP in rats\(^{17,18}\) or combination of lower doses of phenobarbital with other antiseizure medications as used in the current study may be needed to improve neuroprotection. These findings demonstrate the efficacy of combining subanesthetic doses of antiseizure medications in reducing cholinergic-induced SE and epileptogenesis.

Triple therapy with phenobarbital, ketamine, and midazolam also provided significant protection from neuropathological damage following soman-induced seizure. Animals that received the triple therapy had less neurodegeneration illustrated with silver stain in fiber tracts, thalamus, amygdala, hippocampus, and piriform cortex compared with those that received midazolam monotherapy. Rats that received phenobarbital monotherapy or dual therapy of phenobarbital with ketamine or midazolam were not significantly different from rats that received midazolam monotherapy. Using antibodies against a neuron-specific marker (NeuN) and a GABAergic marker (GAD67), we visualized the overall populations of mature neurons and inhibitory interneurons to determine whether treatment with combination antiseizure medications would reduce soman-induced neuronal loss compared with midazolam...
monotherapy. Animals treated with delayed midazolam had extensive neuronal loss as evidenced by reduced NeuN+ cells in the thalamus, amygdala, and piriform cortex. Treatment with triple therapy of phenobarbital, ketamine, and midazolam protected neurons in the thalamus and piriform cortex, while treatment with phenobarbital in combination with midazolam reduced loss of neurons in the piriform cortex. In agreement with our findings of reduced neuronal loss 2 weeks after soman exposure in rats treated with phenobarbital (30 mg/kg, IP) as an adjunct to midazolam (3 mg/kg, IP), higher doses of phenobarbital (56 or 100 mg/kg, IP) in combination with midazolam (1.8 mg/kg, IM) reduced cell death (FluoroJade B) measured 24 hours after soman exposure. In addition, rats treated with phenobarbital (50 mg/kg) 30 minutes prior to pilocarpine exposure had reduced brain damage shown with cresyl violet. The current findings show added protection of neurons when ketamine is used as combination with phenobarbital and midazolam.

GABA interneurons play a crucial role in excitatory and seizure activity. An antibody against GAD67, commonly used as a selective marker of neurons that synthesize the neurotransmitter GABA, was used to quantify loss of inhibitory neurons after soman exposure. Our laboratory previously reported that delayed benzodiazepine treatment of soman exposure in rats led to loss of GAD67+ cells in the amygdala, vital for seizure regulation, and in the piriform cortex, implicated in seizure propagation. The loss of GABA interneurons in these regions following soman exposure may contribute to the development of epileptogenesis. We currently report that midazolam-treated rats had significant loss of GAD67+ cells in the amygdala and piriform cortex and that triple therapy with phenobarbital, ketamine, and midazolam attenuated loss. In the piriform cortex, phenobarbital dual therapy with midazolam also reduced the loss of GAD67+ cells following soman exposure. Protection from loss of GABA interneurons by phenobarbital in combination with midazolam and ketamine may reduce the potential to develop epileptogenesis.

Following exposure to a seizure-inducing dose of soman, animals develop a biphasic change in motor activity, with reduced motor activity and startle response in the first 24 hours followed by increased activity and startle and perseverative behaviors in the weeks after exposure, with increased hyperactivity predominant during the dark cycle when rodents are most active. Soman-exposed animals also are hyper-reactive, to include biting during handling and exaggerated startle to air-puff. In the current study, triple therapy with phenobarbital, ketamine, and midazolam prevented the development of hyperactivity following soman exposure, while rats treated with midazolam monotherapy had increased activity by postexposure day 9.
In sum, our current findings suggest that the antiseizure medication phenobarbital is more beneficial than midazolam monotherapy at treating soman-induced SE when treatment is delayed and that the addition of ketamine and midazolam provided increased benefit over that of monotherapy or dual therapy in preventing epileptogenesis. In addition, although dual therapy of phenobarbital with midazolam provided some neuroprotection in the piriform cortex, triple therapy with phenobarbital, ketamine, and midazolam protected additional brain regions from excitatory and inhibitory neuronal loss.

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CONFLICT OF INTEREST
Jerome Niquet and Claude Wasterlain have a patent on polytherapy of cholinergic seizures (UC Case No. 2012-172-2). Other authors have no conflict of interest to disclose.

ETHICAL APPROVAL
We confirm that we have read the Journal’s position on issues involved in the ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

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