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Novel neuroprotective effects with *O*-benzyl derivative of pralidoxime in soman-intoxicated rodents

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Abstract

Pharmacological properties of oxime reactivators, not related to its ability to regenerate or reactivate nerve agent-inhibited acetylcholinesterase located at nerve synapses, have been reported to be important in protecting against poisoning by the nerve agent soman. Such non-reactivation effects have thus far been associated only with bispyridinium oximes. This study investigated the possibility of creating similar non-reactivation therapeutic effects in the mono-pyridinium ring oxime, pralidoxime (2-PAM) through attachment of alkyl groups of increasing chain length to the oxime functional group. Of the 4 derivatives investigated, only the *O*-benzyl derivative displayed strong sedative effects in mice and mitigated the development of motor convulsions following soman challenge ($1.8 \times LD_{50}$, subcutaneous). Anticonvulsant effects of this compound were enhanced by co-administration of a non-anticonvulsant dose of atropine sulfate. Administration of equivalent amount of other *O*-derivatives of pralidoxime failed to elicit similar anticonvulsant actions. Electroencephalographic (EEG) and histopathological studies using the rat model, intoxicated with a lethal dose ($1.6 \times LD_{50}$, s.c.) of soman, confirmed *O*-benzyl derivative neuroprotective capabilities when used as a pretreatment drug. Microdialysis studies revealed that its neuroprotective effect is related to its ability to attenuate soman-induced increase in acetylcholine.

Keywords: Pralidoxime derivative; Sedative effect; Soman poisoning; Anticonvulsant effect; Acetylcholine; Non-reactivation effect

1. Introduction

Oxime reactivators are used in nerve agent therapy mainly for their ability to reactivate nerve agent-inhibited acetylcholinesterase. Soman intoxication is a difficult nerve agent poisoning to treat as soman-inhibited human acetylcholinesterase rapidly (half-life of 2 min) becomes non-reactivable in a phenomenon known as "aging". HI-6 or otherwise known by its IUPAC name of 1-(4-carbamoylpyridino) methoxymethyl-2- (hydroxyiminomethyl) pyridinium dichloride, a bispyridinium oxime, is reported to be significantly more effective in treating soman toxicity than other oximes, such as pralidoxime-2-chloride (2-PAM) and obidoxime. It has been reported to be effective in protecting primates against lethal doses ($5 \times LD_{50}$) of soman without any evidence of acetylcholinesterase reactivation (Hamilton and Lundy, 1989). Consequently, the protective effects of HI-6 has been ascribed to a variety of non-reactivation effects including anti-nicotinic, anti-muscarinic, competitive acetylcholinesterase inhibition, and presynatic modulatory effects (van Helden et al., 1996). While the isonicotinamide component and the intermediate ether linkage of HI-6 have been studied and found to provide little contributions to the non-reactivating therapeutic effects, the effects from modifications to the "pralidoxime component" of HI-6 on the observed "direct" effects are relatively less well studied. Pralidoxime itself is known to possess similar weak anti-muscarinic and acetylcholinesterase inhibition effects as HI-6 (Shih et al., 1991; Loke et al., 2002), yet it possesses little

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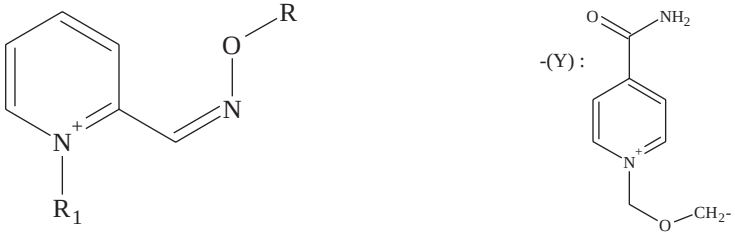
Table 1
Structures and nomenclature of *O*-substituted derivatives of pralidoxime and HI-6



Compound code	Identity of R group	Identity of of R_1 group	Name of compound
1	–(CH_3)	–(CH_3)	*O*-methylpyridine-2-carbaldoxime methidodie (MeP)
2	–(CH_2–C_6H_5)	–(CH_3)	*O*-benzylpyridine-2-carbaldoxime methiodide (BzP)
3	–($CH_2C\ell CH_2$)	–(CH_3)	*O*-prop-2-ynylpyridine-2-carbaldoxime methiodide (PrP)
4	–($CH_2C\ell C$–CH_3)	–(CH_3)	*O*-but-2-ynylpyridine-2-carbaldoxime methiodide (BuP)
5	–(H)	–(CH_3)	Pralidoxime (PAM)
6	–(H)	–(Y)	HI-6

of HI-6 protective effects against soman poisoning. This study investigated the possibility of increasing the anticholinergic effects, and hence "direct" effects, of pralidoxime through attachment of alkyl groups of increasing chain length to the oxime functional group. Previous investigation has shown that direct intracereboventricular administration of *O*-substituted derivatives of pralidoxime compounds (Table 1) protected against respiratory depression and bradycardia effects of soman poisoning (Loke et al., 2002). The observed therapeutic effects is unlikely to be related to reactivation of acetylcholinesterase as *O*-substitution would have resulted in the loss of the hydrogen bond donating property of the oxime functional group required for enzyme reactivation reactions. This present study is a continuation of the previous effort to investigate if similar protective effects could be observed with systemic administration of these compounds into soman-poisoned rodents. The present findings showed that, although enhanced animal survival against lethal challenges with soman was not observed, *O*-benzyl derivative of pralidoxime was observed to provide novel neuroprotection effects against a lethal soman challenge. Possible neurochemical mechanisms related to the neuroprotective properties in terms of neurotransmitter release were also investigated.

2. Materials and methods

2.1. Chemistry

The synthesis and characterisation of the pralidoxime derivatives (*O*-methylpyridine-2- carbaldoxime methiodide (**1**); *O*-benzylpyridine-2-carbaldoxime methiodide (**2**); *O*-prop-2- ynylpyridine-2-carbaldoxime methiodide (**3**); and *O*-but-2-ynylpyridine-2-carbaldoxime methiodide (**4**)) have been reported earlier (Loke et al., 2002). These synthetic variants of the pralidoxime parent structure are illustrated in Table 1 for better clarity on the structural changes made to pralidoxime. In this report, *O*-benzyl derivative of pralidoxime will be used as the abbreviated form for the compound *O*-benzylpyridine-2-carbaldoxime methiodide. Atropine sulfate and 2-pyridinealdoxime methochloride (2- PAM) were obtained from Sigma Chemical Co., St. Louis, MO. HI-6 dichloride was obtained from Astra, Sweden. Saline (0.9% NaCl) was prepared in-house, 0.2 μm filtered, autoclaved and sealed in Wheaton serum bottle. Soman (pinacolyl methyl phosphonofluoridate), synthesized in-house at the Centre for Chemical Defence (DSO National Laboratories), was freshly diluted in ice-cold saline prior to injection. All other pharmacological compounds used in this study were similarly prepared in ice-cold 0.9% saline. The volume of injection was 0.5 ml/kg and all drug solutions were prepared and injected separately to avoid drug interaction. Soman was administered subcutaneously (s.c.), HI-6 intraperitoneally (i.p.) while the rest of the drugs were administered intraperitoneally (i.p.) with the mice study and intramuscularly (i.m.) in the rats study.

