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Research Paper

Neuroprotective effects of a catalytic antioxidant in a rat nerve agent model

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ABSTRACT

Persistent inhibition of acetylcholinesterase resulting from exposure to nerve agents such as soman, is associated with prolonged seizure activity known as status epilepticus (SE). Without medical countermeasures, exposure to soman and resultant SE leads to high morbidity and mortality. Currently available therapeutics are effective in limiting mortality, however effects on morbidity are highly time-dependent and rely on the ability to suppress SE. We have previously demonstrated significant protection from secondary neuronal injury in surrogate nerve agent models by targeting oxidative stress. However, whether oxidative stress represents a relevant therapeutic target in genuine nerve agent toxicity is unknown. Here, we demonstrate that soman exposure results in robust region- and time-dependent oxidative stress. Targeting this oxidative stress in a post-exposure paradigm using a small molecular weight, broad spectrum catalytic antioxidant, was sufficient to attenuate brain and plasma oxidative stress, neuroinflammation and neurodegeneration. Thus, targeting of oxidative stress in a post-exposure paradigm can mitigate secondary neuronal injury following soman exposure.

1. Introduction

Soman and the closely related sarin are highly toxic chemical warfare agents, exposure to which has the potential to result in indiscriminate and mass casualties. This was highlighted recently by the 2013 attack on Syrian civilians with a chemical agent later determined to be sarin by the United Nations [1]. Thus, despite the best efforts to control the use of these weapons by the Organization for Prohibition of Chemical Weapons (OPCW), intentional or accidental exposure to chemical weapons remains a threat to military personnel and civilians alike. Therefore, the development of medical countermeasures to prevent mortality and secondary effects caused by exposure to chemical weapons, is much needed [2].

Nerve agents exert their early toxicity through irreversible inhibition of acetylcholinesterase (AChE). This persistent hyperstimulation of cholinergic receptors rapidly leads to one of the most critical manifestations of nerve agent toxicity: prolonged seizure activity known as status epilepticus (SE). Secondary to AChE inhibition and SE, nerve agent toxicity is associated with neuroinflammation, neuronal loss and high mortality rates. Current standard therapeutics such as oximes and benzodiazepines target early toxicity events i.e. they can reduce mortality and attenuate SE however their ability to prevent neuronal injury can be modest and highly time-dependent [3–5]. These agents do not target the secondary processes that lead to functional outcomes such as neuropathology and cognitive impairment. Thus, pursuit of novel neuroprotective countermeasures that target secondary events following nerve agent exposure is necessary and supported by the trans-NIH initiative known as the Counter Measures against Chemical Threats (CounterACT) program.

Prolonged seizure activity such as SE is associated with increased oxidative stress leading to neuronal loss and interventions aimed at attenuating seizure-induced oxidative stress have been demonstrated to be neuroprotective [6–11]. AEOL 10150 is a small molecular weight, superoxide dismutase (SOD) mimetic with the capability to catalytically detoxify a broad range of reactive species including superoxide, hydrogen peroxide, lipid peroxides and peroxynitrite [12]. We have previously demonstrated efficacy of the catalytic antioxidant, AEOL 10150, against oxidative stress, neuroinflammation and neurodegeneration associated with exposure to surrogate nerve agents including pilocarpine and diisopropylflurorphoshate (DFP) [6,7]. However whether 1) nerve agent exposure results in significant oxidative stress and 2) treatment with AEOL 10150 has the ability to inhibit oxidative stress indices in a genuine nerve agent model is yet to be determined. The goal of the current study was to determine if post-treatment with AEOL 10150 could attenuate indices of oxidative stress, neuroinflammation and neurodegeneration associated with exposure to soman. Pharmacokinetic data in vehicle and soman-exposed rats was used to optimize a dosing paradigm. A therapeutic window of the efficacy of AEOL 10150

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treatment against indices of oxidative stress was used to guide further evaluation of neuroinflammation and neurodegeneration. Our findings indicate that suppression of oxidative stress by AEOL 10150 can attenuate soman-induced microgliosis, release of pro-inflammatory cytokines, and neurodegeneration. These results suggest that targeting of oxidative stress in a post-exposure treatment paradigm may provide a novel therapeutic avenue to improve secondary neuronal injury after nerve agent exposure.

2. Material and methods

2.1. Reagents

Soman was sythesized and obtained by MRI Global (Kansas City, MO). Midazolam was purchased from Hopira Inc. (Lake Forest, IL). Manganese (III) meso-tetrakis (di-N-ethylimidazole) porphyrin designated as AEOL 10150 (also known in the literature as Mn^{III}TDE-2-ImP^{5+}) was pharmaceutical grade and obtained from Aeolus Pharmaceuticals. All other compounds were purchased from Sigma Aldrich unless otherwise noted.

2.2. Animals

Animal studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23). All procedures were approved by the Institute Animal Care and Use Committee (IACUC) of MRI Global, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Due to hormonal influences on the LD_{50} of soman, only males were included in the studies [13]. Male, Sprague-Dawley (SD) rats (300–350 g) were purchased from Harlan Laboratories (Indianapolis, IN) and used for all experiments. Upon arrival, rats were group housed on a 12/12 light/dark cycle with ad libitum access to both food and filtered water. Rats were allowed one week of acclimation before the initiation of any studies.

2.3. Soman and AEOL 10150 administration

Soman exposure producing prolonged convulsive seizures was performed following methods previously described [14,15]. SD rats were injected with soman (154 μg/kg, s.c., ~1.4 × LD_{50}) and standard therapy including, HI-6 (125 mg/kg, i.m.) 30 min prior to soman challenge and atropine methyl nitrate (2 mg/kg, i.m.) 1 min after soman. Approximately 10 min following soman administration, rats showed signs of seizure onset, including salivation, tremors, wet dog shakes, seizures with rearing and rolling over. Midazolam (2 mg/kg, i.m.) was administrated 1 min, 5 min or 15 min after status epileptic (SE) initiation. This group of rats will be referred to as the soman + standard therapy group. Control rats received saline in place of soman but still received standard therapy (HI-6, atropine and midazolam). This group of rats will be referred to as control + standard therapy. In some soman + standard therapy rats, AEOL 10150 (7 mg/kg s.c.) was also administered 1 min, 5 min or 15 min after SE onset and every 4 h thereafter until sacrificed. This group of rats will be referred to as soman + standard therapy + AEOL 10150. All rats were visually monitored during SE and behavioral seizures were scored using a modified Racine scale [16]. Briefly, seizures were scored on the following scale: P1-immobilization and staring, P2-head nodding and/or “wet dog shakes”, P3- unilateral forelimb clonus, P4- bilateral forelimb clonus, and P5-bilateral forelimb clonus with rearing, falling and loss balance. Only rats experiencing SE defined as: at least P3 or higher seizures followed by a period of continuous seizure activity were included in further analyses.

2.4. Pharmacokinetic analyses of AEOL 10150

Rats with jugular vein catheter cannulation (obtained from Harlan) and treated as either the control + standard therapy group or the soman + standard therapy group were administered AEOL 10150 dissolved in sterile saline via catheter (i.v., 1 mg/kg) or subcutaneous (s.c., 5 mg/kg) route. Blood samples (0.1 ml) were withdrawn from the rat's jugular catheter at 1 h, 3 h, 6 h, 12 h, 24 h and 48 h after AEOL 10150 administration. Blood samples were centrifuged at 8000 rpm for 10 min and 50 μl of the supernatants were collected as plasma. The plasma samples were stored at −80 °C for pharmacokinetic analysis. AEOL 10150 levels were measured by a HPLC equipped with a spectrophotometric detector (Elite LaChrom System; Hitachi) and a YMC-Pack ODS-A column (4.6 × 150 mm, 3 μm) as previously described [17]. Mobile phase consisted of 20 mM trimethylamine, 20 mM trifluoroacetic acid, pH 2.7% and 70% acetonitrile. Flow rate was 1 ml/min. Plasma and brain samples were extracted and precipitated with 50% methanol and 0.05 N perchloric acid centrifuged at 16,000*g* for 20 min. The resulting supernatant was filtered through a 0.22-μm filter. An aliquot of supernatant (10 μl) was injected into the HPLC set at 466 nm. Recovery of metalloporphyrins from plasma samples was determined to be greater than 95%.

