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Anticonvulsants for Nerve Agent-Induced Seizures: The Influence of the Therapeutic Dose of Atropine

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ABSTRACT

Two guinea pig models were used to study the anticonvulsant potency of diazepam, midazolam, and scopolamine against seizures induced by the nerve agents tabun, sarin, soman, cyclosarin, O-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate (VX), and O-isobutyl S-(2-diethylamino)ethyl)-methyl phosphonothioate (VR). Animals instrumented for electroencephalogram recording were pretreated with pyridostigmine bromide (0.026 mg/kg i.m.) 30 min before challenge with $2 \times LD_{50}$ (s.c.) of a nerve agent. In model A, atropine sulfate (2.0 mg/kg i.m.) and pyridine-2-aldoxime methylchloride (2-PAM; 25.0 mg/kg i.m.) were given 1 min after nerve agent challenge, and the tested anticonvulsant was given (i.m.) 5 min after seizure onset. In model B, a lower dose of atropine sulfate (0.1 mg/kg i.m.) was given along with 2-PAM 1 min after nerve agent challenge, and the anticonvulsant was given at seizure onset.

With the lower dose of atropine, seizure occurrence increased to virtually 100% for all agents; the time to seizure onset decreased for sarin, cyclosarin, and VX; the signs of nerve agent intoxication were more severe; and coma resulted frequently with cyclosarin. The anticonvulsant ED_{50} doses for scopolamine or diazepam were, in general, not different between the two models, whereas the anticonvulsant ED_{50} values of midazolam increased 3- to 17-fold with the lower atropine dose. Seizure termination times were not systematically effected by the different doses of atropine. The order of anticonvulsant effectiveness within each model was scopolamine $\geq$ midazolam > diazepam. The findings indicate that the dose of atropine given as antidotal therapy can significantly influence measures of nerve agent toxicity and responsiveness to anticonvulsant therapy.

The potential for exposure to nerve agents exists on the battlefield and as a terrorist threat (e.g., 1995 Tokyo subway incident). Nerve agent exposure causes a progression of toxic signs (hypersecretions, muscle fasciculations, tremors, convulsions, and respiratory distress) that are due to inhibition of acetylcholinesterase (AChE) by the agent and the subsequent increase in the concentration of the neurotransmitter acetylcholine (ACh) and hyperstimulation of the cholinergic system at central and peripheral sites (Taylor, 2001). A combined regimen of prophylaxis and therapy is the most effective medical countermeasure for dealing with the threat of nerve agent poisoning to military personnel (Moore et al., 1995; Sidell, 1997; Aas, 2003). Pretreatment with carbamate cholinesterase inhibitors, such as pyridostigmine bromide

(PB), shields a fraction of cholinesterase in the periphery from irreversible inhibition by the nerve agents (Berry and Davis, 1970; Dirnhuber et al., 1979). In the event of nerve agent poisoning, immediate treatment with an anticholinergic drug, such as atropine sulfate, will antagonize the effects of excess ACh at muscarinic receptor sites, and an oxime, such as pyridine-2-aldoxime methylchloride (2-PAM), is used to reactivate inhibited AChE (Moore et al., 1995; Taylor, 2001).

This regimen, however, does not prevent nerve agent-induced seizures. Prolonged epileptiform seizures in a nerve agent casualty can produce irreversible brain damage (McDonough et al., 1995; Shih et al., 2003) that can result in long-term deficits in cognitive function and behavior. Concomitant administration of an anticonvulsant drug such as diazepam is considered essential to optimize the regimen of carbamate pretreatment plus atropine and oxime therapy for severely exposed casualties (Dunn and Sidell, 1989; Aas, 2003). However, i.m. diazepam produces inconsistent bioavailability, it is slow to act, and it can

ABBREVIATIONS: AChE, acetylcholinesterase; ACh, acetylcholine; PB, pyridostigmine bromide; 2-PAM, pyridine-2-aldoxime methylchloride; GA, tabun, ethyl N,N-dimethyl phosphoramidocyanidate); GB, sarin, isopropyl methylphosphonofluoridate; GD, soman, pinacolyl methylphosphonofluoridate; GF, cyclosarin, cyclohexylmethyl phosphonofluoridate; VX, O-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate); VR, O-isobutyl S-(2-(diethylamino)ethyl)methyl phosphonothioate); pyridostigmine bromide; EEG, electroencephalogram.

provide less than total protection against the neuropathological consequences of nerve agent exposure (Hayward et al., 1990; McDonough et al., 1995). Thus, there has been an active research effort to find better drugs to treat nerve agent-induced seizures.

In our previous studies, anticonvulsant ED_{50} values of diazepam, midazolam, and several anticholinergics to antagonize seizures produced by a number of nerve agents, such as tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), VX, or VR, were determined in a guinea pig model (Shih and McDonough, 1999, 2000; Shih et al., 2003). In this original model (designated here as model A; Fig. 1, top), guinea pigs were pretreated with PB and then challenged with $2 \times LD_{50}$ of a nerve agent, followed 1 min later by 2-PAM (25 mg/kg i.m.) and atropine sulfate (2.0 mg/kg i.m.) therapy. The drug tested for anticonvulsant activity was given (i.m.) 5 min after the onset of seizures. When given 5 min after seizure onset, atropine could terminate nerve agent-induced seizures, even though the anticonvulsant ED_{50} required was high (i.e., ~10.0 mg/kg for most nerve agents) (Shih and McDonough, 1999, 2000; Shih et al., 2003). Thus, the inclusion of 2.0 mg/kg atropine as part of the immediate treatment with 2-PAM may have overestimated the efficacy of the tested anticonvulsant drugs. Furthermore, a single nerve agent antidote kit (the MARK I; Meridian Medical Technologies, Columbia, MD) contains autoinjectors containing 2 mg of atropine and 600 mg of 2-PAM, and each military service member carries three MARK I kits. After a severe nerve agent exposure, three MARK I nerve agent kits are supposed to be administered to the casualty by his or her buddy or a medic. The delivered dose for a 70- to 75-kg soldier would be approximately 0.1 mg/kg for atropine and 25 mg/kg for 2-PAM. Thus, the approximate atropine dose would be substantially lower than that used in model A. Furthermore, a delay in anticonvulsant treatment after seizure onset often altered the effectiveness of anticonvulsant drugs (McDonough and Shih , 1993), and in practice, service members are taught