For neurochemical analyses, acetylcholine and choline were obtained from Sigma Chemical Co., St. Louis, MO. Norepinephrine (NE), epinephrine (E), dopamine (DA), dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine or serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) were purchased from Fluka. Other chemicals (analytical and HPLC grades) for high performance liquid chromatography (HPLC) analysis were purchased commercially and used without further purification. Ringer perfusion solutions (140 mM NaCl, 1.2 mM $CaCl_2$, 3.0 mM KCl, 1.0 mM $MgCl_2$ and 0.04 mM ascorbic acid, pH 6; with 1 μM pyridostigmine added to prevent acetylcholine (ACh) hydrolysis) for the microdialysis experiments were prepared in-house.

2.2. Apparatus

Catecholamine neurotransmitters were analysed using an Inertsil C18 column (particle size 5 μm; 100 × 1 mm I.D.)

from Micro-Tech protected with a Inertial C18 guard column (10 × 1 mm I.D.). Acetylcholine and choline were analysed after resolving through a polymeric microbore guard column (14 × 1 mm) and analytical column (530 × 1 mm) (MF-8903, BAS) connected to an enzyme reactor column (IMER) (50 × 1 mm) containing acetylcholinesterase and choline oxidase. Microdialysis probes (CMA-12) were purchased from Carnegie Medicine, Sweden. EEG recordings were enabled using ART version 2.01 receiver and implanted telemetry probe system from Datascience International.

2.3. Animals

Female Swiss albino mice (average body weight 23 g) and male Wistar rats (average body weight range 250–300 g), obtained from the National University of Singapore Laboratory Animal Centre, were used for the experiments. The animals were handled according to international guidelines for animal research (Howard-Jones, 1985). They were adequately housed at temperatures of 22 ± 2 °C, humidity 55 ± 5% and a 12 : 12 h light/dark cycle with lights on at 7:00 a.m. Food and water were provided ad libitum.

2.4. Surgery

All animals were implanted with two cortical stainless steel connectors (female ends; Elfa EB) under halothane anaesthesia 1 week prior to drug experimentation. Connectors were placed, with the aid of dental cement and anchor screws, approximately equidistant between bregma and lamda and bilaterally ±2.0 mm from the midline. The male ends of the connectors were attached directly to lead wire ends of a TA11C-F40 telemetry implant placed in a subcutaneous pouch on the side of the animal. A magnet was used to activate the telemetry implant when recording of EEG signals was required.

For catecholamine and acetylcholine sampling, a microdialysis guide cannula (CMA/12; Carnegie Medicine, Sweden) was implanted in the left striatum using a stereotaxic frame (David Kopf Instruments, USA) at the following coordinates relative to the bregma: (A) +1.2, (L) −2.4, and (V) −3.5 mm. During surgery, the body temperature was kept at 37.5 °C using a thermoregulatory pad (CMA/150). After surgery, the animals were allowed to rest for 5 days at room temperature of 24 ± 2 °C, humidity 55 ± 5% and a 12 : 12 h light/day cycle with lights on at 7:00 a.m. Food and water were provided ad libitum and the animals trained to adapt to staying in the microdialysis activity bowl for 1 h prior to actual microdialysis sampling. The probe was gently inserted into the brain through the cannula space, vacated by the dummy probe, on the day of the experiment. To avoid influences of anaesthetic procedure on neurochemistry of animal, it was not anaesthetized when the dummy probe was exchanged with the microdialysis probe. Its head was instead held in place gently and firmly with the researcher's left hand while the right hand effected the exchange of the microdialysis probes.

2.5. EEG recordings

EEG recordings were enabled using Datascience International ART Telemetry System, version 2.01. EEG signals from implanted telemetry probes were collected by a receiver placed below each animal cage. The EEG recording did not require any form of animal restraints and the resultant stress-free animal model provides for a better approximation to a physiological mammalian system.

2.6. Chromatographic conditions

The microdialysate samples were analysed by a dual-column microbore liquid chromatography system fitted with two Antec Decade Electrochemical Detectors (ECD). 10 μl aliquots of sample were injected using a 10-port valve fitted to two separate liquid chromatography columns. Catecholamine separation was carried out on an Inertsil C18 column (particle size 5 μm; 100 × 1 mm I.D.) using an isocratic flow rate set of 80 μl/min. Detection was through electrochemical means with applied potential set at +0.75 V with respect to Ag/AgCl reference electrode. Detection limit was 5 fmol or 1 nM catecholamine in perfusate.

For separation of acetylcholine and its precursor choline, a polymeric guard column (14 × 1 mm) (MF-8935, BAS) was connected in series with an analytical column (530 × 1 mm) (MF-8903, BAS). The enzyme reactor column (IMER) (50 × 1 mm) (MF-8904, BAS) containing acetylcholinesterase and choline oxidase was inserted between the analytical column and the electrochemical detector. The temperature of all the columns was maintained at 37 °C. The mobile phase used for analysis contained 6.9 g $NaH_2PO_4 \cdot H_2O$ and 5 ml/l Kathon CG reagent (CF-2150, BAS) at pH8.5 (6 N NaOH) while the flow rate was maintained at 100 μl/min. Amperometric detector settings used for acetylcholine analysis were set at +100 mV and a picoampere integrator range was used. Detection limit was 100 fmol or 20 nM acetylcholine (ACh) or choline (Ch) in the perfusate.

2.7. Therapeutic and anticonvulsant studies on soman-treated mice

Two series of experiments were conducted to determine the therapeutic effects of these derivatives of pralidoxime. In the first series of experiments, the animals were pretreated (−30 min) intraperitoneally with 10 mg/kg of atropine sulfate and increasing doses of the test drug prior to soman administration ($1.8 \times LD_{50}$; subcutaneous). Groups of 10 mice were used per set of experiment. Based upon 24-h survival rates following soman administration, the therapeutic effects of pralidoxime and its derivatives were compared.

In the second series of experiment, the anticonvulsant capabilities of these pralidoxime derivatives were compared at a single equivalent dose (0.145 mmol/kg). This comparative dose was selected from the results of the first series of experiment, on the basis of a derivative dose that yielded the maximum sedative action and minimal toxicity. The derivatives of pralidoxime (**1**–**4**) were administered intraperitoneally, in the presence and absence of atropine (10 mg/kg; i.p.) co-administration, 30 min before soman injection ($1.8 \times LD_{50}$; s.c.). Survival rates and occurrence of motor convulsions over 24 h were determined.