2.5. Measurement of oxidative markers

Glutathione (GSH), glutathione disulfide (GSSG), tyrosine and 3-nitrotyrosine (3-NT) were measured in hippocampus and piriform cortex 6 h, 12 h, 24 h, and 48 h after soman exposure and in frontal cortex 24 h after soman in control + standard therapy and soman + standard therapy groups. Additionally, to establish a therapeutic window for AEOL 10150 a separate experiment measured these markers 24 h after soman in control + standard therapy, soman + standard therapy, and soman + standard therapy + AEOL 10150 groups. For these experiments, AEOL 10150 (7 mg/kg) was given 1 min, 5 min and 15 min after soman and every 4 h thereafter. Midazolam was given at the same time point as AEOL 10150. All assays were performed using an ESA (Chelmsford, MA) 5600 CoulArray HPLC equipped with eight electrochemical cells following the company instruction (ESA Application Note 70–3993) with small modification [18]. The potentials of the electrochemical cells were set at 400/450/500/570/630/690/810/860 mV vs. Pd. Analyte separation was conducted on a TOSOHAAS (Montgomeryville, PA) reverse-phase ODS 80-TM C-18 analytical column (4.6 mm × 250 mm; 5 μm).

2.6. Measurement oxidative stress markers in plasma

The levels of cysteine (Cyss), cysteine (Cys), GSH and GSSG were measured in plasma with an ESA (Chelmsford, MA) 5600 CoulArray HPLC equipped with eight electrochemical cells following company instruction (Dionex Application Brief 131) and with small modifications [19,20]. Briefly, a 1:1 ratio of plasma and 2% PCA/0.2 M boric acid was centrifuged at 13,000*g*, 4 °C for 10 min and 20 μl of the supernatant was injected into the HPLC. The potentials of the electrochemical cells were set at 400/500/600/650/700/750/800/850 mV vs. Pd. Analyte separation was conducted on YMC-Pack ODS-A C-18 analytical column (Waters Inc., 4.6 mm × 250 mm; 5 μm particle size). A two-component gradient elution system was used with component A of the mobile phase composed of 50 mM NaH_2PO_4 pH 2.7, 1.0 mM 1-octanesulfonic acid and component B composed of 50 mM NaH2PO4 pH 2.7, 1.0 mM 1-octanesulfonic acid and 50% methanol.

2.7. Fluoro-Jade B analysis

Formalin-fixed, paraffin-embedded brain sections (20 μm) containing hippocampus, piriform cortex and amygdala were cut coronally and stained with Fluoro-Jade B (FJB; Histo-Chem) as previously

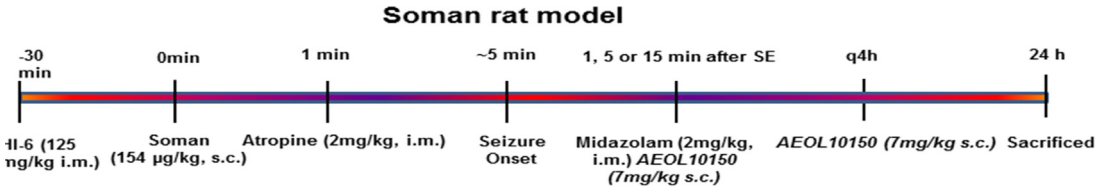

Fig. 1. Schematic of treatment paradigm.

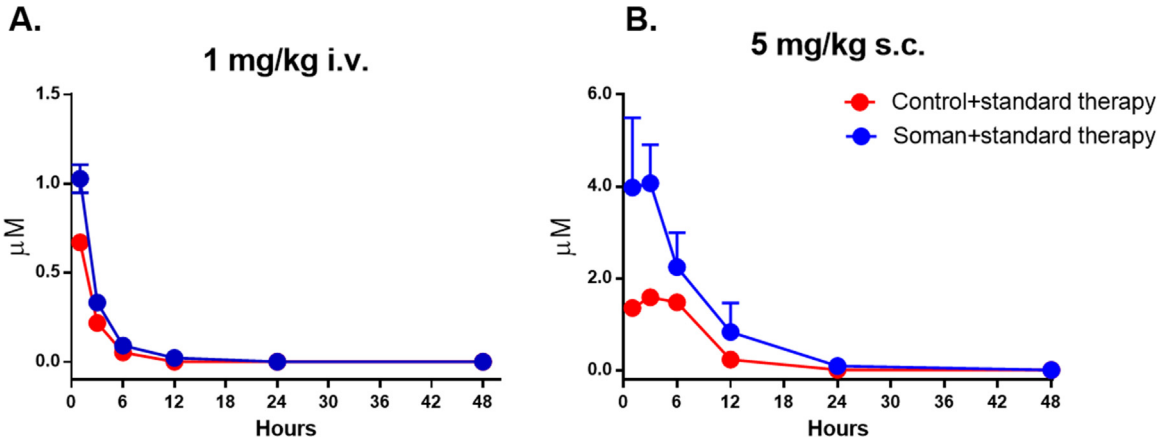

Fig. 2. The concentration of AEOL 10150 in plasma at 1, 3, 6, 12, 24 and 48 h after AEOL 10150 5 mg/kg, s.c. (A) or 1 mg/kg i.v. (B) with saline + standard therapy or soman + standard therapy treatment.

described [21]. FJB selectively stains neurons undergoing neurodegeneration. Images were captured using a Nikon Eclipse Ti2 microscope. The number of positively stained neurons in hippocampus (CA1, CA3 and hilus), piriform cortex and amygdala (10 × axis) were quantified with Nikon NIS-Elements Advanced image analysis software (version 4.6) in six sections 100 μm apart from each other and from both hemispheres of each animal. The average of FJB positive neurons within a given area (10x axis) from six sections of each animal and six animals of each group were expressed as positive cell number/HP (high power, 10 × axis).

2.8. IBA1 staining for microglial activation

Formalin-fixed, paraffin-embedded brain sections (20 μm) containing whole hippocampus, piriform cortex and amygdala were deparaffinized and rehydrated in sequential steps. After blocking in normal goat serum for one hour, sections were immunostained with anti-IBA1, (ionized calcium adaptor molecule 1, Rabbit. Wako, Japan) for identification of microglial activation, followed by incubation with the secondary antibody, goat anti-rabbit conjugated Rhodamine Red (1:100, Jackson Immuno Research Inc.). Images were captured using a Nikon Eclipse Ti2 microscope. Average fluorescence intensity within a given area of the hippocampus (CA3 and hilus), piriform cortex and amygdala was quantified with by Nikon NIS-Elements Advanced image analysis software (version 4.6) in six sections 100 μm apart each other from both hemispheres of each animal. The average of IBA1 staining positive fluorescent intensity within a given area (10 × axis) from six sections of each animal and six animals of each group were expressed as positive cell number/HP (high power, 10 × axis).

2.9. Multiplex pro-inflammatory cytokine measurement

Levels of the pro-inflammatory cytokines, TNF-α, IL-1β, IL-6 and KC/GRO, were measured using a rat multiplex pro-inflammatory cytokine array Kit from Mesoscale Discovery (MSD) according to manufacturer's instructions and method described before [22]. Briefly, the assay was run as follows. One side of hippocampus or piriform cortex from each rat was lysed in MSD Tris Lysis buffer supplemented with protease and phosphatase inhibitors in a 10% (w/v) ratio (0.1 g tissue/ml). The lysates were then centrifuged at 13,000 rpm for 10 min and supernatants collected. Prior to incubation with samples, wells were blocked for 30 min. 25 μl of each sample or standard was loaded per well in duplicate followed by incubation with shaking (700 rpm) for 2 h at room temperate. Standard calibration curves were prepared with a range of 10,000–2.45pg/ml. Then 25 μl of detection antibody was added per well and incubated for an additional 2 h. Finally, the plate was washed, MSD read buffer was added to the wells and the plate was read using Sector Imager 2400. Concentrations of analytes were determined by the measuring the intensity of light emitted at 620 nm and Softmax Pro software using curve-fit models.