to administer anticonvulsant treatment (10 mg of diazepam; convulsant antidote, nerve agent) at the first sign of seizures. Therefore, model B (Fig. 1, bottom) was developed, where 0.1 mg/kg atropine was given along with 25 mg/kg 2-PAM 1 min after nerve agent challenge, and the test anticonvulsant drug was given at the time of seizure onset. Model B closely mimics the doses and timing of current United States military pretreatment and therapy regimen in the event of nerve agent exposure. The present study determined the potency of diazepam, midazolam, and scopolamine in terminating seizures induced by GA, GB, GD, GF, VX, or VR in this refined model B. A comparison was then made with respect to these two doses of atropine on a number of parameters: seizure occurrence, latencies for seizure onset, time to seizure termination, 24-h lethality, and potency of anticonvulsant drugs.

Materials and Methods

Subjects. Male Hartley guinea pigs [Crl: (HA) BR COBS; Charles River Laboratories, Inc., Kingston, NY], weighing 250 to 300 g, served as subjects. Upon arrival, the animals were quarantined for a week and tested for evidence of disease. They were individually housed in polycarbonate cages in temperature- ($21 \pm 2°C$) and humidity-controlled ($50 \pm 10\%$) animal quarters maintained on a 12-h light-dark full-spectrum lighting cycle with lights on at 6:00 AM. Laboratory chow and water were freely available whenever the animals were in home cages. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Materials. Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs, Inc. (Berkeley, CA). Atropine sulfate, scopolamine hydrobromide, and sodium pentobarbital were purchased from Sigma-Aldrich (St. Louis, MO). Buffered formalin (10%) was purchased from Fisher Scientific (Hampton, NH). PB, diazepam, and midazolam were obtained from Hoffman-La Roche Inc. (Nutley, NJ), and 2-PAM was purchased from Ayerst Labs, Inc. (New York, NY). Attane (isoflurane, USP) was purchased from Minrad, Inc. (Bethlehem, PA). The six chemical warfare nerve agents studied were GA, GB, GD, GF, VX, and a Russian V-type agent designated VR. They were obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agents were diluted in ice-cold saline before injection. PB, atropine sulfate, scopolamine hydrobromide, 2-PAM, and midazolam were prepared in saline. Diazepam and pentobarbital were prepared in a vehicle containing 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, and 48.5% distilled water. Atropine sulfate and 2-PAM were admixed in a solution; all other solutions were prepared and injected separately. Injection volume was 0.5 ml/kg for all nerve agents and treatment drugs.

Surgery. All animals, while under isoflurane anesthesia, were prepared for recording of electroencephalogram (EEG) approximately 1 week before experimentation by implanting cortical stainless steel screw electrodes using previously described procedures (Shih and McDonough, 1999; Shih et al., 2003).

Experimental Procedure. On the day of the experiment, guinea pigs were placed in individual recording chambers and continuously monitored for EEG activity. EEG recordings were made using amplifiers and QND software supplied by Neurodata, Inc. (Pasadena, CA) (low-frequency filter, 0.3 Hz; high-frequency filter, 40 Hz; and sampling rate, 128 Hz) and displayed on a computer monitor. After
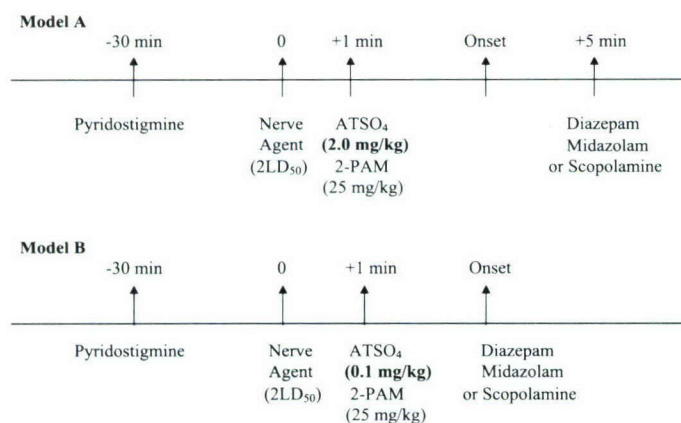

Model A

-30 min	0	+1 min	Onset	+5 min
Pyridostigmine	Nerve Agent (2LD$_{50}$)	ATSO$_4$ **(2.0 mg/kg)** 2-PAM (25 mg/kg)		Diazepam Midazolam or Scopolamine

Model B

-30 min	0	+1 min	Onset
Pyridostigmine	Nerve Agent (2LD$_{50}$)	ATSO$_4$ **(0.1 mg/kg)** 2-PAM (25 mg/kg)	Diazepam Midazolam or Scopolamine

Fig. 1. Experimental models for measuring anticonvulsant drugs against nerve agent-induced EEG seizures. In model A (top), used in previous studies, guinea pigs were pretreated with PB (0.026 mg/kg i.m.) 30 min before challenge with $2 \times LD_{50}$ of a nerve agent s.c., followed 1 min later by 2-PAM (25 mg/kg i.m.) and atropine (2.0 mg/kg i.m.) therapy. The drugs tested for anticonvulsant activity were given (i.m.) 5 min after the onset of seizures. In model B (bottom), used in this study, guinea pigs were pretreated with PB 30 min before challenge with $2 \times LD_{50}$ of a nerve agent, followed 1 min later by 2-PAM (25 mg/kg i.m.) and atropine (0.1 mg/kg i.m.). The anticonvulsant treatment was given immediately following EEG seizure onset.

