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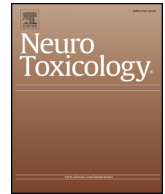
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## Full Length Article

# Potential of antiseizure and neuroprotective efficacy of standard nerve agent treatment by addition of tariquidar



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## ABSTRACT

Organophosphate (OP) induced seizures are commonly treated with anticholinergics, oximes and anticonvulsants. Inhibition of P-glycoprotein (PgP) has been shown to enhance the efficacy of nerve agent treatment in soman exposed rats. In the present study, the promising effects of the PgP inhibitor tariquidar were investigated in more detail in rats s.c. exposed to 150 µg/kg soman. Treatment with HI-6 and atropine sulfate (125 and 3 mg/kg i.m. respectively) was administered 1 min after exposure. Diazepam (0.5 mg/kg i.m.) and/or tariquidar (7.5 mg/kg i.v.) were included either at 1 min or 40 min following onset of seizures.

Animals that received tariquidar, in addition to HI-6 and atropine, at 1 min, displayed a rapid normalization of EEG activity and cessation of seizure-associated behaviour. This improvement by addition of tariquidar was even more substantial in animals that also received diazepam, either immediately or delayed. Animals exhibiting lower intensity seizures displayed less severe neuropathology (neuronal loss, microglia activation and astrogliosis), primarily in the piriform cortex, and to a lesser extent amygdala and entorhinal cortex.

The present findings suggest that the interaction of tariquidar with atropine may be the decisive factor for enhanced treatment efficacy, given that atropine was previously found to be a PgP substrate. A more thorough understanding of the interactions of nerve agent antidotes, in particular the actions of central anticholinergics with benzodiazepines, could contribute to a future optimization of treatment combinations, particularly those aimed at later stage medical interventions.

## 1. Introduction

Organophosphorus nerve agents are substances that irreversibly inactivate acetylcholinesterase (AChE), leading to accumulation of acetylcholine in the synaptic cleft resulting in epileptic seizures and often death (Shih and McDonough, 1997). Pharmacotherapy of nerve agent poisoning is based on anticholinergic drugs, AChE reactivators and anticonvulsants to prevent lethality. Several agents, in particular soman, result in rapid ageing of AChE, rendering the enzyme resistant to reactivation. Consequently, *de novo* enzyme synthesis in that case is the only mechanism for AChE regeneration. As anticholinergic blockade with atropine is inadequate by itself, oximes are generally employed to aid in AChE reactivation before ageing of the enzyme-nerve agent complex occurs. Due to the low brain penetration of oximes, high doses are currently needed to achieve sufficient AChE reactivation in the brain (Cassel et al., 1997). Minor reactivation is presumably sufficient to be lifesaving, but a higher level of reactivation is assumed to be

beneficial to mitigate of initiation and/or reduce or inhibit seizure propagation (Shih et al., 2011; Bueters et al., 2003).

Whereas passive transport through the blood-brain-barrier is largely determined by drug properties like lipophilicity, active drug transport is regulated by specific blood-brain-barrier (BBB) transporters such as the ATP binding cassette (ABC) transporters P-glycoprotein (PgP), and breast cancer resistance protein (BCRP). Both transporters actively lower bioavailability of PgP substrates in the brain. Many drugs, including cytotoxic drugs for cancer treatment, are PgP substrates and may therefore reach an insufficient availability at their site of action inside the brain. It has previously been proposed that a similar active brain efflux of nerve agent antidotes, possibly involving PgP action, also leads to a reduction of their therapeutic potential. Indeed, in a soman exposure model, pretreatment with tariquidar or coadministration of tariquidar, a potent, non-competitive inhibitor of PgP, successfully increased therapeutic efficacy of HI-6 and atropine (Joosen et al., 2011, 2016). In the present study, we set out to investigate in more detail the

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**Table 1**  
Experimental groups. Soman was administered at t = 0.

Group	Soman (150 µg/kg) s.c.	Treatment at 1 min after exposure			Treatment at 40 min. after seizure onset		n per group	
		HI-6 (125 mg/kg) AS <sup>1</sup> (3 mg/kg) i.m.	Tariquidar (7.5 mg/kg) i.v.	Diazepam (0.5 mg/kg) i.m.	Tariquidar (7.5 mg/kg) i.v.	Diazepam (0.5 mg/kg) i.m.	ChE activity	immuno
1	•	•	–	–	–	–	6	12
2	•	•	•	–	–	–	6	12
3	•	•	–	•	–	–	–	6
4	•	•	•	•	–	–	–	6
5	•	•	–	–	•	–	–	8
6	•	•	–	–	–	•	–	8
7	•	•	–	–	•	•	–	8
8	–	–	–	–	–	–	–	6

<sup>1</sup> Atropine sulfate.

promising effects of tariquidar on enhancing nerve agent treatment efficacy over the first 24 h after soman exposure in rats. In contrast to earlier studies, the atropine dose was decreased, and the combined treatment of HI-6 and atropine was supplemented with diazepam. Additionally, we investigated whether a delayed treatment with tariquidar could interfere with ongoing seizure activity 40 min after seizure onset, or whether it would augment effects of diazepam administered at the same time. Continuous EEG recordings served as a direct measure of seizure related brain activity and behavioral expressions of intoxication were assessed using a modified Racine scale. The extent of neuropathology, neuronal loss, microglia activation and astrogliosis were studied histologically 24 h after soman exposure in the piriform cortex, entorhinal cortex, amygdala, hilus and presubiculum, the main regions of the brain involved in seizure initiation and propagation.

## 2. Materials & methods

### 2.1. Animals

Male Wistar WU rats (240–260 g at arrival, n = 60) were obtained from Charles River Laboratories (Sulzfeld, Germany). The animals were housed 2–3 animals per cage in Macrolon Type 4 cages for at least one week to acclimate to the conditions in the facility. A 12 h light cycle (7 AM–7 PM) was employed and temperature was kept between 19–22 °C with a relative humidity of 55–65%. Standard rodent chow (Teklad Global Diet, Harlan, Horst, The Netherlands) and acidified water were available *ad libitum*. All experiments were in agreement with EU Directive 2010/63/EU for animal experiments and approved by the Ethical Committee on Animal Experimentation of TNO and USAMRMC Animal Care and Use Review Office (ACURO).

### 2.2. Chemicals

Soman (1,2,2-Trimethylpropyl methylphosphonofluoridate) and HI-6 (1-2-hydroxyiminomethyl-1-pyridino-3-(4-carbamoyl-1-pyridino -2-oxapropane dichloride) were obtained from the stocks of TNO Rijswijk, The Netherlands and were of > 98% purity. Diazepam solution (5 mg/ml) was obtained from AUV Veterinary Service (Cuijck, The Netherlands). Tariquidar (98%) was obtained from MedKoo Biosciences (Chapel Hill, NC, USA), and solubilized by adding 2.2 M equivalents of methanesulphonic acid in milli-Q water/acetonitrile (50/50). The solution was lyophilized overnight, washed with diethyl ether (3 × 2 ml) and lyophilized overnight to produce a final dimethylsulphonate salt that was quantified using NMR and LC/MS. All other chemicals (atropine sulfate salt monohydrate, 97%) and standard chemicals were obtained from Sigma Aldrich and of standard purity.

