Novel Catalytic Antioxidant Formulation Decreases Oxidative Stress, Neuroinflammation and Cognitive Dysfunction in a model of Nerve Agent Intoxication

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Running Title:

Oxidative Stress and Nerve Agent Toxicity

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ABSTRACT

Reactive oxygen species have an emerging role in the pathological consequences of status epilepticus (SE). We have previously demonstrated the efficacy of a water-for-injection formulation of the meso-porphyrin catalytic antioxidant, AEOL10150 against oxidative stress, neuroinflammation, and neuronal death initiated by kainic acid, pilocarpine, diisopropylfluorophosphate (DFP) and soman. This previous dose and dosing strategy of AEOL10150 required smaller multiple daily injections, precluding our ability to test its efficacy against delayed consequences of nerve agent exposure such as neurodegeneration and cognitive dysfunction. Therefore, we developed formulations of AEOL10150 designed to deliver a larger dose once daily with improved brain pharmacodynamics. We examined four new formulations of AEOL10150 that resulted in 8 times higher subcutaneous dose with lower acute toxicity, slower absorption, longer half-life, and higher maximal plasma concentrations compared to our previous strategy. AEOL10150 brain levels exhibited improved pharmacodynamics over 24h with all four formulations. We tested a subcutaneous dose of 40 mg/kg AEOL10150 in two formulations (2% CMC and 4% PEG-4000) in the DFP rat model and both formulations exhibited significant protection against DFP-induced oxidative stress. Additionally, and in one formulation (4% PEG-4000), AEOL10150 significantly protected against DFP-induced neuronal death, microglial activation, delayed memory impairment and mortality. These results suggest that reformulation of AEOL10150 can attenuate acute and delayed outcomes of organophosphate neurotoxicity.
Significance Statement:

Reformulation of AEOL10150 allowed higher tolerated doses of the compound with improved pharmacodynamics. Specifically, one new formulation allowed fewer daily doses and improvement in acute and delayed outcomes of organophosphate toxicity.
INTRODUCTION:

Organophosphorus (OP) agents, which include some nerve agents have been one of the most important credible terrorist threat in the United States Homeland Security list due to their availability, potency, and lethality (Jett and Yeung, 2010; Jett et al., 2020). The mechanism of OP nerve agent toxicity is the rapid and irreversible inhibition of acetylcholinesterase (AChE). Excessive acetylcholine (ACh) accumulation in the central nerve system (CNS) induces prolonged seizures that quickly advance to status epilepticus (SE) (Tattersall, 2009). Nerve agents can cause acute lethal SE or lead to delayed neuropsychiatric damage (Figueiredo et al., 2018; Ganie et al., 2022; Jokanovic et al., 2023). Early treatment with anticholinergic drugs (Jett and Yeung, 2010), oximes, AChE reactivators, and anticonvulsants such as midazolam, can be used as the standard therapy for OP exposure (Chen, 2012; Kaur et al., 2014; Pereira et al., 2014; Siso et al., 2017). OPs easily cross the blood–brain barrier (BBB) due to their high lipophilicity. However, because of poor BBB permeability, atropine and oximes do not have a significant therapeutic effect on CNS ACh accumulation and resultant SE (Alozi and Rawas-Qalaji, 2020). Survivors of OP exposure given standard therapy continue to have long-term comorbidities, such as persistent neurological damage that may eventually initiate spontaneous recurring seizures (epileptogenesis), long-lasting cognitive deficits and electroencephalographic abnormalities (Jett, 2007; Jett, 2016; Guignet et al., 2020; Jett et al., 2020; Calsbeek et al., 2021). The United States government through programs such as the National Institutes of Health (NIH) Countermeasures against Chemical Threats (Counter ACT) Program supports the
development of countermeasures against chemical threats such as nerve agents (Chen, 2012; Jett, 2016; Jett et al., 2020).