2.8. Therapeutic and anticonvulsant studies on soman-treated rats

Wistar rats fitted with cortical EEG screws and microdialysis probes in the striatum, were pretreated with candidate drugs 30 min prior to a lethal injection of soman ($1.6 \times LD_{50}$; s.c.). Candidate drugs investigated were atropine sulfate (2 mg/kg), *O*-benzyl derivative of pralidoxime (50 mg/kg dose; 0.145 mmol/kg), *O*-benzyl derivative of pralidoxime (50 mg/kg dose; 0.145 mmol/kg) with 2 mg/kg atropine sulfate, and saline vehicle controls. HI-6 (125 mg/kg, i.p.) was co-administered 30 min prior to soman to enhance animal survival while atropine methyl nitrate (4 mg/kg) was administered 1 min after soman administration to reduce respiratory distress due to excessive bronchial secretions.

The test drug was administered intramuscularly, in standard injection volumes of 0.5 ml/kg. EEGs and behavioural alterations were monitored continuously for the first 4 h following soman administration and periodically at 15 min intervals thereafter for the next 20 h. Status epilepticus is considered as 30 min of continuous high-amplitude rhythmic spikes or sharp wave activity (Treiman et al., 1990). Seizure is rated as terminated by the absence of continuous high-amplitude rhythmic spikes or sharp wave activity. A seizure has to be suppressed within 4 h after anticonvulsant administration and remained suppressed for 24 h for the anticonvulsant therapy to be considered successful. Isolated spiking and/or short spike trains recurring in some records were not considered as seizures recurrence unless these trains progressed to become continuous rhythmic spikes (Shih et al., 1999). Survival rates and occurrence of status epilepticus of 4-h duration over the following 24 h were recorded.

The microdialysis probe was perfused continuously with Ringer solution at a flow-rate of 2 μl/min. During the experiment on the freely moving animal, dialysates collected for the first 60 min immediately after insertion of the probe are discarded before regular collections of 30 μl fractions at 15-min interval were carried out in sealed micro-vials (200 μl volume). Four fractions of basal perfusate were collected prior commencement of drugs pretreatment and subsequent soman intoxication 1/2 h later. Collected samples were either analysed immediately or snap-frozen in liquid nitrogen till analysis. The collected microdialysate samples were analysed for catecholamines and acetylcholine content.

All animals that survived for 24 h following soman administration were anaesthetised with hypnorm : dormicum : water analeptic mixture (2 : 1 : 1 ratio) and perfused transcardially for 15 min with physiological saline followed by paraformaldehyde (4% (*w*/*v*); 0.1 M phosphate buffer, pH 7.4; 30 min). The brains were removed and post-fixed in the same fixative for 4 h at 4 °C. Additional overnight cryoprotection treatment was carried in sucrose (30% (*w*/*v*); 0.1 M phosphate buffer) at 4 °C. Serial coronal sections of 40-um thickness were cut from frozen samples using a cryostat and mounted on silane-coated (i.e., Vectabond; APES) slides. Slides were air-dried for 30 min and Nissl-stained with Cresyl violet for general histology studies.

2.9. Neuropathological quantification

Coronal sections at bregma −2.8 mm were chosen to determine the level of neuronal loss in the piriform cortex. Quantification was carried out with a computerised image analysis (Image Pro Plus, Media Cybernetics, USA). Sections were viewed through a microscope (Leica DMLB) connected via video camera to a computer monitor, with images captured and saved into the hard disk. Cell quantification at the piriform cortex was expressed as the percentage of total area occupied by the cells in Layer II and III, respectively, over the total section area in the image at ×100 magnification.

2.10. Statistical analysis

All data given in the text and figures are expressed as means ± SEM. Time-to-seizure onset was compared between experimental groups and soman-treated animals (with saline post-treatment) by two-tailed *t*-test. Perfusate data were subjected to repeated measures analysis of variance (ANOVA) with time as factor for comparing various treatment groups. With histology data, one-way ANOVA was used for comparison of statistical difference between treatment groups. Statistically significant differences compared to basal level or control treatment group were further assessed by Dunnett's *t*-test. Statistical significance was set at $P < 0.05$.

3. Results

The initial mouse protection study involved parenteral administration of candidate drugs 30 min prior to soman intoxication to exclude considerations of drug pharmacokinetics on resultant therapeutic effects against systemic soman poisoning. The results revealed that while none of the *O*-derivatives of pralidoxime (data not shown) nor atropine protected against 1.8 LD_{50} dose of soman in mice, the *O*-benzyl derivative of pralidoxime potentiated the therapeutic effects of atropine by significantly increasing the survival time over that achieved with atropine alone (Table 2). It was

Table 2
Dose–response protective effects of *O*-benzyl substituted derivative of PAM against 1.8 × LD_{50} dose of soman in mice

Treatment [a]	Dose of atropine (mg/kg)	Dose of *O*-benzyl derivative of PAM (mg/kg)	Sample size (*n*)	Time-to-death (min) [b]	24 h mortality rate	Remarks
Saline	–	–	8	6.4±0.8	8/8	None sedated
Atropine sulfate	10	–	10	11.42±1.1	10/10	None sedated
Atropine + *O*-benzyl	10	1	10	21.8±5.1	10/10	None sedated
derivative of PAM	10	25	10	100.0±40.0	10/10	None Sedated
(compound code 2) [c]	10	50	10	100.5±35	9/10	9/10 animals sedated
	10	75	10	45.5±9.1	10/10	All 10 animals sedated
	10	100	10	19.5±0.3	10/10	All 10 animals sedated
	10	125	10	46.89±5.0	10/10	1 died before soman; all 10 animals sedated

[a] Test drugs (equimole dose of 0.145 mmol/kg) were administered prophylactically (−30 min) prior soman, in the presence and absence of atropine co-prophylactic administration. Survival rates and number of observed motor convulsions over 24 h were determined.
[b] Values refer to (mean±S.E.M.) where S.E.M. refers to the standard error of the mean.
[c] Structures and nomenclature of *O*-substituted derivatives of pralidoxime.

also observed that mice pretreated with this compound displayed potent and dose-dependent hypoactivity, decreasing both locomotion and rearing within 15 min of systemic administration, at intraperitoneal doses of 50 mg/kg and higher. These animals remained deeply sedated and died quietly in this manner about 4 h from soman intoxication without regaining consciousness.

To assess the anticonvulsant potentials arising from its unusual sedative effects, 50 mg/kg of *O*-benzyl derivative of pralidoxime was selected as the testing dose for further studies since this was the minimal dose that produced strong sedative effects without concomitant increase in mortality rates. Higher doses of this compound were not studied as they produced severe respiratory depression, which could compound the toxicity of soman poisoning. All *O*-derivatives of pralidoxime were administered in equivalent molar quantities to 50 mg/kg of *O*-benzyl derivative of pralidoxime, 30 min prior to 1.8 LD_{50} (s.c.) soman. The dose of soman was selected for its ability to ensure 100% seizures occurrence in all poisoned animals. At this high soman dose, only HI-6 pretreatment ensured animal survival over 24 h. However, *O*-benzyl derivative of pralidoxime significantly lengthened the survival duration over atropine-treated animals (Table 3). Amongst the derivatives of pralidoxime, *O*-benzyl derivative of pralidoxime was the only compound to display strong sedative (14/16) and anticonvulsant effects (2/16 in the absence of atropine and 8/16 in combination with atropine).