2.10. Statistical analysis

All data are expressed as mean ± SEM. Pharmacokinetic parameters were determined by fitting the data to a one compartment model with first-order input and first order output (Model #3, PK Analyst, MicroMath). Statistical differences were analyzed by *t*-test, one-way ANOVA or two-way ANOVA with post hoc multiple comparison test corrected for multiple comparisons where appropriate. P values less than 0.05 were considered statistically significant. All analyses were performed using Prism 5 software (Prism 5. GraphPad Software, San Diego, CA).

3. Results

3.1. Soman exposure induces status epilepticus

Within ~ 10 min of exposure to 1.4 × LD_{50} soman (154 μg/kg) all rats exhibited signs of nerve agent toxicity including hypersalivation, lacrimation and diarrhea. In all rats exposed to soman, this was followed with tremors, wet dog shakes and limbic seizures with rearing and rolling. Each animal exhibited at least one P3 or greater seizure before rapidly progressing to SE. AEOL 10150 treatment had no observable effect on the frequency or severity of behavioral seizures

Table 1
Comparison of plasma pharmacokinetic profiles (5 mg/kg, s.c.).*

PK parameters	Control (n = 3)	Soman (n = 3)	p-value
Half life (h)	2.3 ± 0.2	2.4 ± 0.1	ns
Tmax (h)	3.0 ± 0.3	2.6 ± 0.2	ns
Cmax (ng/ml)	1960 ± 60	5100 ± 1220	0.03
AUC (ng x h/ml)	15,270 ± 1030	32,640 ± 9310	0.03

* Data are means + SEM., non-parametric t-test (Mann-Whitney). Pharmacokinetic data of AEOL 10150 was obtained using a one compartment model with first order input and first order output (PK analyst, model #3, MicroMath, Salt Lake City, UT)

Table 2
Comparison of plasma pharmacokinetic profiles (1 mg/kg, i.v.).*

PK Parameters	Control (n = 2)	Soman (n = 6)	p-value
Half-life (h)	0.4 + 0.1	1.1 + 0.2	0.021
Cmax (ng/ml)	1150 + 50	1867 + 88	0.0005
AUC (ng x h/ml)	3410 + 601	3885 + 279	0.57

* Data are means + SEM., non-parametric t-test (Mann-Whitney). Pharmacokinetic data of AEOL 10150 was obtained using a one compartment model with first order input and first order output (PK analyst, model #3, MicroMath, Salt Lake City, UT).

induced by soman. Additionally, we have previously demonstrated that treatment with AEOL 10150 does not alter electrographic power in pilocarpine-induced SE [23]. All of the animals (100%) achieved SE with this dose of soman. Due to optimization of adjuvants, mortality was minimal. Fig. 1.

3.2. AEOL 10150 pharmacokinetic analyses

To determine the pharmacokinetic profile of AEOL 10150 and whether this profile was altered in soman-treated rats, AEOL 10150 was administered by i.v. (Fig. 2A; 1 mg/kg) or s.c. routes (Fig. 2B, 5 mg/kg) and its concentration in plasma was evaluated at various time points following administration. AEOL 10150 by s.c. and i.v. route concentration-time data best fit a one compartment PK model with first-order input and output. AEOL 10150 plasma elimination half-life ($T_{½}$) in saline and soman treated groups was not significantly different between groups and estimated to be 2.3 ± 0.2 h and 2.4 ± 0.1 h, respectively. Time to achieve maximum plasma concentration (T_{max}) of AEOL 10150 by s.c. route was not significantly different between saline and soman treated groups and was estimated to be 3.0 ± 0.3 h and 2.6 ± 0.2 h, respectively (Table 1). The maximum plasma concentrations (C_{max}) and area under the time curves (AUC) were significantly higher in the soman + standard therapy group compared to control + standard therapy group (Table 1 and Table 2). While it isn't entirely clear why these parameters are altered in the soman group, it is possible

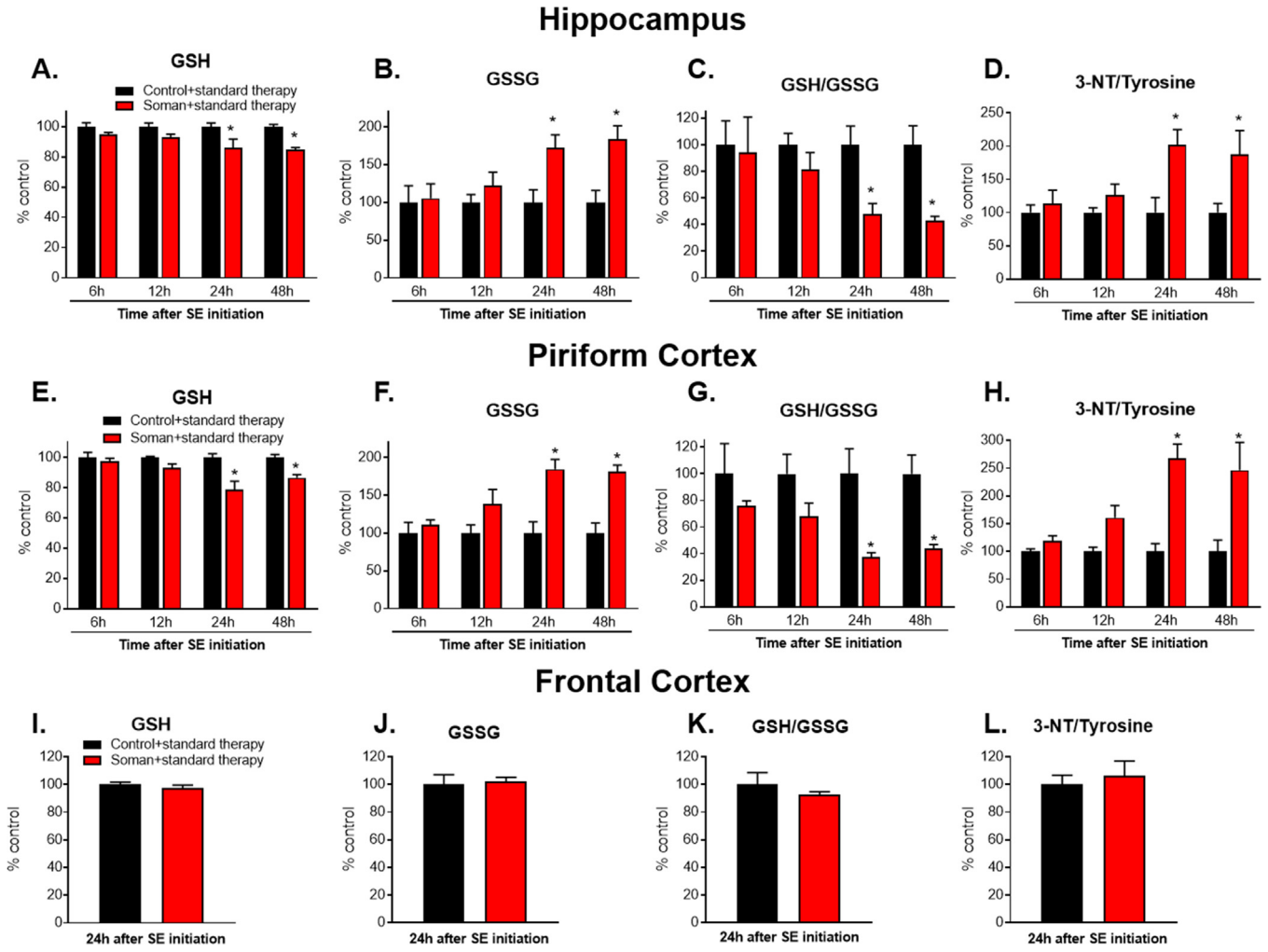

Fig. 3. Oxidative makers in brain at different time points after soman treatment. The levels of GSH (A, E, I), GSSG (B, F, J), GSH/GSSG (C, G, K) and 3-NT/tyrosine (D, H, L) in the hippocampus (A-D), piriform cortex (E-H) or frontal cortex (I-L) of rats at 6, 12, 24 and 48 h after initiation of SE. Bars represent mean + S.E.M., *$p < 0.05$ vs. saline, two-way ANOVA or *t*-test, n = 6 per group.