a 15-min recording baseline EEG, animals received a dose of PB (0.026 mg/kg i.m.) to produce 20 to 30% whole blood cholinesterase inhibition (Lennox et al., 1985). Thirty minutes later, animals were challenged s.c. with $2 \times LD_{50}$ of GA (240 μg/kg), GB (84 μg/kg), GD (56 μg/kg), GF (114 μg/kg), VX (16 μg/kg), or VR (22.6 μg/kg). One minute after nerve agent challenge, the animal was treated with atropine sulfate (0.1 mg/kg i.m.) plus 2-PAM (25 mg/kg i.m.). This dose of 2-PAM approximates the total dose of 2-PAM in three auto-injectors (600 mg per injector) given to a 70- to 75-kg human. The 0.1-mg/kg dose of atropine sulfate approximates the total dose of atropine sulfate in the three autoinjectors (2.0 mg per injector) given to a 70- to 75-kg human. Immediately (usually within 15 s) after the onset of EEG seizure activity, diazepam, midazolam, or scopolamine was given intramuscularly.

Animals were observed continuously for the 1st h following exposure and treatment and periodically thereafter for at least 6 h. EEG was recorded continuously throughout this time and for another 30 min at 24 h after exposure. Seizure onset was operationally defined as the appearance of ≥10 s of rhythmic high-amplitude spikes or sharp wave activity in the EEG tracing. Each animal was rated as having the seizure terminated (OFF) or not terminated (NOT OFF) based on the overall appearance of the EEG record at the end of the experimental day and during the 24-h observation. (Note: an animal was rated as OFF if the seizure was terminated, and the EEG remained normal at all subsequent observation times.) Mortality was recorded 24 h after nerve agent exposure. Animals that survived 24 h were euthanized with an overdose of sodium pentobarbital (75 mg/kg i.p.) and then perfused through the aorta with saline, followed by 10% neutral-buffered formalin.

The brain was blocked, embedded in paraffin, cut 6 to 10 μm thick, stained with hematoxylin and eosin, and then evaluated by a board-certified pathologist who was unaware of the experimental history of a given subject. The procedures and criteria used for pathological evaluation have been published previously (McDonough et al., 1995, 2000; Shih et al., 2003). In brief, six brain areas (cerebral cortex, pyriform cortex, amygdala, hippocampus, thalamus, and caudate/putamen) were evaluated in each animal, and each area was rated on a scale from 0 (no damage) to 4 (severe, >45% tissue involvement) for neuropathological damage. In addition to the individual brain areas, a total brain lesion score was obtained for each animal by summing the scores of the six areas.

Data Analysis. Dose-effect curves and the median effective dose (ED_{50}) for anticonvulsant activity of each individual drug were determined by probit analysis (Bliss, 1952) using four to seven doses with five to six animals per group. A probit regression analysis (SPSS for Windows, version 10.0; SPSS Inc., Chicago, IL) was used to estimate the ED_{50} values along with the 95% confidence intervals for each drug treatment and nerve agent combination. Furthermore, the SPSS program was used to determine the relative mean potency of each drug treatment for each nerve agent and each nerve agent for each drug treatment at the 50% response along with the respective 95% confidence intervals. These relative mean potencies are the ratio of the 50% response for two treatments for a given agent or two agents for a given treatment. With this determination, if the 95% confidence interval of the resultant ratio included the value of 1, the ED_{50} values for the two treatments (or agents) were considered similar; if the 95% confidence interval excluded the value of 1, then the ED_{50} values for the treatments (or agents) were significantly different. Using this statistic, the anticonvulsant ED_{50} values for each of the three drugs were also compared among different nerve agents. Latencies for seizure onset between the different nerve agents were not normally distributed, and they were evaluated using the Kruskal-Wallis analysis of variance on ranks followed by Dunn's multiple comparison test. Latencies for seizure terminations were evaluated using both one- and two-way analysis of variance procedures followed by Tukey's multiple comparison tests. The differences between proportions of animals surviving challenge with each

nerve agent, the proportion of animals surviving as a function of successful control of the seizure, and the incidence of neuropathology as a function of seizure control all were evaluated using the Chi-square procedure with Yates correction (Winer, 1971). In all of the statistical analyses, $P < 0.05$ was considered significant.

Results

Seizure Occurrence. The percentage of seizure occurrence was approximately 100% in GA-, GD-, GF-, and VR-exposed guinea pigs with either dose of atropine. When the atropine dose was lowered from the 2.0- to 0.1-mg/kg dose, seizure occurrence increased significantly ($P < 0.001$) in animals exposed to GB [from 86% ($n = 100$) to 100% ($n = 85$)] and VX [from 51.5% ($n = 202$) to 95.4% ($n = 87$)].

Seizure Onset. Seizure onset times (in minutes) after $2 \times LD_{50}$ of a nerve agent are shown in Fig. 2. The seizure onset times (mean ± S.E.M.) were significantly shorter ($P < 0.01$) when the dose of atropine was lowered (2.0 and 0.1 mg/kg, respectively) in animals exposed to GB [8.45 ± 0.28 ($n = 102$) and 6.08 ± 0.30 ($n = 103$)], GF [5.67 ± 0.38 ($n = 109$) and 4.53 ± 0.25 ($n = 101$)], or VX [20.75 ± 0.71 ($n = 103$) and 16.93 ± 0.82 ($n = 100$)]. However, times to seizure onset remained constant between the two doses of atropine sulfate in GA-, GD-, and VR-exposed animals.