However, based on earlier reports of status epilepticus occurring in the absence of motor convulsions, a study with

Table 3
Comparison of protective effects of *O*-substituted derivatives of PAM against 1.8 × LD_{50} dose of soman (subcutaneous) in mice

Atropine prophylaxis (−30 min; i.p.)	Treatment drug [a] (−30 min; i.p.)	Sample size (*n*)	Motor convulsions		Time-to-death [b] (min)	24 h mortality rate	Remarks
			Response fraction	Time-to-seizure onset (min) [b]			
Yes	Saline	9	9/9	2.7±0.3	7.1±0.9	9/9	
(10 mg/kg)	HI-6 (55 mg/kg)	8	6/8	4.6±1.0	7.3±0.3	5/8	2 animals died after 6 h
	PAM (25 mg/kg)	8	8/8	2.9±0.3	13.3±2.5	8/8	
	MeP (40 mg/kg)	8	8/8	1.9±0.2	7.5±0.9	8/8	
	PrP (44 mg/kg)	8	8/8	4.5±1.6	11.4±4.5	8/8	
	BuP (46 mg/kg)	8	8/8	2.0±0.4	20.4±11.4	8/8	
	BzP (50 mg/kg)	16	8/16	7.5±4.5	66.1±10.9	16/16	14 animals sedated after drug pretreatment
No	HI 6 (55 mg/kg)	8	7/8	1.7±0.4	5.2±0.9	6/8	
	Atropine (10 mg/kg)	7	7/7	2.7±0.2	9.9±0.9	7/7	
	PAM (25 mg/kg)	8	8/8	1.8±0.3	5.0±0.6	8/8	
	MeP (40 mg/kg)	8	8/8	1.8±0.3	5.1±1.0	8/8	
	PrP (44 mg/kg)	8	8/8	1.9±0.3	6.6±0.9	8/8	
	BuP (46 mg/kg)	8	8/8	3.9±0.7	17.6±7.4	8/8	
	BzP (50 mg/kg)	16	14/16	11.4±3.1 [c]	21.7±3.6 [c]	16/16	14 animals sedated after drug prophylaxus

[a] Test drugs (equimole dose of 0.145 mmol/kg) were administered prophylactically (−30 min) prior soman, in the presence and absence of atropine co-prophylactic administration. Survival rates and number of observed motor convulsions over 24 h were determined.
[b] Values refer to (mean±S.E.M.) where S.E.M. refers to standard error of the mean.
[c] Indicates statistical significance ($p<0.05$) by paired, two-tailed *t*-test, compared to soman-intoxicated rats that received atropine for pretreatment.

Table 4
Anticonvulsant and protective effects of *O*-benzyl derivative of pralidoxime against lethal dose of soman ($1.6 \times LD_{50}$, s.c.) in rats

Pretreatment drug regimen [a]	Dose (mg/kg)	Sample size (*n*)	4 h status epilepticus [b]		24 h mortality	
			Response fraction	Time-to-onset (mean±S.E.M.) (min)	Response fraction	Time-to-death (mean±S.E.M.) (h)
Saline	NA	17	17/17 (100%)	8.27±0.7	7/17 (41.2%)	3.74±0.7
Atropine sulfate	2	11	11/11 (100%)	8.05±1.2	2/11 (18.2%)	1.73
O-benzyl derivative of pralidoxime	50	14	4/14 (28.6%)	16.43±2.5	5/14 (35.7%)	2.79±0.4
O-benzyl derivative of pralidoxime+atropine sulfate	50, 2	9	0/9 (0%)	29.27±9.8	2/9 (22.2%)	5.04±1.3

[a] Test drugs were administered prophylactically (−30 min) prior soman, in the presence of HI-6 (125 mg/kg; i.p.) co-prophylactic (−30 min) administration.
[b] EEG signals were monitored for 4 h following soman administration and periodically for 3 min every 0.5 h thereafter for the next 20 h. Seizure onset was defined as appearance of >10 s of rhythmic high-amplitude spikes or sharp wave activity in the EEG. Animals were rated for presence of 4 h of continuous seizure or status epilepticus as well as survival rates over 24 h. Figures in parenthesis refer to the ratio of rats that responded over the total number of rats tested, expressed in percentage.

the EEG-wired rat model was carried out to validate the mouse results (Sparenborg et al., 1993). Soman administration, at the selected dose of $1.6 \times LD_{50}$, resulted in 100% occurrence of status epilepticus in non-treated animals (i.e., indicated as "Saline") with a mean seizure onset time of 8.27±0.7 min (Table 4). Despite prophylactic administration of HI-6 oxime and atropine methyl nitrate to minimize bronchial secretions, a high mortality rate of 41.2% was still observed amongst the treated animals. Addition of a non-anticonvulsant dose of atropine (2 mg/kg) (Shih et al., 1999) to the HI-6 pretreatment regimen significantly enhanced survival rates (81.8%) without attenuating the high seizure rates (100% occurrence, mean seizure onset time of 8.05± 0.7 min). However, a pretreatment dose of 50 mg/kg of *O*-benzyl derivative of pralidoxime was able to prevent 4 h status epilepticus in 10 of the 14 treated animals and mean seizure onset time was doubled (16.43±2.5 min). However, as observed in the initial mice study, *O*-benzyl derivative of pralidoxime did not significantly enhance the survival rates over saline-treated animals.

The anticonvulsant effect of *O*-benzyl derivative of pralidoxime was further enhanced when co-administered with 2 mg/kg dose of atropine. None of the treated animals displayed 4 h of status epileptics (Table 4). Seizure onset was further delayed up to 30 min post-soman administration and observed survival rates at 24 h were comparable to that achieved with atropine alone. Rats pretreated with this drug combination did not display the expected sequel of full-blown status epilepticus in the first 4 h, with only intermittent ictal activities of 10–20 s duration observed every 3–5 min in most of the treated rats. Even in rats that did develop the usual train of continuous ictal spikes, status epilepticus typically do not last more than 2 h (Fig. 1). In all the treated animals, ictal spikes were also much reduced in intensity (0.4 mV as compared to 1.2 mV in saline-treated animals). Similar anticonvulsant properties were however not observed when compound was administered post-seizure (5 min after seizure onset) to 3 animals, even in the presence of co-administered 10 mg/kg atropine (data not shown). None of these post-seizure animals treated survived beyond 4 h and no further post-seizure therapeutic evaluations were carried out with this compound.