### 2.3. Surgery

During cortical EEG probe implantation, rats were anesthetized with 2–3% isoflurane. The EEG electrodes (two stainless steel screws) were placed on the dura mater at A1.0 and P6.0 mm relative to Bregma and at 1 mm from the sagittal suture, and fixed in place with dental cement and connected to an adaptor. In the same surgical procedure, an indwelling jugular vein catheter was routed to the opening for the head stage. The cannula was filled with heparinized glycerol (500 IU/ml) and capped. Subcutaneous analgesia (carprofen, 5 mg/kg) and antibiotics (Borgal® -Trimethoprim 4 mg/kg and Sulfadoxine 20 mg/kg) were given prior to surgery and 24 h post operatively (1 ml/kg). Following surgery, the animals were individually housed and allowed to recover for one week prior to soman exposure.

### 2.4. Treatments

One week after surgery, baseline EEG values were recorded for at least 30 min before rats were administered soman at a dose of 150 µg/kg (2LD50 s.c. according to Marrs et al., 2007). An overview of the experimental groups is presented in Table 1. One minute after soman exposure, atropine sulfate and HI-6 were given (3 and 125 mg/kg, i.m., respectively 0.5 ml/kg, groups 1–7). In predetermined groups, animals were co-administered diazepam (0.5 mg/kg i.m.) with atropine/ HI-6 in the same injection (groups 3 and 4). For the delayed treatment groups, animals received diazepam 40 min after seizure onset (groups 6 and 7). Tariquidar (7.5 mg/kg i.v.) or its vehicle (propylene glycol/5% sucrose/ethanol 4:5:1) were also administered at 1 ml/kg 1 min after soman (groups 1–4) or 40 min after seizure onset (groups 5–7). Half of the animals were subjected to transcardial perfusion with formaldehyde fixative to allow for brain immunohistochemistry, while cholinesterase activity was assessed in the brains of the remainder of each group. In the groups where tariquidar and diazepam were co-administered 40 min after seizure onset (5–7), only immunohistochemistry was conducted.

### 2.5. EEG signal acquisition and analysis

Cortical EEG data were acquired using Ponemah software (Data Science Inc, DSI) using the TL11M2-F40-EET transmitter, which was attached to the adapter fixed on the skull. The transmitter data were sampled at 250 Hz. Simultaneously, video capture of the animal's behavior was stored in a synchronized manner with the EEG data. The behavior and EEG data were analyzed offline using Neuroscore software. The onset of seizures was scored by visual inspection of the online signal and was defined as high frequency spiking of at least 3 times elevation from baseline over a period of 20 s. Total EEG power (expressed as EEG-AUC) was defined as the summed power in different frequencies obtained via FFT spectral analysis, which also served as a

semi-automated analysis of seizure presence. In order to test for differences in seizure activity during the subacute/subchronic time window (8 h post-exposure), area-under-the-curve (total EEG power\*min) was calculated.

## 2.6. EEG readouts and seizure rating

Following soman exposure, signs of cholinergic toxicity (chewing, shivering, salivation, tremor, convulsions and respiratory distress) were visually scored according to procedures described previously (Joosen et al., 2016, 2011). Convulsions were defined as involuntary movements of the entire body while the animal is seemingly dissociated from the environment. Each sign of toxicity was scored as present or not in the animal, irrespective of its duration. Furthermore, seizure related behaviors were visually rated using a modified Racine scale (Racine, 1972). Briefly, 0 – No behavioral seizures; 1 – Masticatory movements, chewing, shivering; 2 – Head myoclonus; 3 – Forelimb myoclonus; 4 – Forelimb myoclonus; followed by rearing or hind limbs involved; 5 – Falling or generalized tonic-clonic convulsions; 6 – Apnea, coma.

## 2.7. Cholinesterase activity determination in blood and brain

Blood samples were drawn from the cannula prior to and at 30 min post soman exposure, or via intracardial puncture prior to perfusion. Animals pre-designated for determination of brain ChE levels were decapitated 24 h after soman exposure; trunk blood and brains were collected to determine cholinesterase activity. Blood samples were diluted 10 times in 1% saponin and snap frozen on dry ice. Fresh brains, stripped of the cerebellum and medulla oblongata, were homogenized (900 rpm, 10% w/v homogenate) in ice-cold TENT buffer (50 mM Tris, 5 mM EDTA, 1 M NaCl and 1% v/v Triton X-100, pH 7.4). Brain tissue homogenates were centrifuged at 12,000g at 4 °C for 10 min and supernatants were immediately frozen on dry ice. All samples were stored at –80 °C until analysis.

Samples were analyzed for ChE activity using a modified version of the method described previously (Ellman et al., 1961). Briefly, 10 µl of diluted blood or homogenized samples were incubated with 0.8 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (Sigma Aldrich B.V.) and 0.8 mM β-methyl acetylthiocholine iodide in 50 mM Phosphate Buffer (pH 8.0). Absorbance at 415 nm was measured using a photospectrometer directly after the addition of the substrate, and again 20–40 min later. The increase in absorbance per minute at ambient temperature served as measurement for ChE activity. Values are represented as percent of baseline value (blood) or as percent of naïve controls.

## 2.8. Immunohistochemistry for NeuN, GFAP and OX42

### 2.8.1. Tissue preparation

Animals designated for immunohistochemistry were deeply anesthetized with intraperitoneal sodium pentobarbital (50–100 mg) 24 h post soman exposure and subsequently transcardially perfused with PBS (Phosphate Buffered Saline, 0.1 M, pH 7.4), followed by 4% paraformaldehyde in PBS. The perfused animals were kept at 4 °C overnight, after which time the brain was removed from the skull and stored in PBS-sodium azide 0.01% at 4 °C. Prior to sectioning, brains were cryoprotected by saturation in 15% and 30% sucrose in PB (Phosphate Buffer). A sliding microtome (R. Jung AG, Heidelberg, Germany) was used to obtain 40 µm thick coronal brain sections in a one-in-eight or one-in-ten series in antifreeze solution (20% glycerol, 30% ethylene-glycol, 50% 0.05 M PBS), slices were stored at –20 °C.

Slices containing the amygdala, hilus, entorhinal cortex, piriform cortex and presubiculum were selected according to their positions relative to Bregma. To assess the extent of tissue damage caused by soman exposure, as well as possible treatment effects of HI-6/ atropine sulfate, tariquidar and/or diazepam, several cell type specific markers were selected. As neuronal loss is frequently observed after seizures, we

performed NeuN immunohistochemistry to stain for mature neurons. A lower NeuN immunoreactivity was considered to represent neuronal loss. The occurrence of astrocytic gliosis was assessed using immunohistochemistry for Glial Fibrillary Acidic Protein (GFAP), a commonly used marker for astrocytes. To detect possible microglial activation in response to damage or loss, we stained for OX42/CD11a/b, a member of the integrin family that is upregulated in activated microglia. In all cases, the observer was unaware of the experimental group studied.