Seizure Termination. Seizure termination times (in minutes) after $2 \times LD_{50}$ of nerve agent are shown in Fig. 3. Terminations were significantly different (longer) when the atropine sulfate dose was lowered from the 2.0-mg/kg to the 0.1-mg/kg dose of atropine only in the GF-exposed animals that were treated with diazepam (14.18 ± 6.80 and 68.74 ± 14.05), but they remained the same in midazolam (10.69 ± 5.00 and 29.47 ± 18.35) and scopolamine (31.50 ± 8.13 and 21.47 ± 8.13) treatment groups. All other termination times were statistically equivalent for a given treatment with a given nerve agent tested between the two doses (2.0 or 0.1 mg/kg) of atropine sulfate. Another notable difference, when the results of these two guinea pig models were compared, was the significantly more rapid action of midazolam than of diazepam in terminating GA-, GD-, and VR-induced seizure
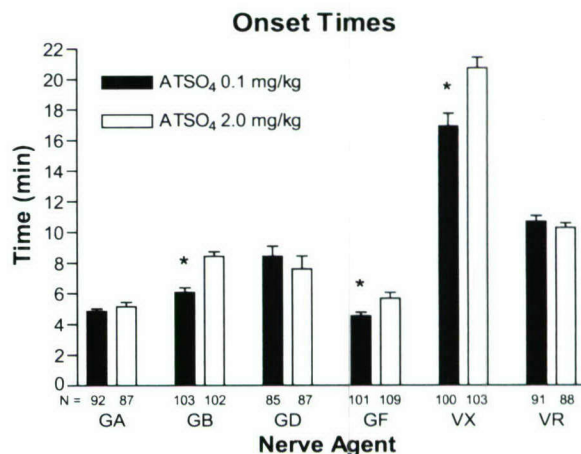
Fig. 2. The time (in minutes) to onset of EEG seizure following $2 \times LD_{50}$ of nerve agents in guinea pig models (see Fig. 1), where atropine was either 0.1 or 2.0 mg/kg i.m. The group size range was 85 to 109. Seizure onset times (in minutes) were significantly ($P < 0.01$) decreased in GB-, GF-, and VX-treated animals when the atropine sulfate dose was lowered from 2.0 to 0.1 mg/kg. *, $P < 0.05$ compared between 0.1 and 2.0 mg/kg atropine.
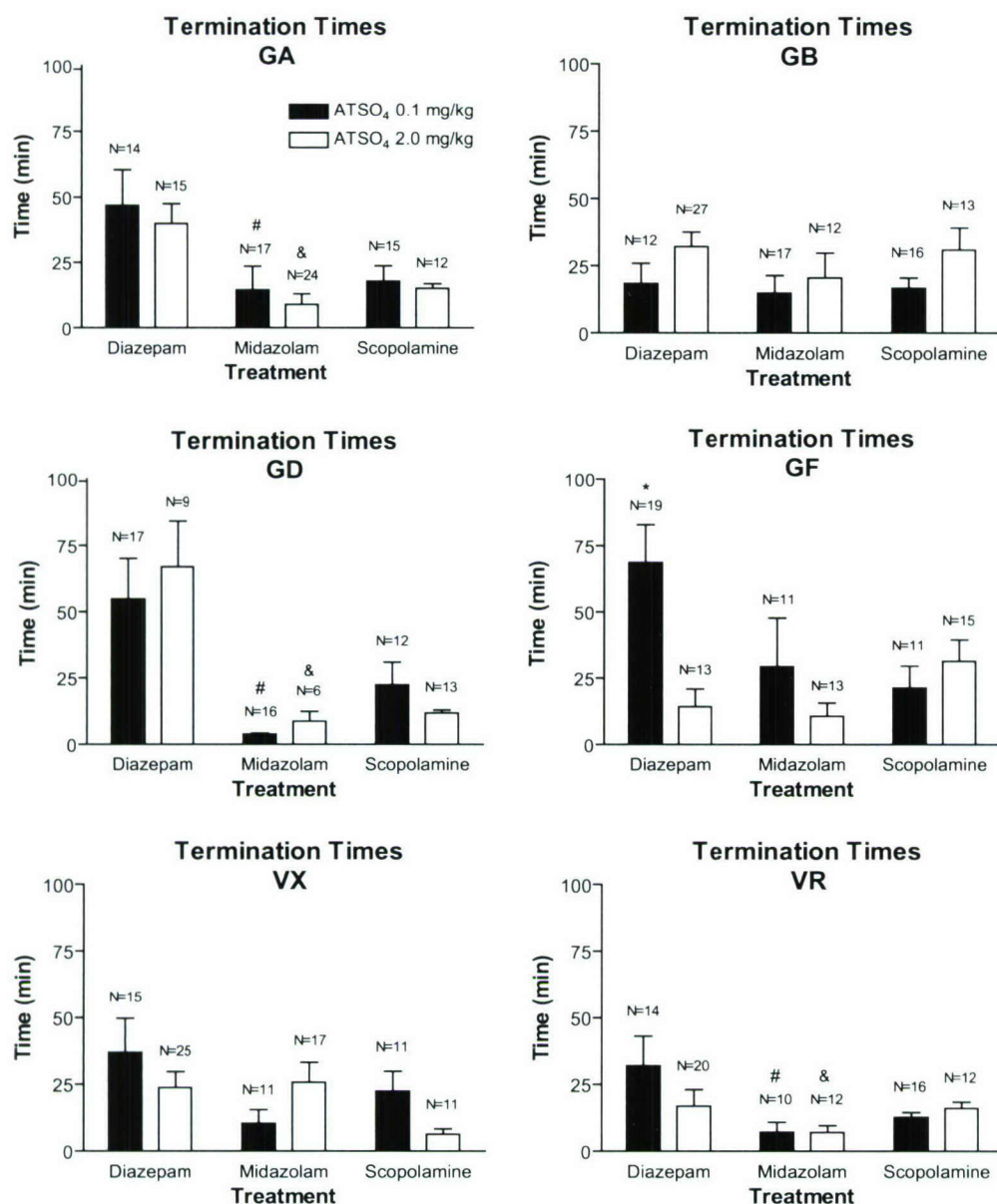
Fig. 3. The latency (in minutes) for termination of ongoing seizure following $2 \times LD_{50}$ of nerve agents and various doses of anticonvulsant drug treatment in guinea pig models (see Fig. 1), where atropine was either 0.1 or 2.0 mg/kg i.m. Mean latency ($\pm$S.E.M.) for seizure termination categorized by treatment drug and nerve agent. All animals whose seizure activity was terminated, regardless of treatment drug dose, were included in each nerve agent-treatment drug combination group. The group size was shown on top of each bar, with a range of six to 27. Seizure termination times (in minutes) were only significantly ($P < 0.05$) longer in diazepam for GF when the atropine dose was lowered from 2.0 to 0.1 mg/kg. *, $P < 0.05$ compared between atropine 0.1 and 2.0 mg/kg. #, $P < 0.05$ compared with diazepam and atropine 0.1 mg/kg treatment group. &, $P < 0.05$ compared with diazepam and atropine 2.0 mg/kg treatment group.