Histopathological data (Figs. 2 and 3) confirmed that pretreatment with *O*-benzyl derivative of pralidoxime protected against loss of neurons from both Layers II and III of the piriform cortex. The observed neuroprotection was significant ($P<0.05$, one-way ANOVA with post-test by Dunnett's *t*-test) as compared to soman-poisoned animals receiving saline or atropine sulfate pretreatment protocols. On the other hand, atropine pretreatment at 2 mg/kg (i.m.) did not provide neuroprotection against soman poisoning.

Microdialysis recovery with HPLC-ECD analysis revealed that following soman intoxication, there was a significant increase (200–400×) in acetylcholine levels in the striatum over the baseline levels (32.53±2.0 nM) from 0.25–3.75 h post-administration. Atropine pretreatment did not prevent soman-induced increase in acetylcholine while *O*-benzyl derivative of pralidoxime pretreatment significantly attenuated soman-induced acetylcholine efflux, both quantitatively as well as on the duration of increased release (Fig. 4A; repeated measures ANOVA with Dunnett post-test, $P<0.05$). Acetylcholine release returned to normal within 1.25 h post-soman intoxication with *O*-benzyl derivative of pralidoxime pretreatment. Combination of *O*-benzyl derivative of pralidoxime with atropine sulfate pretreatment attenuated acetylcholine efflux in a similar manner and return to normal levels occurred within 1 h post-soman intoxication. Extracellular level of choline, on the other hand, was significantly reduced (25–50×) from 3.25–3.5 h and at 4 h post-soman intoxication. Pretreatment with *O*-benzyl derivative of pralidoxime did not attenuate this decrease while atropine pretreatment resulted in an earlier reduction of choline level below baseline levels (2.25–4.0 h). Combination of atropine with *O*-benzyl derivative of pralidoxime pretreatment prevented the late decrease in choline level in soman-poisoned rats.

With the catecholamines, a delayed but significant increase (200–250× above basal 31.34±7.2 nM) in striatal

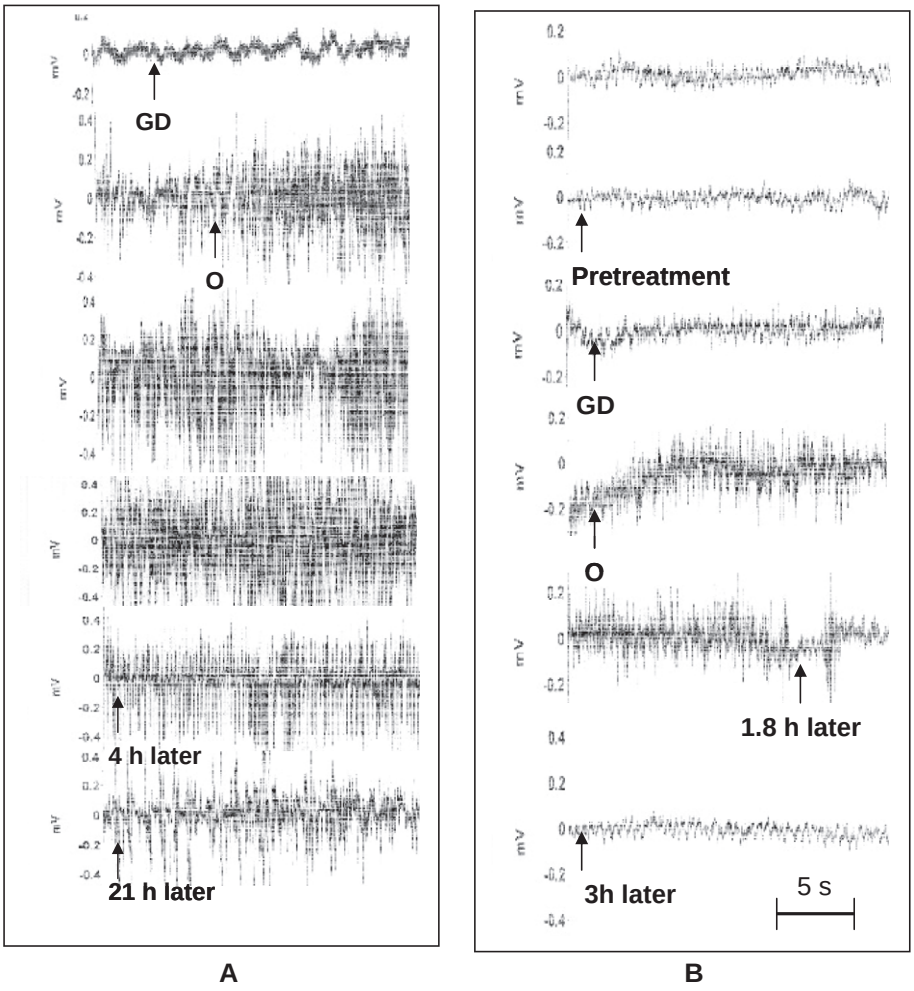


Fig. 1. Effects of pretreatment with (A) saline vehicle or (B) a combination of *O*-benzyl derivative of pralidoxime (50 mg/kg) and atropine sulfate (2 mg/kg) on rat electroencephalography (EEG) during soman-induced status epilepticus ($1.6 \times LD_{50}$; 176 μg/kg, subcutaneous). On the recorded rat EEG traces, the label "pretreatment" refers to time point where HI-6 and other treatment drugs are administered; the "GD" label refers to point of soman administration; "O" label refers to point for onset of seizure following soman administration; while the annotations "1.8 h later", "3 h later", "4 h later" and "21 h later" refer to various time intervals post-soman administration.

dopamine (Fig. 4B; repeated measures ANOVA with Dunnett's post-test, $P<0.05$) was observed at 3.0–3.5 h post-soman poisoning. HVA, a metabolite of dopamine, increased significantly (120–150×) above basal levels (0.78±0.06 μM) over the period 2.5 to 4 h post-soman intoxication. There was no observed change in DOPAC level (basal 1.35±0.2 μM) following soman poisoning. Pretreatment with any of the antidotes prevented post-soman-induced increase in dopamine efflux. With dopamine metabolites, pretreatment with *O*-benzyl derivative of pralidoxime pretreatment alone or in combination with atropine prevented soman-induced increase in HVA efflux. However, pretreatment with atropine alone further enhanced soman-induced increase in dopamine turnover as reflected by higher level of HVA and DOPAC in microdialysate samples collected over 1.5 to 4 h following soman administration.

Modest increase (150×) in 5-HT (basal level 3.50±0.5 nM) was observed at 2.75–3 h post-soman intoxication. Similar delayed but significant increases (200–300× above basal) in striatal 5-HIAA (basal level 0.289±0.02 μM) were observed in microdialysate samples collected over 3.5–4 h post-soman administration (Fig. 4C; repeated measures ANOVA with Dunnett's post-test, $P<0.05$). Overall, an increase in 5-HIAA/5-HT ratio indicating increased turnover of 5-HT was observed following soman intoxication in accordance with prior published study (McDonough and Shih, 1997). Atropine sulfate pretreatment did not modulate soman-induced increase in either 5-HT or 5-HIAA efflux. On the other hand, pretreatment with *O*-benzyl derivative of pralidoxime, in the absence and presence of atropine co-administration, was able to prevent changes in 5-HT and 5-HIAA in soman-poisoned rats.