### 2.8.2. NeuN immunohistochemistry and analysis

Sections were washed with PBS to remove antifreeze solution. Residual endogenous peroxidase activity was blocked for 15 min with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS. Sections were then incubated at room temperature (RT) for 1 h with monoclonal mouse anti-NeuN (EMD Millipore, MAB377) diluted 1:1000 in 0.4% Bovine Serum Albumin (BSA), 0.1% Triton X-100 in PBS and stored overnight at 4 °C. The next day, sections were allowed to adjust to RT, and following washing with PBS the sections were incubated for 2 h with biotinylated sheep anti-mouse (1:200, RPN1001V, GE Healthcare) in 0.4% BSA and 0.1% Triton X-100 in PBS. The signal was then amplified by incubating for 2 h in Avidin Biotin Complex (1:800, Vectastain Elite, Vector Labs) in PBS following washing with PBS. Finally, sections were subsequently washed with Tris-buffered Saline (TBS, 0.05 M pH7.6) and 3x TB. The staining was developed with 0.05% 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma, no D-5637) in 0.01% H<sub>2</sub>O<sub>2</sub> in TB. Sections were mounted with 0.2% gelatin in TB and air dried overnight. Following dehydration in a graded alcohol-series and clearing in xylene, the sections were coverslipped using Entellan.

NeuN immunostained sections were bilaterally imaged per brain region at 5x magnification using a Zeiss Axiophot brightfield microscope. For image acquisition, the Optronics Microfire camera was controlled through Stereoinvestigator software (MicroBrightfield, Magdeburg, Germany). ImageJ (FiJI) software was used for analysis of immunoreactivity (NeuN coverage). The acquired images were made binary and watershed and a selection of the respective brain region of interest was made from which NeuN coverage was determined expressed as percentage of the total surface area. Furthermore, the piriform and entorhinal cortices were divided in layer I-III and an inner- and outer layer, respectively, to allow identification of subregion-specific effects in NeuN immunoreactivity. For instance, in the piriform cortex, the most outward layer (I) in control animals has NeuN<sup>+</sup> coverage of about two percent, whereas layers II and III are more densely immunostained with average coverages of 55 and 25 percent, respectively.

### 2.8.3. OX42 immunohistochemistry and analysis

Sections were washed initially with PBS to remove antifreeze solution as well as between incubation steps, however PBS wash was not used between protein blocking and primary antibody incubation. Endogenous peroxidase activity was blocked for 15 min with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS. Following that, a-specific binding was blocked by incubating for 30 min in 5% Normal Goat Serum (NGS) and 0.3% Triton X-100 in PBS. After three PBS wash cycles, the sections were incubated at RT for 1 h and O/N in 4 °C with monoclonal mouse anti-OX-42 (Abcam, AB1211) diluted 1:400 in 1% NGS, 0.3% Triton X-100 in PBS. The next day, sections were first acclimatized to RT before washing with PBS. The secondary antibody incubations until mounting were performed as described above. After haematoxylin counterstaining according to Ehrlich, the sections were dehydrated with alcohol-xylene and covered with Entellan.

Microglia immuno-positive for OX42 were bilaterally counted and classified according to different subtypes in all investigated brain regions. Entorhinal and piriform cortices were treated as a whole, as no layer-specific OX42 activation was observed. Microglia were counted at 5x magnification using a Zeiss Axiophot microscope equipped with a

LEP MAC5000 motorized stage (Ludl Electronic Products, Hawthorne, NY) controlled through StereoInvestigator software. Regions of interest were outlined and OX42<sup>+</sup> cells were counted and then classified based on their morphological characteristics and staining intensity. Type 0 (inactive/resting) microglia were weakly stained with advanced and nondistinct branching, but no apparent soma and were therefore not counted. Type 1 microglia (in between resting and intermediate activation) displayed higher OX42 immunoreactivity compared to resting microglia and had a more distinct appearance and more retracted, already slightly condensed extensions. Type 2 microglia (intermediately active microglia) had a much more dense and bulkier appearance, but still possess (few) extensions, mostly unbranched. Fully activated, type 3 microglia are characterized by a completely round and dense cellular profile appearance. In all regions, microglia cell subtype counts were expressed separately as density per mm<sup>2</sup>.

#### 2.8.4. GFAP immunohistochemistry and analysis

Sections were washed with TBS to get rid of antifreeze solution initially as well as in between follow on incubation steps. Endogenous peroxidase activity was blocked by a 20 min incubation in 0.5% H<sub>2</sub>O<sub>2</sub> in TBS. Rabbit anti-GFAP antibody (DAKO, Z0334) was diluted 1:2000 in 1% milk powder (Elk, Campina foods, the Netherlands) and 1% Triton X-100 in TBS and the sections were incubated at RT for 1 h and stored O/N at 4 °C. The next day, sections were allowed to acclimatize to RT before washing and were subsequently incubated for 2 h with biotinylated goat anti-rabbit antibody (1:200, 6-BA-1000, Vector) in 1% BSA and 1% Triton X-100 in TBS. Next, the sections were incubated for 2 h at RT with avidin biotin complex (ABC, Vectastain Elite, Vector Labs) at 1:800 in 1% BSA in TBS solution. The rest of the staining and coverslip procedure was similar to the OX42 protocol as described earlier.

For each brain region studied, the extent of astrogliosis was evaluated subjectively using a light microscope according to the criteria in Table 2.

#### 2.9. Data analysis

Results from a parametric data analysis are based on average numbers ± SEM. Effects were analyzed using one- or two way ANOVA. This test was followed by an appropriate post-hoc test to make pairwise comparisons between groups treated with or without tariquidar (Sidak's method for one-way ANOVA and Tukey's Honest Significant Difference (HSD) test for two way ANOVA). As astrogliosis classification concerned ordinal data, a Kruskal-Wallis test was used for analysis. Results were considered statistically significant when  $p < 0.05$ . Statistics was performed using GraphPad Prism 6.0 for Windows.

### 3. Results

#### 3.1. Cholinesterase activity

The extent of soman exposure was confirmed by measurement of

**Table 2**  
Astrogliosis classification.

Class	Description
0	Low GFAP expression and part of the cell surface does not express GFAP
1	Low GFAP expressing astrocytes covering the surface but generally do not overlap each other
2	Strong GFAP expression per astrocyte, together, they cover the surface but do not overlap
3	Strong GFAP expression per astrocytes, many cover the surface and overlap each other
4	Very strong staining of many astrocytes including in protrusions from other cells giving rise to an overall abundance of immuno-reactive signal and fuzzy appearing astrocytes.

cholinesterase activity in blood and brain homogenates. All rats received HI-6 (125 mg/kg) and atropine sulfate (3 mg/kg) i.m. as a standard anticholinergic/oxime treatment) at 1 min post exposure. Select groups of rats received additional tariquidar and/or diazepam at 1 min after soman, or 40 min after seizure onset. Thirty minutes after soman exposure, blood cholinesterase activity was profoundly inhibited, with < 10% remaining (Fig. 1A). At this time point, animals treated with diazepam at 1 min tended to show higher, but not statistically significant, blood cholinesterase activity. Cholinesterase activity in blood was still inhibited > 70% up to 90% in all groups 24 h after exposure (Fig. 1B). At this time point, brain cholinesterase activity of animals exposed to soman was less than 25% of activity compared to unexposed animals, in both tariquidar and vehicle treated animals (Fig. 1C).