regardless of the therapeutic doses of atropine (Shih et al., 2003).

Toxicity and Lethality. The signs of nerve agent intoxication were lacrimation, salivation, rhinorrhea, muscle fasciculations, and tonic-clonic convulsions. These signs were more severe after 0.1 mg/kg atropine sulfate for GA, GB, GF, VX, and VR than with 2.0 mg/kg atropine. However, signs of intoxication were not changed in the GD studies when the atropine sulfate was lowered to 0.1 mg/kg. Shortly after agent administration (5–10 min), GF induced a coma in some animals. The coma was a physical state in which respiration rate lowered to a point where the animal grew cyanotic, and EEG activity decreased in amplitude to a point where it could not be detected; some animals died after reaching this state, whereas in others, respiration rate progressively increased, cyanosis was resolved, and EEG activity resumed. GF-induced coma occurred in 19.8% of animals in the 2.0 mg/kg atropine model, whereas in the 0.1 mg/kg atropine sulfate model, the occurrence of coma increased significantly to 56.2%.

The effects of anticonvulsant treatment on 24-h lethality

after $2 \times LD_{50}$ of a nerve agent are shown in Fig. 4. By lowering the dose of atropine from 2.0 to 0.1 mg/kg, the 24-h lethality increased significantly ($P < 0.01$) when midazolam was used as treatment after GF (23.7 and 60.0%), VX (4.6 and 26.7%), and VR (20.8 and 61.3%) and with diazepam as treatment after GB (0.0 and 25.0%), GF (3.3 and 75.8%), and VR (8.3 and 36.0%). The 24-h lethality remained statistically equivalent, regardless of atropine dosage for the diazepam treatment of GA, GD, and VX, for midazolam treatment of GA, GB, and GD, and for all nerve agents after scopolamine.

As reported previously for the higher dose of atropine in model A (Shih et al., 2003), overall there was a strong relationship between the control of seizure activity and protection against nerve agent-induced lethality with the lower dose of atropine in model B. Only 10% ($n = 25$) of the animals that had their seizures stopped by the anticonvulsant treatment died within 24 h, whereas 52% ($n = 129$) animals died within 24 h if drug treatment failed to stop seizures. This difference was highly significant ($\chi^2 = 95.8$,
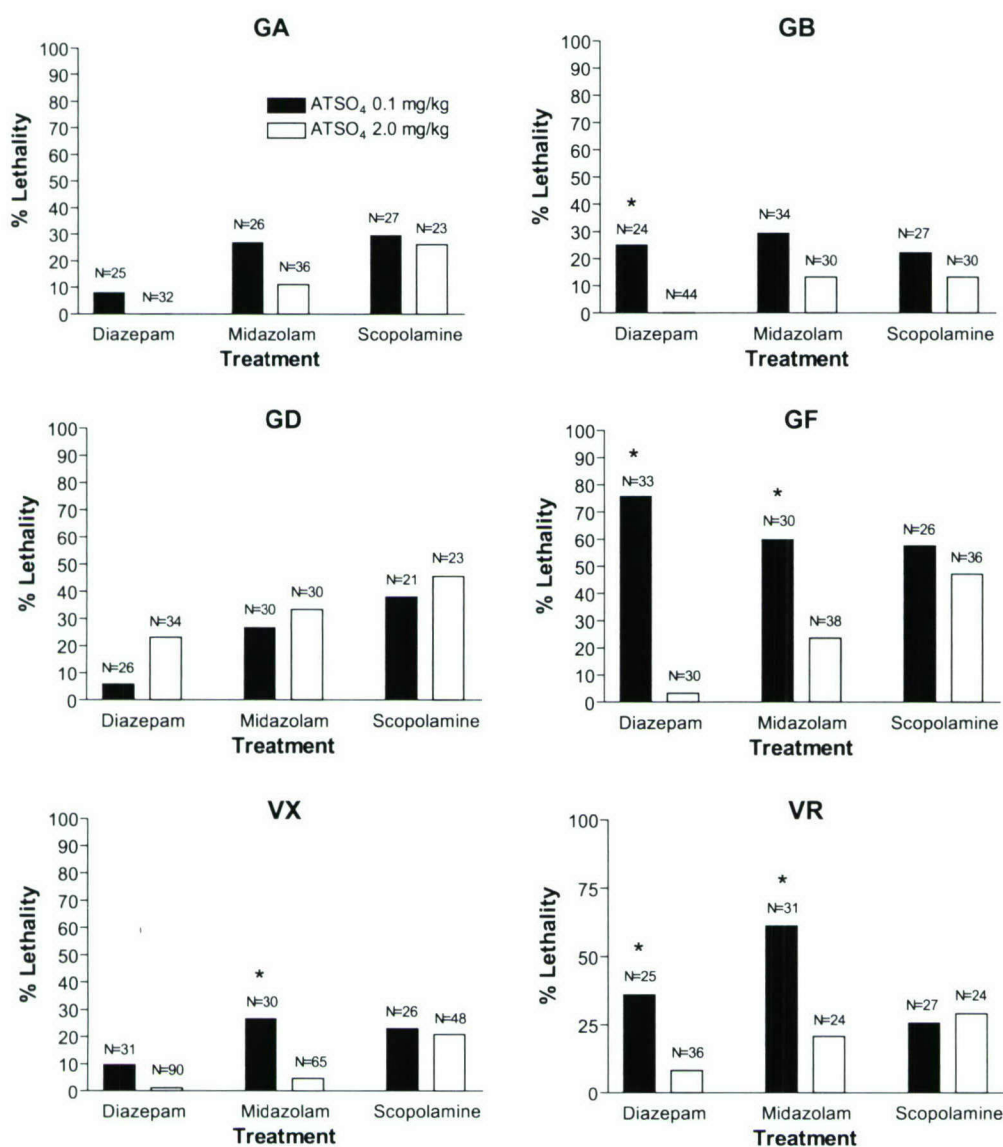
Fig. 4. The 24-h lethality (percentage) following $2 \times LD_{50}$ of nerve agents and various doses of anticonvulsant drug treatment in guinea pig models (see Fig. 1), where atropine was either 0.1 or 2.0 mg/kg i.m. All animals that died at 24 h, regardless of treatment drug dose, were included in each nerve agent-treatment drug combination group. The group size was shown on top of each bar, with a range of 21 to 90. The percentage of lethality was significantly ($P < 0.05$) greater when the atropine dose was lowered from 2.0 to 0.1 mg/kg in diazepam for GB, GF, and VR, and in midazolam for GF, VX, and VR. *, $P < 0.05$ compared between atropine 0.1 and 2.0 mg/kg.