4. Discussion

While prophylactic administration of *O*-substituted pralidoxime derivatives failed to enhance the survival rates

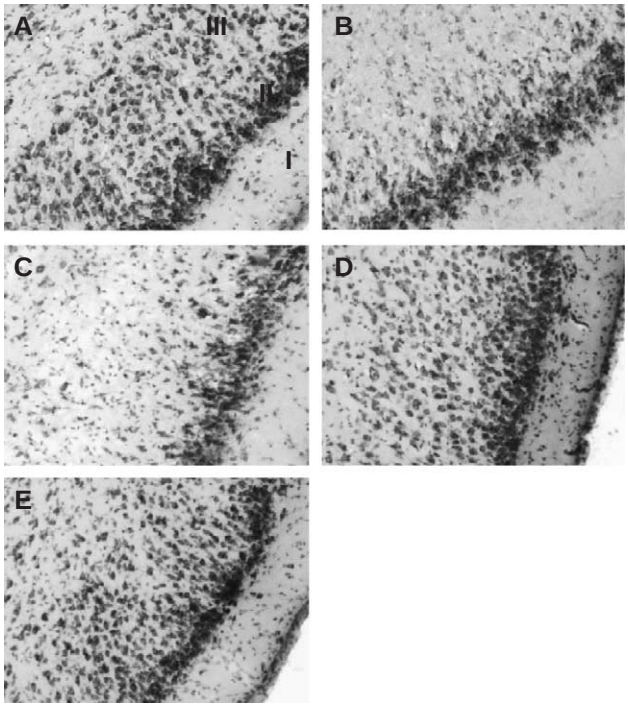


Fig. 2. Effects of various pretreatment solutions in mitigating cellular pathology in rat piriform cortex (bregma −2.8 mm A–P Level) following soman-induced status epilepticus ($1.6\times LD_{50}$; 176 μg/kg, subcutaneous). Images (40 μm thickness), captured with objective lens ×10, refer to brain slices of a (A) control, non-intoxicated rat; soman-poisoned rat pretreated with (B) saline vehicle; (C) atropine sulfate; (D) *O*-benzyl derivative of pralidoxime and (E) a combination of *O*-benzyl derivative of pralidoxime with atropine sulfate.

amongst mice intoxicated by a lethal dose of soman, the survival duration of mice pretreated with *O*-benzyl derivative of pralidoxime was significantly extended beyond that of atropine-treated mice. In rats, however, *O*-benzyl derivative of pralidoxime did not enhance the survival rates of atropine-pretreated rats during lethal soman poisoning ($1.6\times LD_{50}$).

The *O*-benzyl derivative of pralidoxime was however observed to provide unexpected anticonvulsive effects against soman poisoning in both rodent species pretreated with this compound. This novel anticonvulsant effect was potentiated by the presence of a non-anticonvulsant dose of atropine. The rapid appearance (<15 min) of strong sedation effects amongst animals receiving an intraperitoneal administration of *O*-benzyl derivative of pralidoxime suggests that penetration into the central nervous system must have occurred. This suggests that the increased lipophilicity with the inclusion of a side chain bearing an *O*-benzyl group could have overcome expected difficulties in crossing the blood–brain barrier by a positively charged compound. Interestingly, while the animals appeared sedated by this compound, their EEG profile prior to soman administration did not correspond to those of sleeping animals, which typically displayed wavy, undulated EEG baselines. This suggests that full sedation, as achieved by GABAergic drugs like benzodiazepines and barbiturates, was not attained with this compound.

Electroencephalographic (EEG) studies with the rat model supported the presence of anti-seizure capabilities in this novel compound when it is used as a pretreatment drug. While with *O*-benzyl derivative of pralidoxime pretreated animals displayed the usual sequence of aimless chewing, head nodding, and tremor leading to tonic-clonic convulsions after soman injection, the effects were much milder in comparison with these sedated animals. EEG seizures in these pretreated animals were also much subdued in its intensity compared to saline (Fig. 1A) or atropine pretreated rats. The presence of periodic short duration of low-amplitude ictal activities (<20 s) instead of continuous

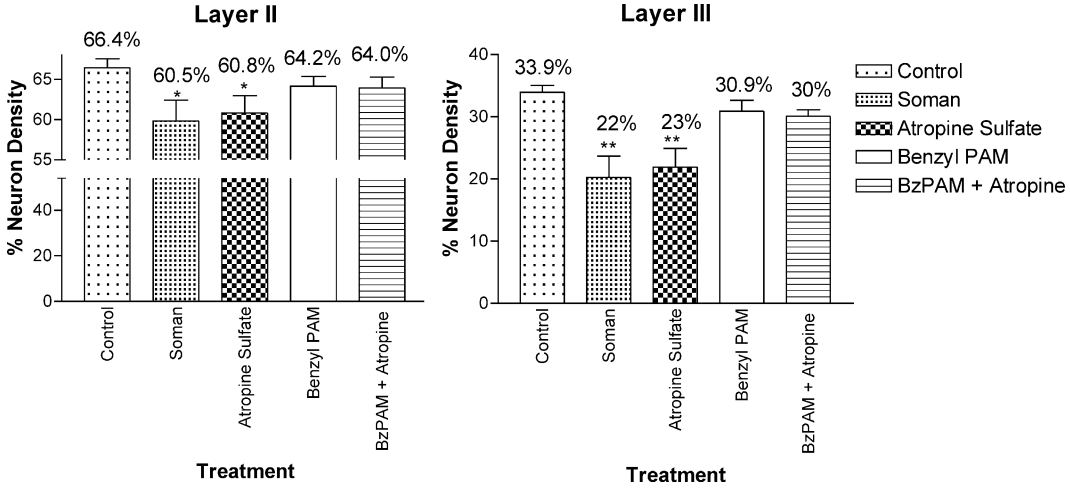


Fig. 3. Effects of various pretreatment options (saline vehicle (soman) ▥; atropine sulfate ▩; *O*-benzyl derivative of pralidoxime (benzyl PAM) □; and combination of *O*-benzyl derivative of pralidoxime (BzPAM) with atropine sulfate ▤) on percentage of neuronal density (±S.E.M.; percentage of neuronal density defined as the total neuron areas as a percentage of the section area counted) in Layers II and III of the rat's piriform cortex following 4 h of soman-induced status epilepticus ($1.6\times LD_{50}$; 176 μg/kg, subcutaneous). Control (non-intoxicated animal) percentage of neuronal density is indicated by ⬚. Pretreatment options were administered intramuscularly 30 min prior to soman. Sample size, $n=6$ per treatment group. Significant differences (one-way ANOVA with post-test by Dunnett's *t*-test) from control are indicated by single ($P<0.05$) and double asterisks ($P<0.01$).