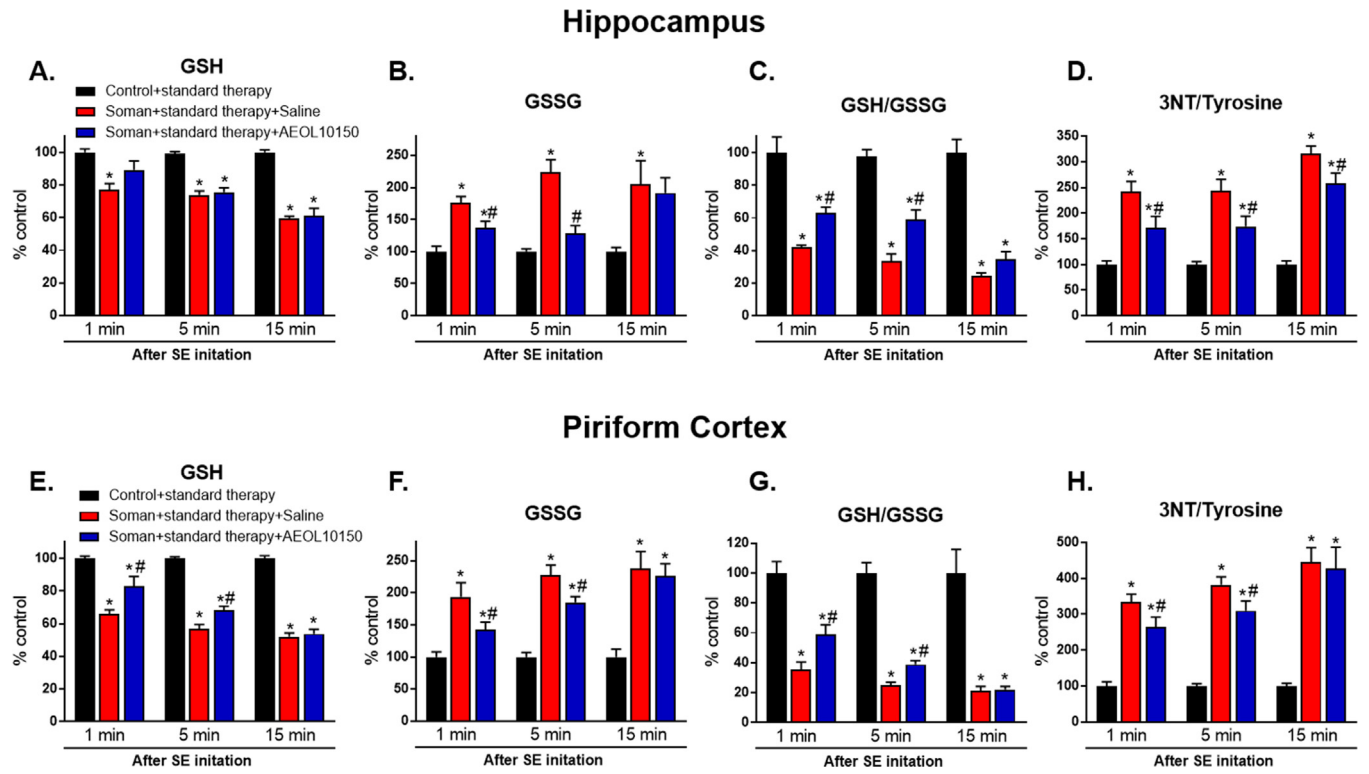

Fig. 4. Therapeutic window of AEOL 10150 against oxidative stress in the soman model. Levels of GSH, GSSG and GSH/GSSG and 3-NT/tyrosine in the hippocampus (A-D) and piriform cortex (E-F) of rats 24 h after soman when treatment with AEOL10150 (7 mg/kg, s.c.) or saline was initiated 1, 5 or 15 min after SE onset and continued every 4 h thereafter. Bars represent mean + S.E.M., *p < 0.01 vs. control, #p < 0.05 vs. soman + standard therapy + saline, one-way ANOVA, n = 6–7 per group.

that peripheral effects of soman on blood pressure, heart rate and renal clearance could affect Cmax and AUC. In particular, since AEOL 10150 is highly dependent on renal clearance, this could be a major factor that altered Cmax and AUC.

3.3. Soman exposure increases indices of oxidative stress in brain regions

To determine the time course and regional distribution of oxidative stress markers, the levels of GSH, GSSG and 3-NT were measured in the hippocampus and piriform cortex 6, 12, 24 and 48 h after soman exposure. These indices were also measured in the frontal cortex 24 h after soman exposure. In the hippocampus, levels of GSH were significantly decreased 24 and 48 h following soman exposure (Fig. 3A, treatment $F_{1,40} = 26.81$, $p < 0.0001$, $\eta^2 = 0.35$; time $F_{3,40} = 1.48$, $p > 0.05$; interaction $F_{3,40} = 1.48$, $p > 0.05$) while levels of its disulfide, GSSG were significantly increased at the same time points (Fig. 3B, treatment $F_{1,40} = 13.75$, $p < 0.001$, $\eta^2 = 0.20$; time $F_{3,40} = 2.38$, $p > 0.05$; interaction $F_{3,40} = 2.38$, $p > 0.05$), resulting in an overall depletion of the GSH: GSSG ratio (Fig. 3C, treatment $F_{1,40} = 10.11$, $p < 0.01$, $\eta^2 = 0.17$; time $F_{3,40} = 1.47$, $p > 0.05$; interaction $F_{3,40} = 1.47$, $p > 0.05$). Similarly, in the hippocampus, the ratio of 3NT/tyrosine was significantly elevated at 24 and 48 h but not 6 or 12 h following exposure (Fig. 3D, treatment $F_{1,40} = 15.35$, $p < 0.001$, $\eta^2 = 0.22$; time $F_{3,40} = 2.24$, $p > 0.05$; interaction $F_{3,40} = 2.24$, $p > 0.05$). In the piriform cortex, indices of oxidative stress followed a similar pattern. At 24 h and 48 h following soman exposure, but not 6 or 12 h, the levels of GSH were significantly decreased (Fig. 3E, treatment $F_{1,40} = 30.11$, $p < 0.0001$, $\eta^2 = 0.32$; time $F_{3,40} = 4.04$, $p < 0.05$, $\eta^2 = 0.13$; interaction $F_{3,40} = 4.05$, $p < 0.05$, $\eta^2 = 0.13$) and GSSG was significantly increased (Fig. 3F, treatment $F_{1,40} = 33.66$, $p < 0.0001$, $\eta^2 = 0.35$; time $F_{3,40} = 3.65$, $p < 0.05$, $\eta^2 = 0.11$; interaction $F_{3,40} = 3.65$, $p < 0.05$, $\eta^2 = 0.11$), resulting in an overall depletion of the GSH: GSSG ratio (Fig. 3G, treatment $F_{1,40} = 21.32$, $p < 0.0001$, $\eta^2 = 0.32$; time $F_{3,40} = 0.96$, $p > 0.05$; interaction $F_{3,40} = 0.96$, $p > 0.05$). Levels of 3-NT/tyrosine in the piriform cortex were increased at the same time points (Fig. 3H, treatment $F_{1,40} = 34.82$, $p < 0.0001$, $\eta^2 = 0.34$; time $F_{3,40} = 4.44$, $p < 0.01$, $\eta^2 = 0.13$; interaction $F_{3,40} = 4.44$, $p < 0.01$, $\eta^2 = 0.13$). In the frontal cortex, there was no significant difference in oxidative/nitrative stress makers measured 24 h after soman exposure (Fig. 3I-L). Based on these results, the ability of the catalytic antioxidant, AEOL 10150, to reverse soman-induced alterations to oxidative stress, neuroinflammation and neurodegeneration was evaluated 24 h following soman exposure.