df = 1, $P < 0.001$), and it was consistent across either the individual nerve agents or individual treatment drugs.

Anticonvulsant Efficacy. Table 1 provides the anticonvulsant ED_{50} results for both doses of atropine sulfate used in model A and model B. When the atropine sulfate dose was lowered from 2.0 mg/kg (model A) to 0.1 mg/kg (model B), the anticonvulsant ED_{50} for midazolam increased for all of the nerve agents tested. The increase for the midazolam ED_{50} in

TABLE 1

Summary of anticonvulsant ED_{50} doses

Anticonvulsant ED_{50} values (with 95% confidence limits in parentheses) for each drug (midazolam, diazepam, or scopolamine) were calculated based on terminating cortical seizure activity induced by a $2 \times LD_{50}$ dose of nerve agents in guinea pig models, where atropine was either 2.0 or 0.1 mg/kg i.m.

	Midazolam	Diazepam	Scopolamine
	mg/kg		
Model A: atropine (2.0 mg/kg)			
GA	0.05 (0.02–0.09)	3.32 (2.38–5.10)	0.04 (0.02–0.06)
GB	0.01 (0.01–0.02)	0.79 (0.32–1.34)	0.01 (0.01–0.02)
GD	0.36 (0.23–0.55)	4.81 (3.01–23.20)	0.12 (0.09–0.16)
GF	0.04 (0.02–0.11)	2.58 (1.92–3.61)	0.11 (0.02–0.13)
VX	0.01 (0.01–0.02)	0.47 (0.26–0.77)	0.05 (0.03–0.07)
VR	0.09 (0.05–0.16)	2.45 (1.79–3.12)	0.08 (0.05–0.11)
Model B: atropine (0.1 mg/kg)			
GA	0.70 (no limits)	2.57 (1.79–3.62)	0.13 (0.09–0.19)
GB	0.13 (0.08–0.22)	0.47 (0.30–0.80)	0.07 (0.04–0.10)
GD	1.67 (1.06–3.06)	2.33 (0.00–4.23)	0.11 (0.07–0.17)
GF	0.71 (0.29–2.01)	1.24 (no limits)	0.14 (0.10–0.20)
VX	0.15 (0.09–0.25)	0.74 (0.53–0.96)	0.06 (0.04–0.09)
VR	0.29 (no limits)	1.07 (0.65–1.68)	0.09 (0.07–0.11)

GA, GB, GD, GF, VX, and VR was 14-, 13-, 5-, 18-, 15-, and 3-fold, respectively. Diazepam as a treatment resulted in an anticonvulsant ED_{50} that was decreased when the atropine sulfate dose was lowered from 2.0 to 0.1 mg/kg in VR by 2-fold (2.45 versus 1.07 mg/kg), but it remained the same in GA, GB, GD, and VX. The anticonvulsant ED_{50} for diazepam to GF was also apparently decreased when the atropine sulfate dose was lowered from 2.0 to 0.1 mg/kg, but it was not statistically significant. The anticonvulsant ED_{50} for scopolamine was not affected by the atropine sulfate doses for GD, GF, VX and VR, but was significantly increased for GA (0.04 versus 0.13 mg/kg; 3-fold) and GB (0.01 versus 0.07 mg/kg; 7-fold) when the atropine sulfate dose was lowered from 2.0 to 0.1 mg/kg.

Neuropathology. All of the nerve agents were capable of producing neuropathology under the conditions of this study model (model B; Fig. 1). As reported earlier for the higher dose of atropine in model A (Shih et al., 2003), there was a strong relationship between the control of seizure activity and both the incidence and severity of brain pathology with the lower dose of atropine in model B. Significantly fewer numbers of animals in which the seizures were controlled by drug treatment displayed brain pathology (71 of 215; 33%) compared with the animals where seizures were not controlled by drug treatment (97 of 119; 82%) ($\chi^2 = 70.1$, df = 1, $P < 0.001$). Moreover, total brain lesion scores were less in animals in which seizures were controlled by drug treatment (mean = 2.54) compared with those in which seizures were not controlled (mean = 8.98). As was with previously studied model A, the basic lesion was one of neuronal necrosis (see Fig. 5 in Shih et al., 2003 for examples of the pathology). There was no qualitative difference in the appearance of a lesion nor the brain areas affected between the two models, among the different nerve agents, or among the different treatment drugs.