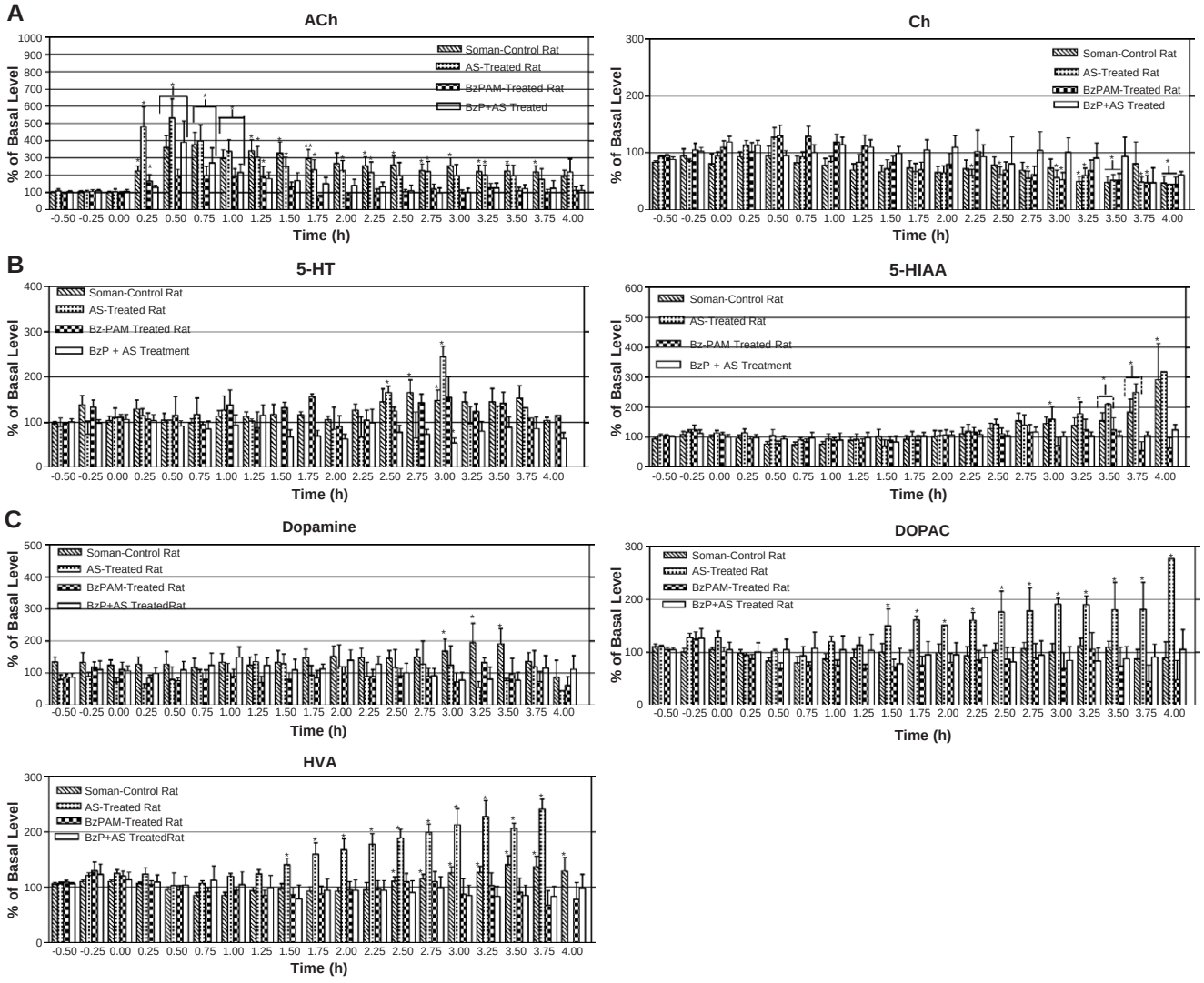


Fig. 4. Effects of various pretreatment options in mitigating status epilepticus-related changes in neurochemistry of soman-intoxicated rat ($1.6 \times LD_{50}$; 176 μg/kg, subcutaneous). Graphs refer to (A) extracellular concentration of acetylcholine and choline in basal ganglia; (B) extracellular concentration of 5-HT and 5-HIAA in basal ganglia; and (C) extracellular concentration of dopamine, DOPAC and HVA in basal ganglia. Pretreatment regimen is indicated in the attached legend where AS refers to atropine sulfate, BzPAM refers to *O*-benzyl derivative of pralidoxime and BzP+AS refers to combination of *O*-benzyl derivative of pralidoxime (BzPAM) with atropine sulfate. Sample size, $n=6$ per treatment group. Significant differences (repeated measures ANOVA with post-test by Dunnett's *t*-test) from basal levels are indicated by a single asterisk ($P<0.05$).

rhythmic ictal spikes complicated our efforts to define seizure onset time points. Consequently, the authors defined seizure onset for animals pretreated with *O*-benzyl derivative of pralidoxime as the appearance of periodic appearance (intervals of 3–5 min) of trains of sharp wave activity in the EEG (Fig. 1B). In the presence of co-administered atropine in the pretreatment regimen, these periodic sharp wave activities decreased in amplitude with time and revert to baseline EEG profile within 2 h.

While EEG data could not provide unequivocal demonstration of neuroprotective effects, neuropathological analysis of the piriform cortex at bregma −2.8 mm (A–P Level), taken at 24 h post-soman exposure, confirmed the neuroprotection achieved by pretreatment with this novel compound. This brain region and timing for assessment of brain tissue was based on the findings of a previous detailed study indicating neural areas between A–P −0.8 to −4.8 mm contain neural structures that have the highest likelihood of being damaged by soman exposure (McDonough et al., 1998). Selection of piriform cortex as a benchmark for assessing neuroprotection is also supported by other research studies suggesting piriform cortex to be the trigger site for initiation and spread of soman-induced seizures in the brain (Zimmer et al., 1997, 1998). Concomitantly, these studies also supported the appearance of profound neuropathology in the piriform cortex by 24 h post-soman intoxication. Our histopathological results confirmed neuroprotective effects of *O*-benzyl derivative of pralidoxime, with or without co-administration of atropine, in both Layers II and III of the piriform cortex of the rat brain.