#### 3.2. Clinical signs

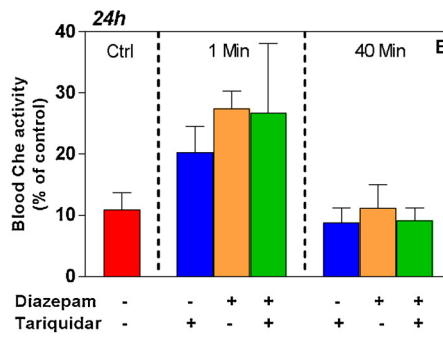
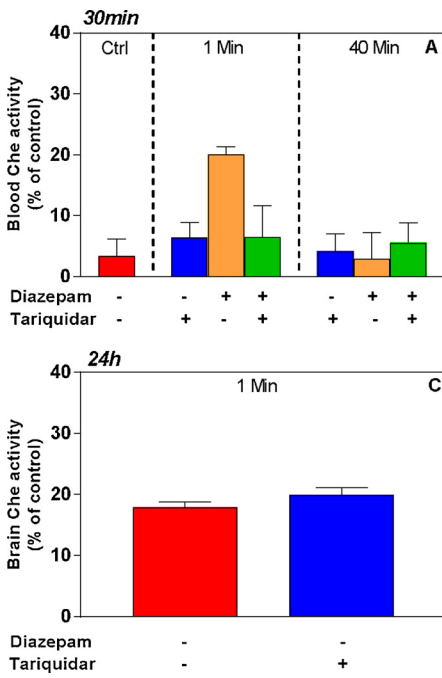
Twenty-four hours after soman exposure and treatment with HI-6 and atropine at 1 min, all animals had lost 5–15% of their body weight overnight. Diazepam alone did not affect weight loss, whether administered at 1 min after exposure or 40 min after the onset of seizures. In contrast, animals that received tariquidar at 1 min post-exposure lost significantly less body weight than animals receiving vehicle. Tariquidar alone, administered at 40 min after seizure onset, did not significantly affect weight loss, but when tariquidar was administered along with DZP at the 40 min time point, weight loss was significantly less (data not shown).

Behavior was recorded for 24 h after exposure and retrospectively scored using a modified Racine scale. All exposed animals quickly expressed behavioral seizure signs as shown by forelimb myoclonus with rearing or rear limb involvement. Animals that only received HI-6 and atropine showed a reduction in Racine scores and reached baseline within 24 h. The behavioral normalization was more gradual in animals that also received diazepam (Fig. 2A). The latter animals still displayed masticatory movements at the time of sacrifice. Animals that received tariquidar in addition, or tariquidar combined with diazepam at 1 min, showed a drop in Racine scores after 4 h, and behavioral signs of seizures had disappeared after 16 h. Delayed interventions (Fig. 2B) did not reduce seizure signs compared to the groups treated with HI-6 and atropine at 1 min. Over time, co-administration of tariquidar and diazepam at 1 min significantly reduced the AUC of Racine scores, whereas no other treatment affected behavioral seizure manifestations (Fig. 2C and D).

#### 3.3. Seizure activity

In accordance with the behavioral manifestations, a subcutaneous injection of soman, followed by treatment after 1 min with HI-6 and atropine sulfate, rapidly led to seizures, reflected by a substantial increase in total EEG power (Fig. 3A). The addition of diazepam at 1 min did not affect seizures. In rats that received also tariquidar, seizure activity gradually decreased and reached baseline levels after 10 h. When tariquidar and diazepam were given simultaneously at 1 min after soman, in addition to HI-6 and atropine, full-blown seizures did not even develop and total EEG power returned to baseline levels within one hour. In both groups, the additional tariquidar treatment led to a significant reduction in the AUC<sub>0-8h</sub> (Fig. 3C).

To address the effect of tariquidar and/or diazepam on ongoing seizures, diazepam or tariquidar were administered 40 min after seizure onset. Whereas neither diazepam nor tariquidar alone effectively mitigated seizure activity, the co-administration of tariquidar and diazepam at 40 min after seizure onset rapidly induced a substantial decline in EEG Total power, which returned to baseline levels within 3 h post treatment (Fig. 3B), leading to a significant reduction in the AUC<sub>0-8h</sub> (Fig. 3C).



**Fig. 1.** Cholinesterase activity in rats at 30 min and 24 h after Soman exposure (150 µg/kg s.c.). All groups were treated with HI-6 (125 mg/kg i.m.) and atropine sulfate (3 mg/kg) i.m. at 1 min after soman injection. Tariquidar (7.5 mg/kg i.v.) and/or Diazepam (0.5 mg/kg i.m.) were administered at either 1 min after exposure or at 40 min after seizure onset. Blood ChE activity was inhibited significantly at 30 min (A) or 24 h (B), irrespective of tariquidar iv treatment at 1 min after Soman exposure. C) Brain cholinesterase activity at 24 h after Soman exposure was inhibited to ~20% of that of naïve animals in both groups treated at 1 min; we did not measure brain ChE in animals treated at 40min. No significant differences were found between treatment groups (Two Way ANOVA with Tukey’s test).

3.4. Neuropathology

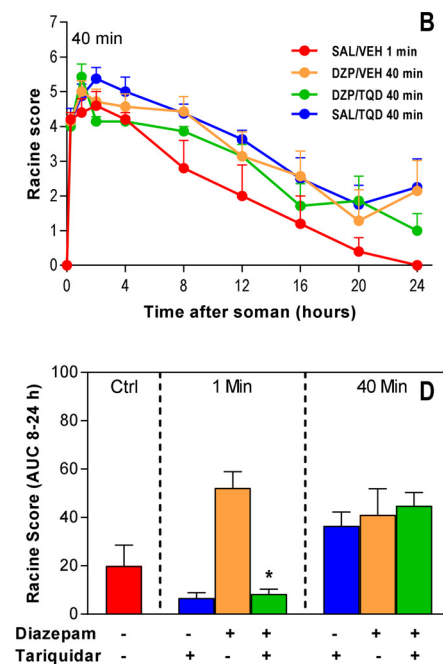
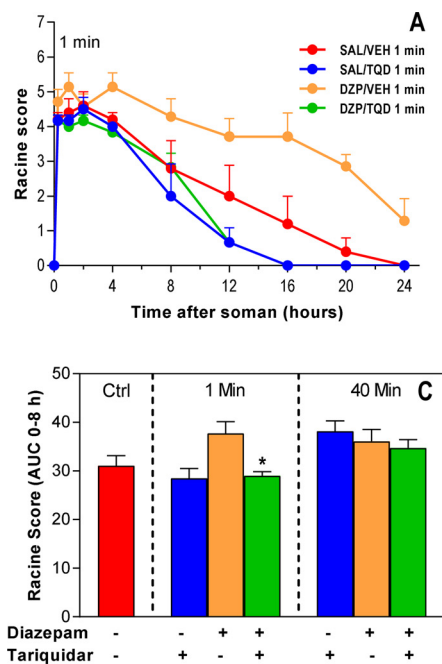
3.4.1. NeuN coverage

The percentage of surface area positive for NeuN immunoreactivity (NeuN coverage) was used as a measure to assess neuronal loss in brain regions known to play a role in seizure propagation. Of the brain regions investigated, the piriform cortex, particularly layer III, appeared to be most affected by soman compared to unexposed controls (Fig. 4A). This was prevented when tariquidar at 1 min, or diazepam combined with tariquidar at 1, or at 40 min into seizures was added to HI-6 and atropine treatment (Fig. 4A). In the amygdala, similar effects were observed although to a lesser extent, still statistically significant (Fig. 4C). In the entorhinal cortex, hilus and presubiculum, NeuN was not affected by either soman nor its treatments (data shown for entorhinal cortex only). Diazepam at 1 min without tariquidar, in addition