Discussion

In the present study, we modified a guinea pig model that had been used previously to evaluate potential anticonvulsant drugs for the treatment of nerve agent-induced seizures (McDonough et al., 1999, 2000; Shih and McDonough, 1999, 2000; Shih et al., 2003). Model A had used a higher dose of atropine (2.0 mg/kg) and anticonvulsant treatment was given 5 min after seizure onset, whereas the refined model B used a lower dose of atropine (0.1 mg/kg), and anticonvulsant treatment was given immediately after EEG seizure onset. Model B uses the total dose of atropine and 2-PAM, equivalent to three sets of the nerve agent antidote kit MARK I autoinjectors, and the anticonvulsant drug was administered at the time EEG seizure activity started. Thus, it more closely simulates the current medical treatment protocol for immediate military treatment of a severe nerve agent exposure. Under these conditions, guinea pigs exhibit moderate-to-severe salivation and lacrimation after seizure, an indication of insufficient atropine. Salivation and lacrimation were rarely observed in guinea pigs treated with 2 mg/kg atropine (McDonough et al., 1999, 2000; Shih and McDonough, 1999, 2000; Shih et al., 2003). Nevertheless, even with the 0.1-mg/kg dose of atropine, diazepam, midazolam, and scopolamine still exhibited excellent anticonvulsant effects against all of the nerve agents. Although two variables were changed in model B, atropine dose and timing of anticonvulsant administration, the data strongly indicate that the lower atropine dose contributed most to the observed differences between the two models. Atropine dose was the only variable that could influence seizure incidence and time of seizure onset. Earlier anticonvulsant administration would be expected to improve clinical outcome and enhance anticonvulsant effectiveness, but these effects were not seen in the results with model B.

When the results with model B were compared with those of model A, there were some predictable differences. With the lowered atropine dose there was a shortened latency to seizure onset and an increased frequency of seizure occurrence when either GB or VX were the challenge agents, and seizure latency was also decreased for GF. However, the latency to seizure onset was virtually the same between these two models in the case of GA, GD, and VR challenge, and the frequency of seizure occurrence for GA, GD, GF, and VR was already near 100% for with the high dose of atropine.

In model A, we found that, for GD and VX, the seizure occurrence was 100 and 56%, respectively, the time to seizure onset was 8.1 and 20.8 min, respectively, and the anticonvulsant ED_{50} for atropine was 11.8 and 4.0 mg/kg, respectively (Shih et al., 2003). The latter indicated that significantly lower atropine doses are required to treat VX-induced seizure activity than GD-induced seizure activity in the therapeutic model. In model B, whereas the latency to seizure and frequency of seizure occurrence for VX were changed, these parameters were not changed for GD. The differences observed between GD and VX in these two models were of particular interest. They may possibly be explained by the different doses of atropine, the different physical properties of the nerve agents, and the different responses of GD and VX to the oxime 2-PAM. It is well documented that the GD-AChE complex ages rapidly and that it is refractory to significant reactivation with 2-PAM (Fleisher and Harris, 1965; Fleisher et al., 1967). Thus, in GD exposure, the use of 2-PAM in the model played no role in reducing the levels of free ACh to compete with atropine for cholinergic receptors. A higher concentration of atropine is therefore required to compete with the excess ACh at synapses. However, VX-inhibited AChE displays negligible aging, and it is readily reactivated by 2-PAM. Because of the availability of reactivated AChE by 2-PAM, less free ACh is available at the synaptic junction, which in turn requires less atropine for competition at the cholinergic receptors. This may also explain the longer seizure latency with VX compared with GD. The lower dose of atropine in model B after administration of VX may have been less effective at blocking ACh receptors as readily as the higher atropine dose did (i.e., mass action) in model A and thus increase seizure frequency and shorten the seizure onset time. However, even at the lower dose of atropine, the latency to seizure onset for VX was still longer compared with GD (17 versus 8 min). This difference is probably due to a difference in physical properties (e.g., chemical structure, pK_a, and lipid solubility) between these two agents, which affects their speed to penetrate the blood-brain barrier and inhibits brain AChE (Shih et al., 2005). There is also the possibility of drug and agent

interactions among the administered pretreatment, therapy, and the nerve agent. It would be of interest to know whether GD and VX when given in the same challenge dose would have similar latency to seizure onset in the absence of PB pretreatment and atropine + 2-PAM therapy in our models. This would more closely mimic the case of a terrorist use of nerve agent against civilians, such as the case of the Tokyo subway sarin attacks, where pretreatment or atropine/oxime/anticonvulsant therapy is not immediately available. The incidence of seizures and other toxic signs and the effectiveness of standard medical countermeasures have not been fully investigated under such delayed treatment circumstances.

When the lower dose of atropine was used, the anticonvulsant ED_{50} values for scopolamine or diazepam (with respect to either high or low doses of atropine sulfate) were, in general, not changed for GD, GF, and VX. The exceptions are that scopolamine became less effective against GA (3-fold) and GB (7-fold), whereas diazepam was more potent (2-fold) against VR. The anticonvulsant ED_{50} values of midazolam were, however, increased 3- to 17-fold for all of the nerve agents tested when the dose of atropine sulfate was reduced from 2.0 to 0.1 mg/kg. Overall, scopolamine and midazolam remained more potent than diazepam in terminating seizures induced by all nerve agents. Time to seizure termination was not changed in either model except in the case of GF challenge, where it took longer for diazepam to terminate seizures when a lower dose of atropine was provided.