3.4. AEOL 10150 treatment attenuates soman-induced oxidative/nitrative markers in brain regions

To determine if treatment with AEOL 10150 could attenuate soman-induced oxidative and nitrative stress, GSH, GSSG and 3-NT were measured in hippocampus and piriform cortex 24 h after soman exposure. To establish the therapeutic window for AEOL 10150, the compound was given 1 min, 5 min, or 15 min after seizure initiation and continuing every 4 h thereafter. No differences were observed between the control group that received vehicle injections and the control group that received AEOL 10150, so these groups were combined to form a single control group. In the hippocampus, treatment with AEOL 10150 did not change levels of GSH (Fig. 4A), whereas levels of GSSG were protected when AEOL 10150 was given at 1 or 5 min (Fig. 4B, $F_{2,16} = 16.37$, $p < 0.0001$, $r^2 = 0.67$ and $F_{2,18} = 25.63$, $p < 0.0001$, $r^2 = 0.74$, respectively), leading to overall protection of the GSH: GSSG ratio when AEOL 10150 was given at 1 min or 5 min after SE initiation (Fig. 4C, $F_{2,16} = 25.48$, $p < 0.0001$, $r^2 = 0.76$; $F_{2,18} = 47.06$, $p < 0.0001$, $r^2 = 0.84$, respectively). Levels of 3NT/tyrosine were protected when AEOL 10150 was given at 1, 5 or 15 min following SE initiation (Fig. 4D, $F_{2,16} = 14.37$, $p < 0.001$, $r^2 = 0.64$; $F_{2,18} = 20.75$, $p < 0.0001$, $r^2 = 0.7$; $F_{2,17} = 50.75$, $p < 0.0001$, $r^2 = 0.86$,

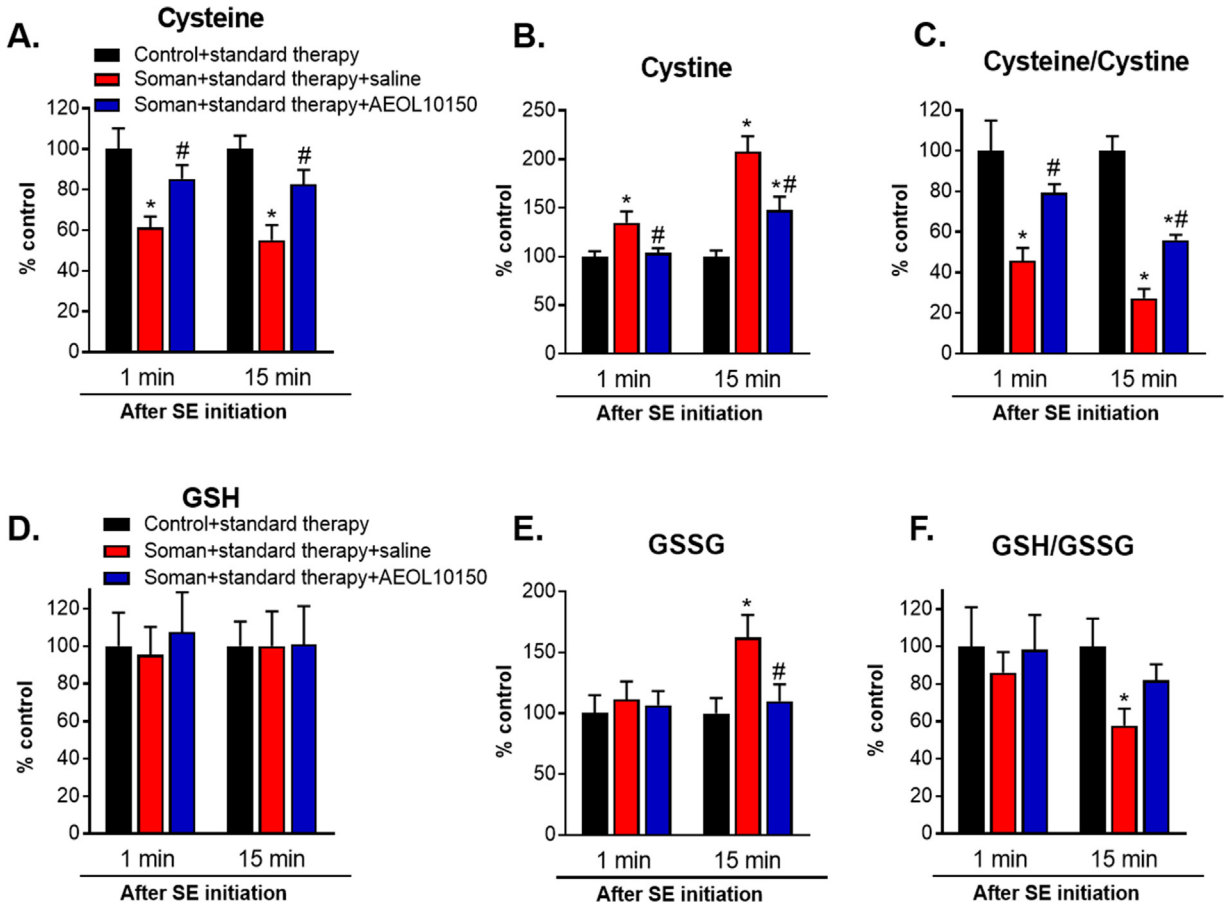

Fig. 5. Plasma oxidative stress makers. The levels of Cysteine (A), Cystine (B), Cys/Cyss (C), GSH (D), GSSG (E), and GSH/GSSG (F) in plasma of rats 24 h after soman-induced SE onset. Treatment with AEOL 10150 (7 mg/kg, s.c.) was initiated 1 min or 15 min following SE onset and continued every 4 h thereafter. Bars represent mean + S.E.M., * $p < 0.01$ vs control, #$p < 0.05$ vs. soman + standard therapy + saline, one-way ANOVA, n = 6–7 per group.

respectively). In the piriform cortex, levels of GSH and GSSG were protected when AEOL 10150 was given 1 and 5 min after SE initiation (Fig. 4E, $F_{2,16} = 16.32$, $p < 0.0001$, $r^2 = 0.67$ and $F_{2,15} = 103.3$, $p < 0.0001$, $r^2 = 0.93$, respectively; Fig. 4F, $F_{2,16} = 9.26$, $p < 0.01$, $r^2 = 054$ and $F_{2,15} = 34.13$, $p < 0.0001$, $r^2 = 0.83$), leading to an overall protection of the GSH: GSSG ratio when treatment was initiated 1 and 5 min after SE initiation (Fig. 4G, $F_{2,16} = 25.15$, $p < 0.0001$, $r^2 = 0.76$ and $F_{2,15} = 80.04$, $p < 0.0001$, $\eta^2 = 0.91$, respectively). Similarly, levels of 3NT/tyrosine were protected when AEOL 10150 was initiated at these same time points (Fig. 4H, $F_{2,16} = 28.48$, $p < 0.0001$, $r^2 = 0.78$ and $F_{2,15} = 52.53$, $p < 0.0001$, $\eta^2 = 0.88$, respectively). Taken together, the data indicate that AEOL 10150 is effective at inhibiting oxidative stress in the soman model. Based on these data, initiation of AEOL 10150 5 min after SE (~ 15 min after soman injection) was chosen as the optimal time to begin treatment with AEOL 10150 to assess its effect on neuroinflammation and neurodegeneration.