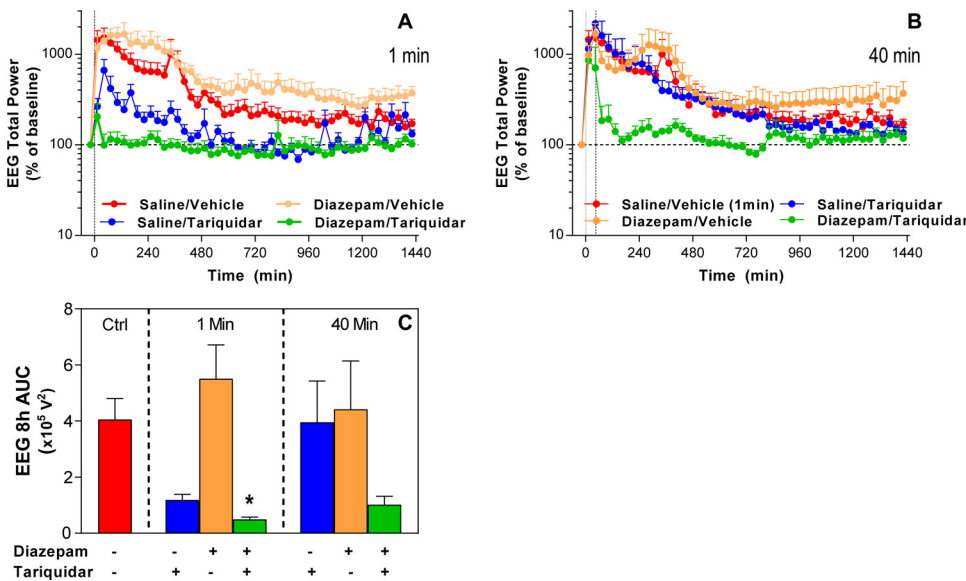
to HI-6 and atropine, failed to prevent neuronal damage and tended to aggravate loss in piriform cortex layer II and amygdala. Whereas co-administration of diazepam and tariquidar at 40 min into seizures prevented neuronal loss in piriform cortex layer III, injury in the amygdala was not prevented.

3.4.2. OX42+ cell counts

In healthy brains, microglia activation is only sparsely observed. In regions of interest, OX42+ stained cell bodies were counted as microglia, classified according to their morphology and their numbers were expressed as a density. In all areas under investigation, soman exposure resulted in microglia activation to some extent. The most profound change was seen in the number of intermediate and fully activated (type II and III) cells, as shown by round dense cells throughout the brain (Fig. 7B).



**Fig. 2.** Racine scores plotted over 24 h after rats received Soman (150 µg/kg s.c.), treated with HI-6 (125 mg/kg i.m.) and atropine sulfate (3 mg/kg) i.m. at 1 min. Tariquidar (7.5 mg/kg i.v.) and/or Diazepam (0.5 mg/kg i.m.) were administered at either 1 min after exposure (Panel A) or at 40 min after seizure onset (Panel B). The Area under curve (AUC) over the first 8 h and the last 16 h of the observation period are shown in panels C and D respectively. Only tariquidar in combination with Diazepam at 1 min significantly improved the AUC of the Racine scores (\*: p < 0.05, Two Way ANOVA followed by Tukey’s test versus vehicle treated animals).

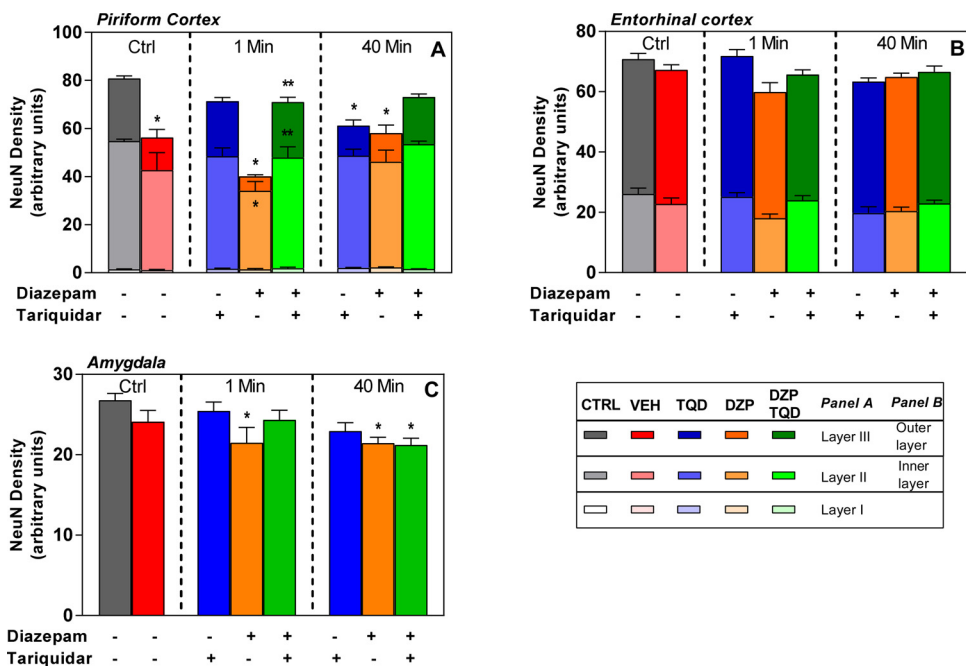


**Fig. 3.** Total EEG power of rats 24 h after Soman exposure (150 µg/kg s.c.), treated with HI-6 (125 mg/kg i.m.) and atropine sulfate (3 mg/kg) i.m. at 1 min. Tariquidar (7.5 mg/kg i.v.) and/or Diazepam (0.5 mg/kg i.m.) were administered at either 1 min after exposure (A), or at 40 min after seizure onset (B). The summed seizure intensity, expressed as AUC over the first 8 h, is represented in (C) Tariquidar administered at 1 min after Soman and diazepam combined with tariquidar both at 1 and 40 min, induced a significant reduction in AUC0-8 h. (\*:  $p < 0.05$ , Two Way ANOVA followed by Tukey test versus vehicle treatment).

Substantial microglia activation in both piriform and entorhinal cortices and in the amygdala was shown in soman exposed animals, treated with HI-6 / atropine. This was attenuated, although not significantly, when tariquidar was co-administered (Fig. 5). Diazepam in addition to HI6/atropine, tended to enhance type III microglia activation in all three areas shown. When diazepam and tariquidar were co-administered with HI-6 and atropine, type II and type III microglia activation was attenuated specifically in the piriform cortex, whereas a reduction in type III microglia was observed in entorhinal cortex and amygdala. At 40 min into seizures, neither diazepam nor tariquidar reduced microglia activation. However, when tariquidar and diazepam were combined, a statistically significant reduction in microglia type III cells in piriform and entorhinal cortices, but not amygdala, was observed.