Another notable difference was the significantly more rapid action of midazolam than of diazepam in terminating GA-, GD-, and VR-induced seizure regardless of the therapeutic doses of atropine. The anticonvulsant potency for midazolam was reduced when the therapeutic dose of atropine was decreased. This implies that the effectiveness of midazolam as an anticonvulsant for nerve agent-induced seizure is also dependent on the ability of atropine to block cholinergic receptors. The importance of cholinergic blockade in stopping nerve agent-induced seizures is also seen from the anticonvulsant potency of scopolamine (Table 1). Scopolamine is an extremely potent anticonvulsant drug against GD-induced seizures when given shortly after seizure onset (McDonough et al., 2000), and it was highly effective in terminating seizures induced by all nerve agents regardless of the therapeutic doses of atropine given. This supports the notion that early and direct cholinergic receptor inhibition is critical and is the most efficient way of alleviating nerve agent-induced toxicity. The reason for the doses of diazepam not changing with the lower atropine dose is not clear. Even though the potency for midazolam was reduced when a lower dose of atropine was used, the absolute anticonvulsant dosage required by midazolam was still lower than that required by diazepam in all cases.

An interesting observation from previous (Shih et al., 2003) and current studies was that with either dose of atropine, the dose of anticonvulsant drugs, whether scopolamine or benzodiazepines, required to stop GD-induced seizure remained at or near the highest among all six nerve agents. This justifies GD as the agent to use when screening or testing potential anticonvulsant drugs (McDonough et al., 1999, 2000).

In conclusion, these findings show that, with the current immediate treatment dose of atropine (0.1 mg/kg) and 2-PAM (25 mg/kg), seizures occur (100%) in guinea pigs for all of the tested nerve agents after a $2 \times LD_{50}$ challenge. These exposed subjects exhibit more severe signs of intoxication and also have a higher incidence of mortality with either benzodiazepine compared with benzodiazepine treatment and the higher atropine dose. The incidence of GF-induced coma would be higher. Furthermore, seizure onset time is shorter for GB, GF, and VX. Diazepam, midazolam, and scopolamine are all effective anticonvulsant drugs for treating seizures induced by nerve agents; midazolam and scopolamine have higher potency than diazepam. An increased dose of midazolam with the lower atropine dose may be required to terminate nerve agent-induced seizure activity. The effectiveness of scopolamine in its anticholinergic and anticonvulsant potency as well as survivability remains the same for either atropine dose. All of these phenomena support the notion that atropine in itself has an anticonvulsant effect, and the dose of atropine given to nerve agent casualties may influence the successful outcomes of survival and long-term neuroprotection. Thus, the current atropine dose for immediate treatment may not be optimal against some nerve agents.

References

Aas P (2003) Future considerations for the medical management of nerve-agent intoxication. *Prehospital Disaster Med* **18**:208–216.

Berry W and Davis DR (1970) The use of carbamates and atropine in protection of animals against poisoning by 1,2,2-trimethylpropyl methylphosphonofluoridate. *Biochem Pharmacol* **19**:927–934.

Bliss CI (1952) The statistics of bioassay with special reference to the vitamins, in *Vitamin Methods*, Academic Press, New York.

Dirnhuber P, French MC, Green DM, Leadbeater L, and Stratton JA (1979) The protection of primates against soman poisoning by pretreatment with pyridostigmine. *J Pharm Pharmacol* **31**:295–299.

Dunn MA and Sidell FR (1989) Progress in medical defense against nerve agents. *J Am Med Assoc* **262**:649–652.

Fleisher JH and Harris LW (1965) Dealkylation as a mechanism for aging of cholinesterase after poisoning with pinacolyl methylphosphonofluoridate. *Biochem Pharmacol* **14**:641–650.

Fleisher JH, Harris LW, and Murtha EF (1967) Reactivation of pyridinium aldoxime methochloride (PAM) of inhibited cholinesterase activity in dogs after poisoning with pinacolyl methylphosphonofluoridate (soman). *J Pharmacol Exp Ther* **156**:345–351.

Hayward IJ, Wall HG, Jaax NK, Wade JV, Marlow DD, and Nold JB (1990) Decreased brain pathology in organophosphate-exposed rhesus monkeys following benzodiazepine therapy. *J Neurol Sci* **98**:99–106.

Lennox WJ, Harris LW, Talbot BG, and Anderson DR (1985) Relationship between reversible acetylcholinesterase inhibition and efficacy against soman lethality. *Life Sci* **37**:793–798.

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

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

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

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

Moore DH, Clifford CB, Crawford IT, and Baggett JM (1995) Review of nerve agent inhibitors and reactivators of acetylcholinesterase, in: *Enzymes of the Cholinesterase Family* (Quinn DM, Balaubramanian AS, Doctor BP, and Taylor P eds), pp 297–304, Plenum Press, New York.

Shih T-M, Duniho SM, and McDonough JH (2003) Control of nerve agent-induced seizures is critical for neuroprotection and survival. *Toxicol Appl Pharm* **188**:69–80.

Shih T-M, Kan RK, and McDonough JH (2005) *In vivo* cholinesterase inhibitory specificity of organophosphorous nerve agents. *Chemicobiol Interact* **157–158**:293–303.

Shih T-M and McDonough JH (1999) Organophosphorous nerve agents-induced seizures and efficacy of atropine sulfate as anticonvulsant treatment. *Pharmacol Biochem Behav* **64:**147–153.

Shih T-M and McDonough JH (2000) Efficacy of biperiden and atropine as anticonvulsant treatment for organophosphorous nerve agent intoxication. *Arch Toxicol* **74:**165–172.

Sidell FR (1997) Nerve Agents, in *Medical Aspects of Chemical and Biological Warfare, Textbook of Military Medicine, Part 1. Warfare, Weaponry, and the Casualty* (Sidell FR, Takafuji ET, and Franz DR eds), pp 129–179, The Office of the Surgeon General, Washington, DC.

Taylor P (2001) Anticholinesterase agents, in *Goodman and Gilman's The Pharma-cological Basis of Therapeutics*, 10th ed. (Hardman JG, Limbird LE, and Gilman AG eds), pp 110–129, McGraw-Hill Companies, New York.

Winer BJ (1971) *Statistical Principles in Experimental Design*, McGraw-Hill Companies, New York.

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