3.5. AEOL 10150 treatment attenuates plasma oxidative stress markers

To determine if treatment with AEOL 10150 could attenuate soman-induced oxidative stress markers in plasma, cysteine, cystine, GSH and GSSG were measured 24 h after soman exposure. The soman-induced decrease in plasma cysteine levels was significantly improved when treatment with AEOL10150 was initiated 1 min or 15 min after SE onset (Fig. 5A, $F_{2,15} = 6.31$, $p < 0.05$, $r^2 = 0.46$ and $F_{2,15} = 10.55$, $p < 0.01$, $r^2 = 0.58$, respectively). Levels of its disulfide, cystine were significantly attenuated when treatment with AEOL 10150 was initiated 1 or 15 min after SE onset (Fig. 5B, $F_{2,15} = 6.11$, $p < 0.05$, $r^2 = 0.45$ and $F_{2,15} = 18.42$, $p < 0.0001$, $r^2 = 0.71$, respectively) resulting in an overall attenuation of the soman-induced depletion of the cysteine/cystine ratio when treatment was initiated 1 min or 15 min after SE (Fig. 5C, $F_{2,15} = 8.0$, $p < 0.01$, $r^2 = 0.52$ and $F_{2,15} = 49.21$ $p < 0.0001$, $r^2 = 0.87$, respectively). Plasma levels of GSH were not significantly altered by exposure to soman (Fig. 5D), while the soman-induced increase in plasma levels of GSSG was attenuated when AEOL 10150 was given 15 min after SE onset (Fig. 5E, $F_{2,15} = 4.87$, $p < 0.05$, $r^2 = 0.39$). Overall, exposure to soman did not alter the plasma ratio of GSH: GSSG (Fig. 5E). Taken together, the data suggest that AEOL 10150 is able to attenuate soman-induced oxidative stress up to 15 min after SE (~ 25 min after soman).

3.6. AEOL 10150 treatment attenuates neuroinflammation

To determine if treatment with AEOL 10150 (7 mg/kg starting 5 min after SE onset and every 4 h thereafter) could attenuate neuroinflammation, microglial activation and levels of pro-inflammatory cytokines were measured 24 h after soman exposure. Microglial activation, as assessed by IBA1 staining, was quantified in various brain regions following soman exposure (Fig. 6A). In the hippocampus, treatment with AEOL 10150 significantly attenuated IBA1 staining in the hilus (Fig. 6B, $F_{2,15} = 27.2$, $p < 0.0001$, $r^2 = 0.78$) while the CA3 region was unaffected (Fig. 6C). IBA1 staining was also significantly reduced by AEOL 10150 treatment in the piriform cortex (Fig. 6D, $F_{2,15} = 18.63$, $p < 0.0001$, $r^2 = 0.71$). Similarly, IBA1 staining in the amygdala was significantly reduced by treatment with AEOL10150, indicative of reduced neuroinflammation (Fig. 6E, $F_{2,15} = 69.4$, $p < 0.0001$, $r^2 = 0.90$).

To further probe the role of oxidative stress in soman-induced

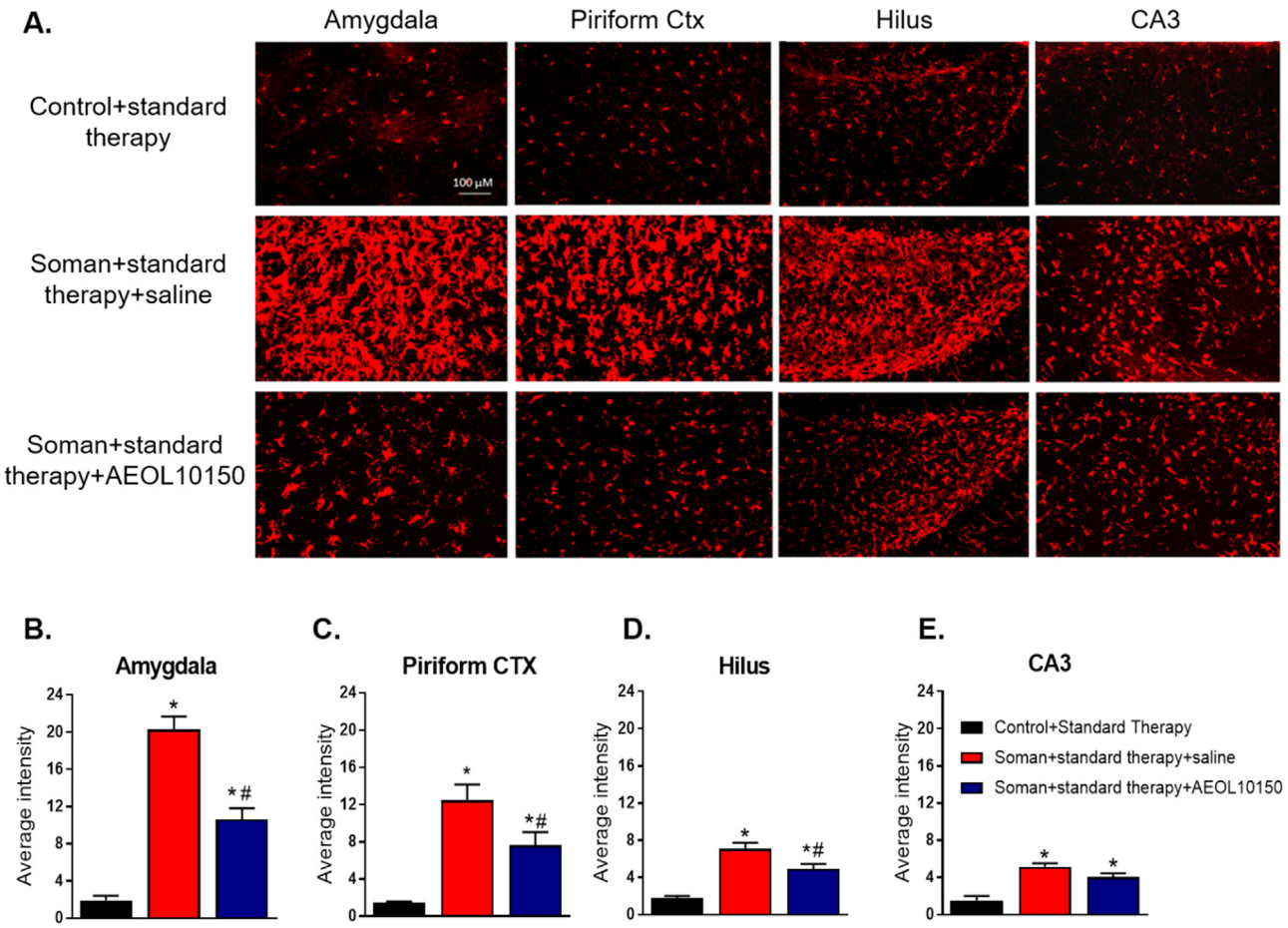

Fig. 6. Microglial activation following soman exposure is attenuated by AEOL 10150. Representative images of IBA1 staining (A) and quantification in amygdala (B), piriform Cortex (C), Hilus (D) and CA3 (E) obtained 24 h after soman exposure. Bars represent mean + S.E.M., *p < 0.01 vs control, #p < 0.05 vs. soman + standard therapy + saline, one-way ANOVA, n = 6–7 per group.

neuroinflammation, the ability of AEOL 10150 to attenuate levels of pro-inflammatory cytokines was assessed by multi-plex assay. In the hippocampus, levels of TNF-α (Fig. 7A) and IL-1β (Fig. 7B) were not affected by AEOL 10150 treatment. However, AEOL 10150 was able to significantly attenuate levels of IL6 (Fig. 7C, $F_{2,15} = 8.4$, $p < 0.01$, $r^2 = 0.52$) and KC/GRO (Fig. 7D, $F_{2,15} = 14.18$, $p < 0.001$, $r^2 = 0.65$). In the piriform cortex, AEOL 10150 treatment significantly attenuated the soman-induced increase in TNF-α (Fig. 7E, $F_{2,15} = 14.18$, $p < 0.001$, $r^2 = 0.65$) and IL-6 (Fig. 7G, $F_{2,15} = 6.8$, $p < 0.01$, $r^2 = 0.48$) while levels of IL-1β (Fig. 7F) and KC/GRO (Fig. 7H) were unaffected by AEOL 10150 treatment. Thus, the data suggest targeting of oxidative stress by treatment with AEOL 10150 is able to attenuate neuroinflammation associated with soman exposure.