3.4.3. Astrogliosis

GFAP-positive astrocytes in the brain regions under investigation were scored based on the rating scale described in Table 2. Of all

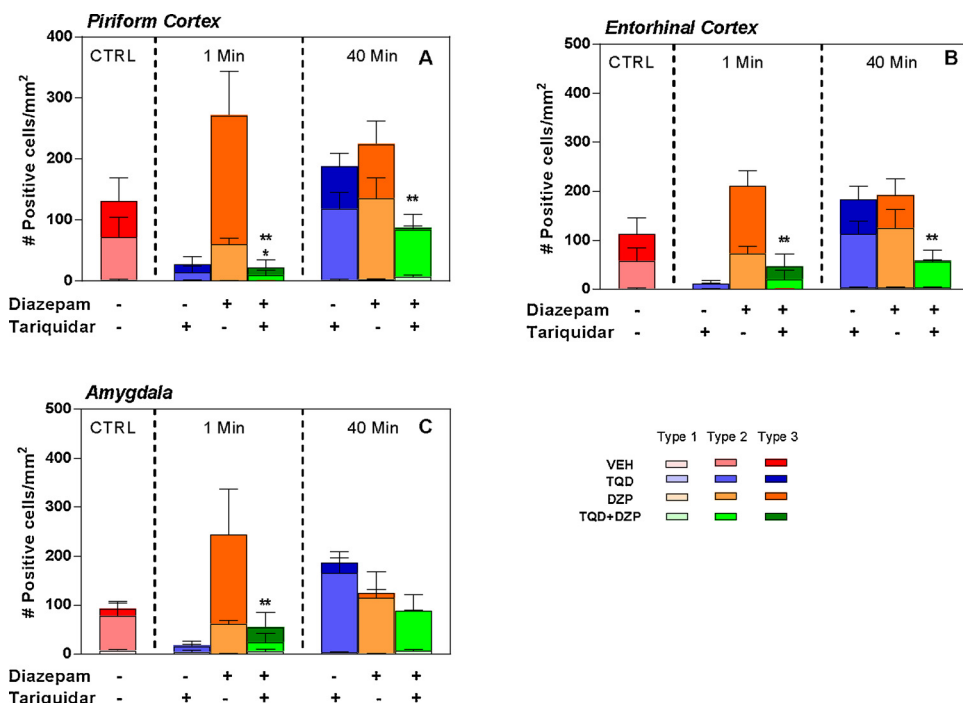


**Fig. 4.** NeuN density in brain areas of rats 24 h after exposure to Soman (150 µg/kg) treated at 1 min with HI-6 (125 mg/kg) and atropine sulfate (3 mg/kg) i.m. with i.v. vehicle or tariquidar (7.5 mg/kg) with or without co-administration of 0.5 mg/kg diazepam i.m. at 1 min or 40 min into seizures. A) Piriform cortex (layer I/II/III, summed area densities). Significantly decreased NeuN immunoreactivity in layer III in soman exposed was normalized by coadministration of tariquidar alone at 1 min, and when diazepam and tariquidar were coadministered at 1 min (also Layer II) or at 40 min after seizure onset. B) Entorhinal cortex (summed area densities) - C) Amygdala NeuN immunoreactivity was significantly lower compared to non-exposed controls in animals treated at 1 min with diazepam and at 40 min in animals treated with diazepam alone and combined treatment with diazepam and tariquidar (\*:  $p < 0.05$ , one-way ANOVA followed by Sidak's post-hoc analysis versus control animals, \*\* versus vehicle treated animals).

regions examined, the GFAP score significantly changed compared to naïve controls (typical scoring 0–1) only in the piriform cortex and entorhinal cortex. In the piriform cortex of soman exposed rats, treated with HI-6 and atropine, the GFAP score was profoundly increased, which was not prevented by diazepam administration, but was decreased by immediately administered tariquidar, or a combination of tariquidar and diazepam. At 40 min into seizures, neither diazepam nor tariquidar reduced astrogliosis, but the combination of both effectively reduced the GFAP rating (Figs. 6 and 7G/H).

4. Discussion

In the present study, we investigated effects of tariquidar on enhancing nerve agent treatment efficacy after soman intoxication in rats. Organophosphate (OP) compounds, including soman, are well known to irreversibly inactivate the enzyme cholinesterase, which results in a systemic cholinergic overload (Shih and McDonough, 1997). In our rat model, profound reductions in blood- and brain cholinesterase activity



**Fig. 5.** OX42<sup>+</sup> cell counts and sub classification in brain areas of rats 24 h after Soman exposure (150 µg/kg s.c.), treated with HI-6 (125 mg/kg i.m.) and atropine sulfate (3 mg/kg i.m.) at 1 min. Tariquidar (7.5 mg/kg i.v.) and/or Diazepam (0.5 mg/kg i.m.) were administered at either 1 min after exposure or at 40 min after seizure onset. (A) Piriform cortex –(B) Entorhinal cortex (C) Amygdala. In both the piriform and entorhinal cortex tariquidar administration with diazepam at 1 min and at 40 min into seizures significantly attenuated microglia activation by Soman exposure, and only at 1 min in the amygdala. (\*, \*\*: p < 0.05, one-way ANOVA followed by Sidak’s post-hoc analysis, Tariquidar versus vehicle treated animals; \*\*: total number of activated microglia, \*: Type 3).

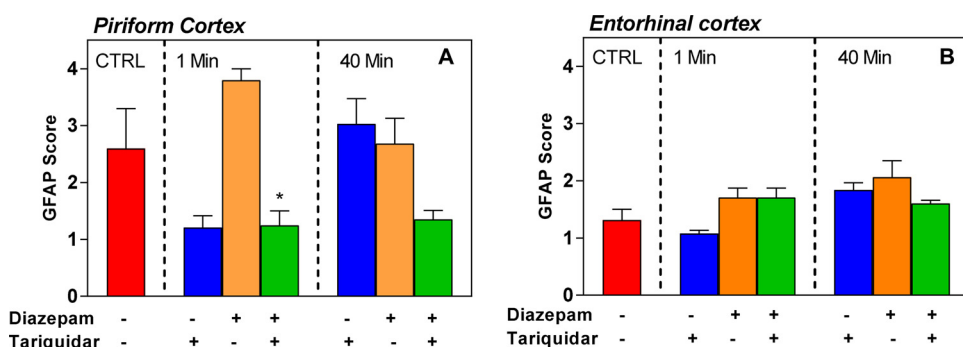
after soman exposure, are indeed accompanied by pronounced seizure activity, irrespective of treatment. Here, we used EEG recordings and brain histology to study (combined) treatment effects of HI-6/atropine and diazepam and investigated whether acute and delayed treatments with tariquidar would improve clinical scores and reduce brain injury.

The main findings of this study were that the addition of the Pgp inhibitor tariquidar decreased seizure duration when administered with intramuscular HI-6 and atropine 1 min after soman exposure. Tariquidar administration at 40 min after seizure onset failed to affect seizure duration. Further, diazepam efficacy was absent in the present model, but the co-administration of tariquidar at either 1 min or 40 min after seizure onset led to a complete and rapid mitigation of seizure activity. Across the experimental groups, the pattern of neuronal loss and microglia activation, Racine scores and bodyweight loss could generally be associated with intensity of seizure activity and epileptiform behavior. In the experimental groups in which seizure intensity was lowered, the body weight loss was largely prevented (Filliat et al., 2007).