Excessive release of acetylcholine in the initial phase of soman poisoning, revealed from microdialysis studies, is in line with previous reports (Shih and McDonough, 1997; Prioux-Guyonneau et al., 1982). Initial cholinergic hyper-excitation has been cited in these reports as the factor for downstream activation of additional neurotransmitter systems, resulting in delayed release of excitatory amino acids and catecholamines (McDonough and Shih, 1997). The immediate increase in striatum acetylcholine efflux from cholinergic inter-neurons may not lead to an immediate enhancement in dopamine efflux as acetylcholine has been reported to induce opposing effects on striatum dopamine release through M_4, M_5 and M_3 muscarinic receptor subtypes (Zhang et al., 2002). The observed delayed increase in striatum dopamine level is likely to be stimulated by glutamate, arising from excitatory corticostriatal projections terminating in the corpus striatum (Bronstein and Cummings, 2001; Borland and Michael, 2004). Since the glutamate-mediated enhanced release of dopamine in the striatum follows from the activation of the cortical glutamate system, which in turn is the follow-on cascade action from the initial cholinergic hyper-drive during nerve agent poisoning, it is plausible to observe the glutamate-mediated increase in striatum dopamine level occurring at a much later time point than the initial acetylcholine efflux within the striatum.

Microdialysis data also indicated a rapid reversion of acetylcholine release to basal levels within 1.5 h of soman poisoning in *O*-benzyl derivative of pralidoxime pretreated animals. This correlated with EEG evidence of seizure termination within 2 h of onset. The magnitude of acetylcholine increase was also significantly attenuated as compared to soman-poisoned rodents. This would account for the observed EEG spikes with subdued amplitude as well as for the absence of neuropathology at 24 h post-soman poisoning. On the other hand, with atropine sulfate pretreated animals, the initial acetylcholine efflux could not be prevented, which accounts for the subsequent status epilepticus and neuropathology in these animals despite atropine pretreatment. The inability to prevent soman-induced cholinergic hyperactivity would also account for the continued enhanced efflux of catecholamine neurotransmitters despite pretreatment with a non-anticonvulsant dose of atropine sulfate. The observed enhanced release of dopamine metabolites without a corresponding enhancement in dopamine efflux within the striatum in these animals may be attributed to rapid conversion of dopamine in microdialysates into dopamine metabolites by the presence of a soluble MAO in the extracellular space (Sim and Lim, 1992). With effective arrest of the initial cholinergic hyperactivity, downstream activation of catecholamine neurotransmitters was hence not observed in soman-poisoned animals pretreated with *O*-benzyl derivative of pralidoxime. These microdialysis data thus suggest that *O*-benzyl derivative of pralidoxime's ability to attenuate excessive acetylcholine release, following a lethal challenge with soman, is related to its ability to prevent neuropathology.

In the previous reported study on these derivatives of pralidoxime (Loke et al., 2002), it was reported that replacement of the hydrogen atom of the oxime function group with alkyl substituents enhanced the anti-muscarinic activity (100–1000×) of these derivatives as compared to the parent pralidoxime compound. However, the final muscarinic receptor affinities remained much lower than that of atropine (600×) and did not display significant selectivity for muscarinic receptor subtypes M_1–M_3. At the current applied pretreatment atropine dose of 2 mg/kg (3 μmol/kg), which was unable to attenuate soman-induced status epilepticus, the equivalent dose of *O*-benzyl derivative of pralidoxime for similar muscarinic antagonistic effects would be 1800 μmol/kg. However, the observed seizure attenuation capabilities of *O*-benzyl derivative of pralidoxime was achieved at only 50× higher pretreatment dose (at 145 μmol/kg) than atropine sulfate, hence its ability in abating soman-induced excessive release of acetylcholine is not likely to be related to its muscarinic receptor antagonist properties. In the same report (Loke et al., 2002), these pralidoxime derivatives were also determined to possess reversible acetylcholinesterase inhibitory properties at micro-molar concentrations. However, such properties would not be of critical consequences in these animal models as complete reversal of enzyme inhibition occurs within 12 min of initial inhibition, which would not have protected synapse acetylcholinesterase from soman injection, which was administered 30 min post-application of *O*-benzyl derivative of pralidoxime.

A possible explanation for the novel ability of this compound in preventing soman-induced excessive release of acetylcholine and its cascade of neurotoxic action may lie instead with its unusual non-competitive muscarinic receptor antagonist properties (Loke et al., 2002). When pre-administered prior to soman, this *O*-benzyl oxime derivative of pralidoxime could have decreased the affinity of muscarinic receptors for acetylcholine in a manner similar to that reported with bisquaternary pyridinium oximes (Kloog et al., 1985). In that study, it was determined that pyridinium compounds with the oxime moiety at position 2 of the ring have the ability to induce selective loss of brain stem muscarinic receptors. The putative mechanism suggested was that oximes could interact with ester bonds and hence the partial inactivation of muscarinic receptors might be attributed to nucleophilic attack on ester bonds present in membranes of neural synapses. Reducing the brain sensitivity to acetylcholine would help modulate the initial central excitement to prevent seizure initiation. This mechanism would require further validation studies.

An alternate mechanism to account for the observed synergistic therapeutic actions between atropine and *O*-benzyl oxime derivative of pralidoxime could arise through the latter mediated allosteric stabilization of atropine binding to muscarinic receptors as reported with the bispyridinium oxime W84 (Jepsen et al., 1988). By stabilizing the anticholinergic–muscarinic complex, a non-anticonvulsive dose of atropine could achieve competitive antagonistic

effects equivalent to a higher anticonvulsive dose of atropine, hence preventing over-excitation of the neural synapses. By minimizing the initial hypercholinergic over-excitation and consequent recruitment of additional neurotransmitter systems, the spread of seizure events would be contained and normal homeostatic mechanisms in the brain would revert the brain neurochemistry back to normalcy and hence prevent the development of status epilepticus. This could hence account for the absence of neuropathology in the treated animals. The allosteric stabilization effect on anticholinergic drug–muscarinic receptor complex has recently been observed in the authors' laboratory (unpublished data).

Hypoactivity-inducing effects have been attributed to the cholinergic system through inhibition of striatal dopamine release by the presence of excessive acetylcholine (Silvestre et al., 1999). This, however, is not likely to be the mechanism for the observed sedative effects of *O*-benzyl derivative of pralidoxime as it neither increased acetylcholine efflux nor was its sedative effects annulled by the presence of an anticholinergic compound, atropine (Table 2). Involvement of the dopaminergic and serotonergic neurotransmitter systems in the observed sedative effects could not be inferred either, as *O*-benzyl derivative of pralidoxime did not induce statistically significant changes in both neurotransmitter systems. Further studies into the changes in neurotransmitters receptor density and distribution in the central nervous system following *O*-benzyl derivative of pralidoxime administration would be required to provide a better insight into its unusual sedative effects.

In this study, a novel *O*-substitute derivative of pralidoxime was synthesised and validated to possess novel sedative and neuroprotective capabilities against a lethal dose of soman when administered as a pretreatment antidote. Therapeutic effects of novel compound appeared to be derived from its ability to attenuate soman-induced increase in acetylcholine in the brain, which is unlikely to be related to its weak direct muscarinic antagonism property. Possible involvement of non-competitive muscarinic antagonistic properties of this compound in relation to its ability to attenuate soman-induced acetylcholine efflux would be evaluated in further studies.

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