3.7. AEOL10150 treatment attenuates neurodegeneration

To assess the ability of AEOL 10150 to attenuate neurodegeneration resulting from soman exposure, Fluoro-Jade B staining of degenerating neurons was quantified in various brain regions 24 h following soman exposure (Fig. 8A). Treatment with AEOL 10150 exerted a neuroprotective effect as evidenced by decreased neuronal FJB staining relative to the soman alone group in the amygdala (Fig. 8B, $F_{2,15} = 48.81$ $p < 0.0001$, $r^2 = 0.87$) and piriform cortex (Fig. 8C, $F_{2,15} = 34.04$, $p < 0.0001$, $r^2 = 0.82$). In the hippocampus, treatment with AEOL 10150 did not affect FJB staining in the hilus (Fig. 8D), however significant protection was observed in the CA1 region (Fig. 8E, $F_{2,15} = 32.34$, $p < 0.0001$, $r^2 = 0.81$) and the CA3 region (Fig. 8F, $F_{2,15} = 37.7$, $p < 0.0001$, $r^2 = 0.83$). Taken together, evidence suggests that treatment with the catalytic antioxidant, AEOL10150, can attenuate neuronal injury associated with soman exposure.

3.8. Discussion

Here, we report that adult male, Sprague Dawley rats exposed to a 1.4xLD_{50} dose of soman exhibit a time- and region-dependent pattern of brain oxidative stress. Similar to our previous studies in surrogate nerve agent models, the small molecular weight, catalytic antioxidant AEOL 10150, demonstrated favorable pharmacokinetics, accessed the blood-brain barrier and inhibited indices of soman-induced oxidative stress in both brain and plasma [6,7]. Inhibition of oxidative stress by AEOL 10150 attenuated soman-induced microgliosis, pro-inflammatory cytokine release and neuronal injury as assessed by FJB staining. Thus, targeting oxidative stress provides a novel therapeutic approach to mitigate acute brain injury resulting from a nerve agent in a post-exposure paradigm. Furthermore, the data presented here together with previous studies demonstrating a neuroprotective effect of AEOL 10150 in pilocarpine and DFP models provide evidence warranting further development of the compound as an adjunct medical countermeasure to prevent deleterious consequences of nerve agent exposure.

We have previously demonstrated that AEOL 10150 accesses the blood-brain barrier and maintains relativly stable levels in brain and plasma of control rats and rats exposed to either pilocarpine or DFP [6,7]. Here, we demonstrate favorable plasma pharmacokinetics of the compound in the soman model, which does not interfere significantly with adjuvants to necessitate dose adjustments between the two groups. This is the first study to our knowledge demonstrating a time- and brain

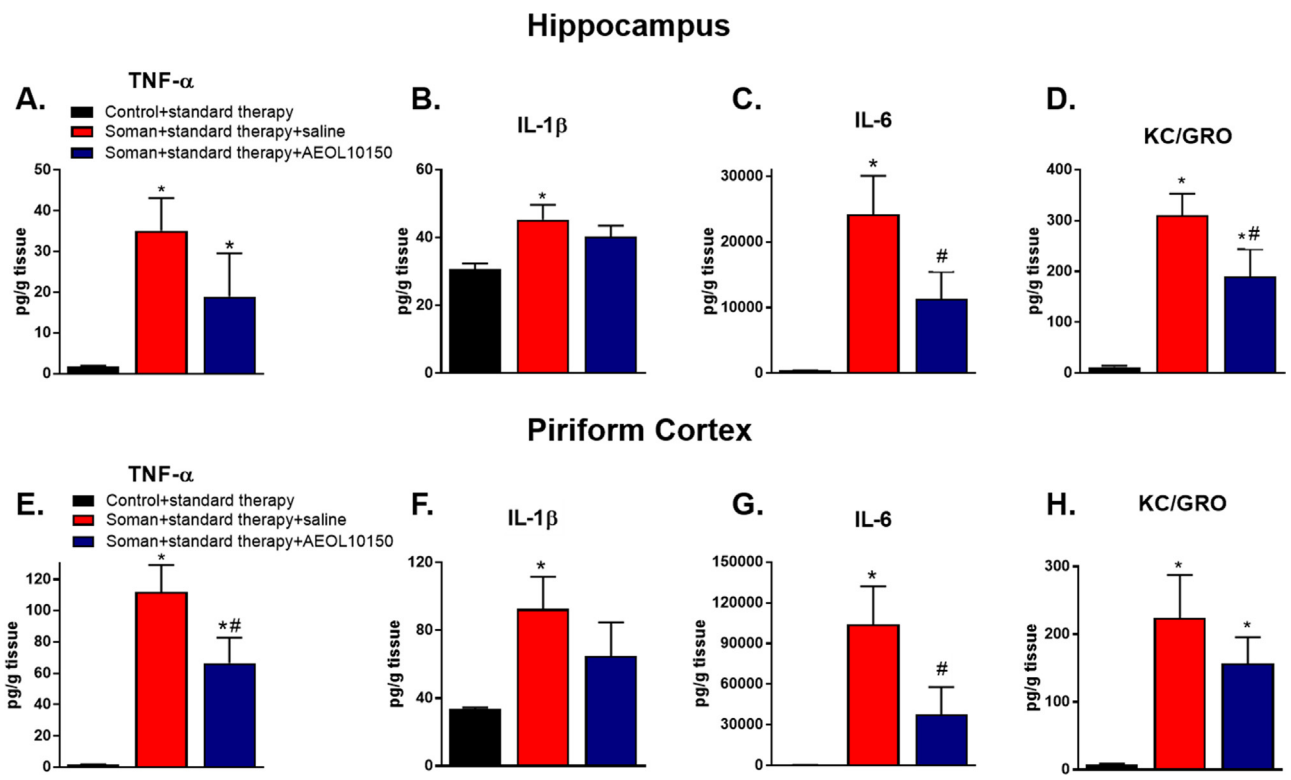

Fig. 7. Soman-induced pro-inflammatory cytokine production is attenuated by AEOL 10150. Hippocampal levels of TNF-α (A), IL-1β (B), IL-6 (C) and KC-GRO (D) and levels of TNF-α (E), IL-1β (F), IL-6 (G) and KC-GRO (H), in the piriform cortex of rats at 24 h after soman. AEOL10150 (7 mg/kg s.c.) treatment was initiated 5 min after SE and continued every 4 h thereafter. Bars represent mean + S.E.M., *p < 0.01 vs. saline + standard therapy, #p < 0.05 vs. soman + standard therapy, One-way ANOVA, n = 6 per group.

region-specific oxidative stress using multiple quantitative biomarkers of oxidative stress i.e. GSH, GSSG, Cys, Cyss and 3NT. Our study demonstrates that oxidative stress is indeed a therapeutic target of nerve agents such as soman as its exposure induced oxidative stress parameters i.e. levels of GSH, GSSG, and ratio of this redox couple (GSH/GSSG) as a measure of redox imbalance and cellular oxidative stress [24]. Additionally, we measured 3NT/tyrosine as a marker of protein nitration resulting from the presence of peroxynitrite [25]. Rats exposed to soman exhibited increased indices of oxidative stress (i.e. lowered GSH, elevated GSSG, lowered GSH:GSSG and elevated 3NT/tyrosine) in a time and brain region-dependent manner. Specifically, in the hippocampus and piriform cortex, markers of oxidative stress were elevated at 24 and 48 h but not 6 or 12 h following soman exposure. This time course of oxidative stress in vulnerable brain regions is identical to what we have previously reported in the DFP model and similar to other reports of decreased GSH levels 24 h following soman but not 1 h [6,26,27]. Conversely and again similar to what we have previously reported in the DFP model, the frontal cortex showed no such evidence of oxidative stress 24 h following soman exposure [6]. It is possible that this data reflects the greater susceptibility of the piriform cortex and hippocampus to soman-induced pathology, particularly in the presence of oximes and benzodiazepines [28,29].