To date, several anticonvulsants and anti-epileptic drugs have been identified as Pgp substrates (Zhang et al., 2012; Luna-Tortos et al., 2008). For example, previous *in vitro* experiments with a MDR1/ MDCK cell line identified atropine, but not HI-6 as a Pgp substrate (Joosen et al., 2016). In the present model with HI6 and atropine administered

at 1 min, administration of tariquidar by itself 40 min into seizures had no effect on seizure activity, Racine scores and weight loss. Pharmacokinetic data of atropine in rats showed that T<sub>max</sub> is reached at ~40–60 min after im dosing (Lieske et al., 1990), which indicates that 40 min after seizure onset, apparently sufficiently high plasma levels of atropine are present to enhance its brain (re)entry. However, it is generally accepted that this stage of nerve-agent induced seizures is less sensitive to subsequent anticholinergic due to extensive involvement of glutamatergic networks (McDonough and Shih, 1997). Recent work has shown that pilocarpine-induced status epilepticus upregulates Pgp expression and activity in brain capillaries 2–3 fold within 48 h, inferring an increased efflux capacity of Pgp substrates (Hartz et al., 2017; Bankstahl et al., 2008; Volk and Loscher, 2005). Given the time frame associated to Pgp upregulation following seizures, the saturating dose of tariquidar and the pharmacokinetics of atropine, the upregulation of Pgp due to seizures is not considered as a contributing factor in the present study.

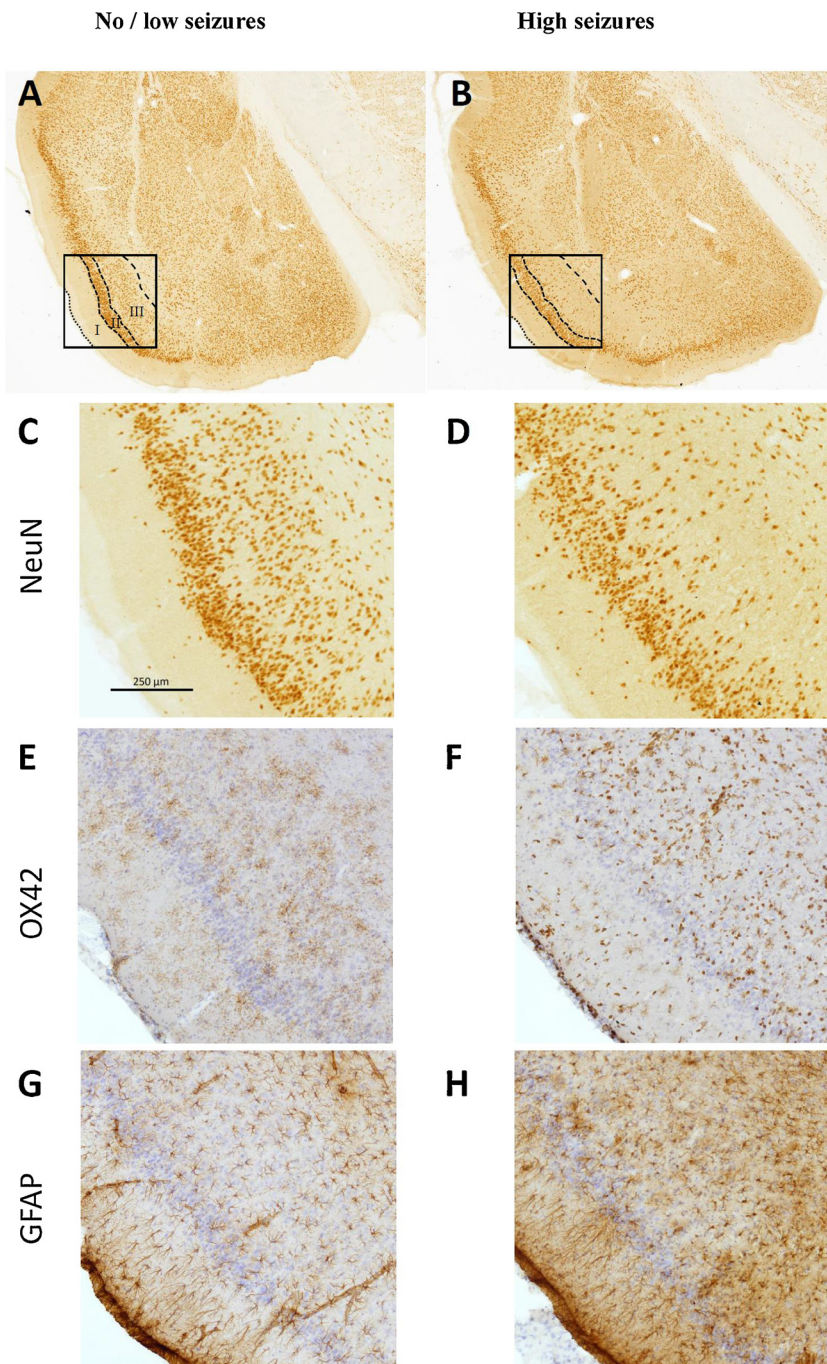
Apart from its main anticholinergic action, atropine can also modulate efficacy of oximes, albeit only when the oxime itself is sufficiently effective (Ligtenstein and Moes, 1991). Atropine can alter HI-6 levels in the brain, although dose itself appears to be not that crucial to oxime efficacy (Jovanovic et al., 1992). This provides a possible explanation for the enhanced suppression of seizure activity by interaction with



**Fig. 6.** GFAP scores in brain areas of rats 24 h after exposure to Soman (150 µg/kg) treated at 1 min with HI-6 (125 mg/kg) and atropine sulfate (3 mg/kg) i.m. and optionally, 0.5 mg/kg Diazepam i.m., either with additional i.v. vehicle or tariquidar (7.5 mg/kg) administered immediately or at 40 min into seizures. (A) Piriform cortex. Astrogliosis scores were increased in piriform cortex in standard treated animals. Tariquidar administration at 1 min combined with diazepam significantly attenuated astrogliosis scores versus diazepam/vehicle treatment. (B) Entorhinal cortex. Astrogliosis scores were slightly elevated from

naïve rats (not shown, scores 0–1) by soman exposure, and not affected by any treatment. (\*: p < 0.05 Kruskal Wallis test, followed by Dunn’s multiple comparisons comparing tariquidar versus vehicle treated animals).





**Fig. 7.** Neuropathological alterations in piriform cortex of rats 24 h after exposure to Soman (150 µg/kg) treated at 1 min with HI-6 (125 mg/kg) and atropine sulfate (3 mg/kg) i.m. and optionally, 0.5 mg/kg Diazepam i.m., either with additional i.v. vehicle or tariquidar (7.5 mg/kg) administered immediately or at 40 min into seizures. (A) NeuN immunostaining in an animal with low intensity seizures was virtually devoid of immunostaining in layer I, but dense immunostaining in layer II and layer III. (B) NeuN staining in high seizure animal shows loss of immunoreactivity predominantly in layer III, indicative of severe neuronal depletion. (C) NeuN immunostaining in an animal with low intensity seizures, high magnification. (D) NeuN immunostaining in an animal with high intensity seizures, high magnification. (E) OX42 staining in an animal with low seizure intensity is fuzzy without distinct cytoplasmic pattern, indicative of resting microglia (F) OX42 staining in high seizure intensity animal reveals a high number of round dense cells throughout the brain, reminiscent of fully activated microglia. (G) GFAP immunostaining in an animal with low seizure intensity shows light staining with a pattern similar to naïve animals. (H) Intense and fuzzy GFAP immunostaining as well as tissue damage due to necrosis and in an animal with high seizure intensity is indicative of severe astrogliosis.

atropine (Joosen et al., 2016). In addition, in the present study, the lower dose of atropine (3 mg/kg i.m.) we used relative to earlier work (16 mg/kg i.m.), resulted in a less pronounced effect of tariquidar alone at 1 min (Joosen et al., 2016, 2011). Taken together these results indicate that interactions with brain entrance of atropine are of critical importance to the treatment enhancing effect of tariquidar.