Given the accumulating data for oxidative stress occurring 24 h after soman exposure, we assessed the ability of the broad spectrum antioxidant, AEOL 10150 to attenuate these indices while at the same time, establishing a therapeutic window for the compound to allow for optimal treatment following soman exposure. Treatment with AEOL 10150 was able to attenuate indices of oxidative stress in brain when given 1 and 5 min after SE onset or ~5–15 min following soman injection. Whereas, the highly sensitive plasma oxidative stress markers, cysteine and cystine were inhibited by AEOL 10150 when the compound was delivered 1 or 15 min following SE. Similarly, we have previously demonstrated the ability of AEOL 10150 to inhibit oxidative stress in surrogate nerve agent models such as the pilocarpine and DFP models [6,7]. Importantly, the efficacy of AEOL 10150 against oxidative stress can be attributed to its antioxidant activities as the compound does not affect acetylcholinesterase activity and thereby does not act to reactivate the enzyme [6].

Neuroinflammation and neurodegeneration are key pathological outcomes resulting from exposure to nerve agents and resultant SE [5,30–32]. In general, neuropathology correlates strongly with the occurrence of overt SE and not merely exposure to sub-convulsive doses of nerve agents [32,33]. Neuropathology can be directly linked to late outcomes such as cognitive impairment observed in human exposures which suggests that it is a valid functional outcome of nerve agent toxicity. Under pathological conditions, oxidative stress and neuroinflammation are often inextricably intertwined and underlie neurodegeneration. Identification of a compound that targets these pathological processes in a post-exposure paradigm may provide a unique opportunity to attenuate neurodegeneration and represents a novel therapeutic strategy. In the DFP model, treatment with AEOL 10150 inhibited oxidative stress, attenuated neuroinflammation and protected vulnerable brain regions from neuronal damage [6]. Here, soman exposure resulted in robust microgliosis and pro-inflammatory cytokine release. Treatment with AEOL 10150 following soman exposure significantly attenuated microglial activation in vulnerable brain regions including the hippocampal hilus, amygdala and piriform cortex. Additionally, treatment with the catalytic antioxidant was able to significantly blunt the levels of some but not all pro-inflammatory cytokines in both hippocampus and piriform cortex. Together, the antioxidant and anti-inflammatory activities of the compound likely contributed to its neuroprotective effects. Soman caused the largest increase in microgliosis and neurodegeneration in amygdala followed by piriform cortex and lastly by hippocampal subregions. This pattern of damage is similar to what has been previously reported in the literature [34]. The greatest protection against microgliosis and neurodegeneration exerted by

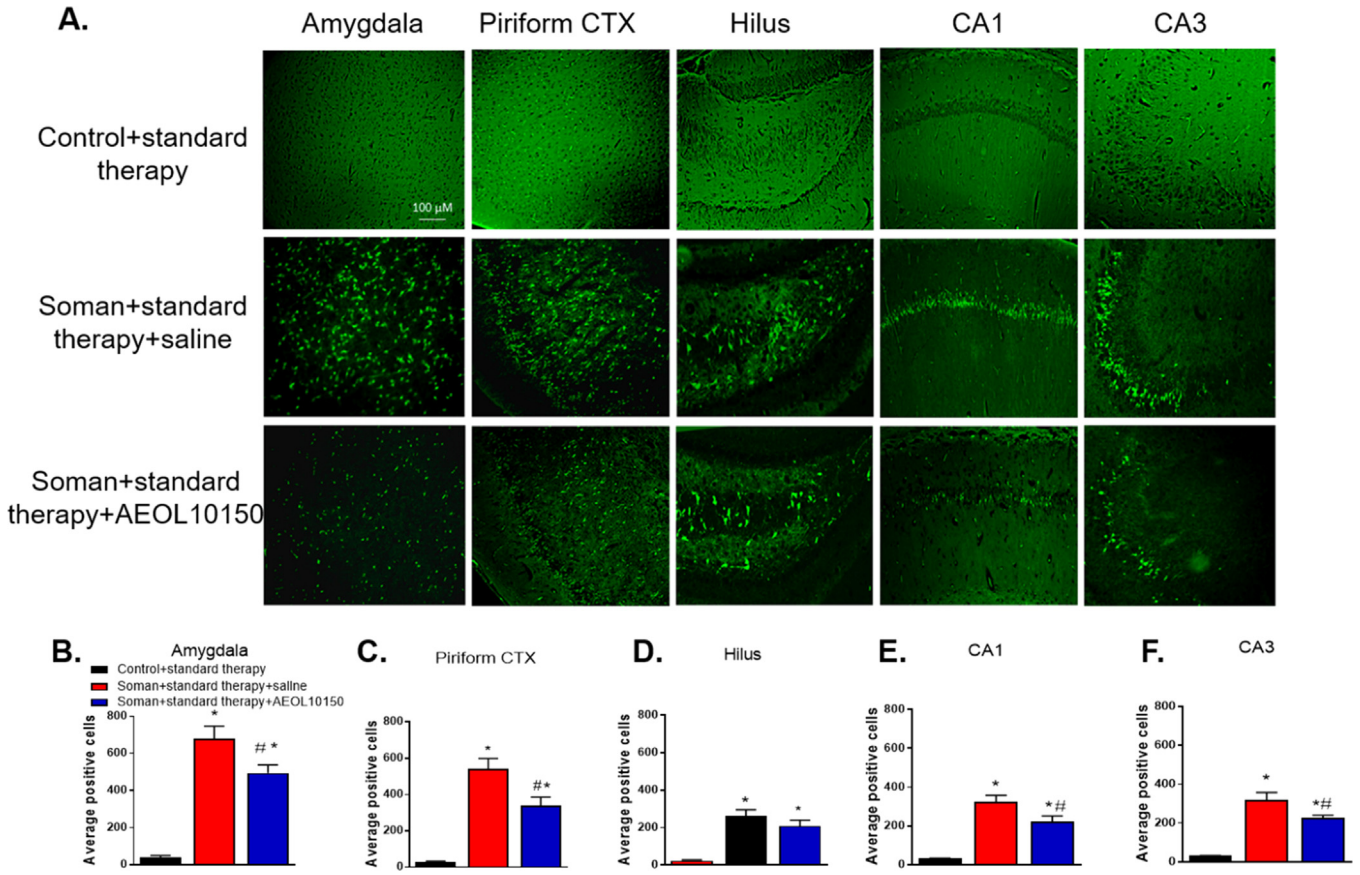

Fig. 8. Treatment with AEOL 10150 attenuates soman-induced neuronal degeneration. Representative FluoroJade B staining images (A) and quantification in the amygdala (B), piriform cortex (C) and hippocampal hilus (D), CA1 (E) and CA3 (F) measured 24 h after soman exposure. AEOL10150 (7 mg/kg) or saline s.c. initiated 5 min after SE initiation and continuing every 4 h thereafter until sacrificed. Bars represent mean + S.E.M., *p < 0.01 vs. saline + standard therapy, #p < 0.05 vs. soman + standard therapy, One-way ANOVA, n = 6 per group.

AEOL 10150 was observed in the areas of greatest soman-induced damage (i.e. amygdala > piriform cortex > hippocampus). With the exception of the amygdala, for which we do not have oxidative stress data, this pattern of damage and protection mirrors what we observed in the oxidative stress markers, with the piriform cortex exhibiting greater levels of oxidative stress markers relative to the hippocampus. Taken together, the data suggest that pharmacological scavenging of reactive species by a catalytic antioxidant can attenuate soman-induced neuroinflammation and neurodegeneration.

In conclusion, the results reported here suggest targeting of oxidative stress in a post-exposure treatment paradigm may be a novel therapeutic avenue to attenuate pathological neurological processes including neuroinflammation and neurodegeneration resulting from soman exposure.

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Conflict of interest statement

During parts of this study, Drs. Day and Patel served as consultants for Aeolus Pharma which developed AEOL 10150 for human use.

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