In line with one of our earlier studies, no effects of tariquidar on cholinesterase activity were found 24 h after soman exposure. Unlike in our previous study, in which significantly higher activity in animals co-administered tariquidar at 4 h after exposure was found, brain cholinesterase activity at earlier timepoints was not assessed in the present study. Such higher activity may also have contributed to an alternate progression of seizure development and mitigation, albeit that we could not corroborate that in the present study. (Joosen et al., 2011, 2016).

Diazepam, an allosteric modulator of  $\gamma$ -aminobutyric acid A

(GABA<sub>A</sub>) receptors, is commonly used as anticonvulsant. In the present study, the addition of a relatively low dose of diazepam (0.5 mg/kg i.m.) failed to lower seizure activity compared to HI-6 and atropine alone, both when administered 1 min after soman or at 40 min into seizures. This is in line with other studies showing continuous seizures using similar treatments (Langston et al., 2012; Moffett et al., 2011). For a delayed treatment, it is important to know that prolonged seizure activity promotes GABA<sub>A</sub> receptor endocytosis and phosphorylation, which reduces surface expression and may diminish the inhibitory effects of GABA and its allosteric modulation by diazepam (Wasterlain and Chen, 2008; Kapur and Coulter, 1995). In contrast to diazepam administration alone, combined administration of tariquidar and diazepam both at 1 min after soman as well as at 40 min into seizures, was highly effective in ablating both the initiation as well as continuation of the seizures in the current model. It is well known that the dose of

atropine, and as such brain levels, possibly in the present study altered by PgP inhibition, influence the anticonvulsant efficacy of anti-seizure agents (Shih, 1991). Whereas the possible increase of brain atropine levels by tariquidar, as proposed earlier, without diazepam appeared to be ineffective to mitigate seizure activity, it appeared sufficient to potentiate GABA-ergic effects leading to a pronounced and rapid culmination of seizures with diazepam.

The patterns of NeuN, OX42 and GFAP immunoreactivity 24 h after soman exposure revealed tissue damage in the piriform and entorhinal cortices and the amygdala. Profound NeuN loss was observed in the piriform cortex layer III specifically, in HI-6 and atropine treated animals 24 h after intoxication, as reported before by others (Zimmer et al., 1997) both based on NeuN or Nissl counts (Collombet et al., 2006; de Araujo Furtado et al., 2010). Generally, a lower extent of neuronal loss was observed in the groups that displayed a lower seizure duration (EEG 8h AUC), which is consistent with the concept that the initial seizure activity triggers neuropathology (Baille et al., 2005; Shih et al., 2003; Philippens et al., 1992).

Microglia activation was observed in several brain regions, which is common after nerve agent exposure (Zimmer et al., 1997). The fact that microglial activation occurred in the entorhinal cortex, a region where neuronal loss was not apparent, suggests that these processes are partly independent. Microglia activation in the piriform cortex was not sub-region-specific, which is in line with the finding that subregion-specificity is apparent only up to 4 h after exposure (Zimmer et al., 1997). In particular full microglial activation was generally absent in animal groups with a lower intensity or duration of seizures. In these animals, mostly intermediate (type II) activated microglia were present, in line with earlier findings that microglial activation correlates well with seizure severity (Avignone et al., 2015).

Astrocytes are sensitive to neuronal hyperactivity and upregulate GFAP expression in response (Zimmer et al., 1998). An increase in GFAP immunoreactivity has previously been observed in piriform cortex one hour after soman exposure, while the amygdala, hippocampus and entorhinal cortex followed hours later. Presumably, the increased staining intensity is thought to be due to an increased antigenicity of the GFAP protein due to cytoskeletal rearrangement, which is again reduced in most brain areas 24 h post-exposure (Collombet et al., 2005). In the present study, an increase in GFAP score (reminiscent of astrogliosis) was apparent in both entorhinal and piriform cortex, but not in other brain regions at the latter time point. Tariquidar administration prevented the increase in GFAP score only in the piriform cortex. As this region predominantly consists of cholinergic neurons, and astrocytes express receptors for acetylcholine, this result is consistent with our hypothesis that tariquidar locally enhances atropine levels.

Body weight loss in these models generally correlates well to the extent of brain damage, and a loss of 12% is considered a threshold for neuropathology (Filliat et al., 2007). Our current neuropathological results were indeed substantially better in the animals that showed a shorter duration of seizure activity and higher body weight. In certain groups, EEG intensity returned to baseline values after approximately 8 h, while overt behavioral signs were gradual to recover up to 24 h. This is in line with recent studies in related models also showing subtle behavioral deficits lasting for a prolonged period of time (Moffett et al., 2011; Langston et al., 2012).

It has been reported that soman-induced neuropathology can already be observed after 20 min of seizure activity (Myhrer et al., 2005). In the present study, seizures were terminated after ~45 min by the combined diazepam/ tariquidar treatment, yielding less neuronal injury. This indicates that in this model, 40 min of seizure activity are required for neuronal loss to become apparent at 24 h. The extent of neuropathology generally is considered the most important determinant in developing behavioral deficits, that may be very long-lasting (Aroniadou-Anderjaska et al., 2016). In our study, the behavioral and physiological consequences of the reduction of NeuN

immunoreactivity, increased microglia and astrocyte activation were not studied. The piriform cortex appeared to be the brain region that was most compromised. Given its possible role in seizure propagation, but also in olfactory sensory integration and associative function between intra/intercortical areas, injury in this region may be expected to be instrumental in the behavioural changes observed.

Summarizing, acute co-administration of tariquidar and delayed tariquidar and diazepam on top of HI-6 and atropine, effectively reduced seizure related brain activity. Animals with a lower seizure duration displayed less severe neuropathology, corroborating previous findings of others (Baille et al., 2005; Shih et al., 2003). Further, the interaction of tariquidar with atropine may be the decisive factor for this enhanced treatment efficacy as (i) both oxime and anticonvulsant therapy efficacy increased with higher doses of atropine (Shih et al., 2007), (ii) atropine is a PgP substrate (Joosen et al., 2016), and (iii) a more pronounced effect of tariquidar was found when a higher dose of atropine (16 mg/kg versus 3 mg/kg) was used in the model (Joosen et al., 2016). Together, these data not only show that improved protection can be achieved by combined treatments, they also highlight that abrogating seizure activity as soon as possible should remain to be a primary goal in treating nerve agent exposure.

#### 4.1. Concluding remarks

In conclusion, these findings warrant further research into a more thorough understanding of the interactions of nerve agent antidotes, and in particular the actions of central anticholinergics such as atropine or scopolamine. Our findings that delayed diazepam, combined with tariquidar, was highly effective in mitigating seizures, even though this occurred in the generally assumed refractory phase, could contribute to a future optimization of treatment combinations aimed at late stage interventions of nerve agent induced seizures.

#### Transparency document

The Transparency document associated with this article can be found in the online version.

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