Oxidative stress, also termed “oxidative distress”, is a redox imbalance with enhanced reactive oxygen and nitrogen reactive species (ROS/RNS) production with disrupted redox circuitry (Sies et al., 2017). ROS/RNS results in damage of vulnerable cellular macromolecules (proteins, lipids, DNA) leading to cellular dysfunction (Kemp et al., 2008; Sies et al., 2017). The brain is especially sensitive to oxidative injury due to various factors that include a higher rate of oxygen consumption based on weight, overabundance of polyunsaturated fatty acids, and autooxidizable neurotransmitters (Patel, 2016). Oxidative stress is both the consequence and cause of SE due to initiation of self-perpetuating mechanisms of oxidant generation (Patel, 2004; Liang et al., 2012; Kalita et al., 2019; Shekh-Ahmad et al., 2019; Guignet et al., 2020). Data from this laboratory and others have consistently demonstrated that targeting ROS/RNS can be a novel countermeasure strategy for neuroprotection against the early consequences of OP-induced SE (Pearson and Patel, 2016; Pauletti et al., 2019; Putra et al., 2020).

AEOL10150, also known as manganese (III) meso-tetrakis (N,N-diethylimidazole) porphyrin, is a broad-spectrum catalytic antioxidant that rapidly catalyzes the removal of various ROS/RNS including hydrogen peroxide, superoxide, hydroxyl radicals, lipid peroxides and peroxynitrite (Day, 2004; Kachadourian et al., 2004)(Figure 1). Several proof-of-concept studies have suggested that AEOL10150 possesses broad efficacy against multiple toxic chemicals and agents, i.e., chlorine gas, sulfur mustard and its analog, 2-chloroethyl ethyl sulfide (CEES) and radiation-induced injury (Rabbani et al., 2007; O'Neill et al., 2010; McGovern et al., 2011; O'Neill et al.,
2011; Zhang et al., 2012; McElroy et al., 2016). AEOL10150 penetrates BBB (Liang et al., 2018) and is safe and well tolerated in human clinical safety studies (Zhang et al., 2018). Our previous data has demonstrated the efficacy of AEOL10150 in a water-for-injection formulation against neurotoxicity initiated by the chemoconvulsant, kainic acid (Rowley et al., 2015), pilocarpine (Pearson et al., 2015; McElroy et al., 2017), DFP (Liang et al., 2018) and soman (Liang et al., 2019). In these model systems, AEOL10150 decreased oxidative stress biomarkers, mitochondrial dysfunction, proinflammatory cytokine release, microglia activation and neuronal damage (Pearson-Smith and Patel, 2020). An issue with the current water-for-injection formulation of AEOL10150 is that it requires six injections daily (i.e. ≤ 5 mg/kg injection/every 4 h) to avoid systemic toxicity and maintain therapeutic levels in the brain which is stressful for the animals and may interfere with behavioral test outcomes. Optimization of the formulation would allow higher doses and less injections/day with better targeting of delayed consequences of nerve agent exposure such as cognitive dysfunction.

The objective of this study was to develop novel formulations of AEOL10150 that were better tolerated and suitable for once or twice-daily dosing, determine the pharmacokinetics and efficacy in DFP-induced ROS/RNS indices, neuronal death, microglia activation, cognitive dysfunction, and mortality.
MATERIAL AND METHODS

Reagents: Diisopropyfluorophosphate (DFP) and all others chemicals, if not specific noted, were purchased from Sigma Aldrich. DFP was stored at -20 ºC and freshly diluted with saline 30 min before use. The pharmaceutical grade AEOL10150 was obtained from Dr. Brian Day.

Animals: Sprague-Dawley rats (male, 300-350 g and ~3 month old) were purchased from Envigo Laboratories (Indianapolis, IN). Prior to any experiment performed, the rats were housed on a 10/14 light/dark cycle with free access to both food (rat chow) and filtered water for one week. All studies were completed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). All behavioral tests were carried out in the Neuroscience Behavioral Core, Animal Facility of University of Colorado Anschutz Medical Campus. Animal studies were designed to minimize pain and distress following NIH regulatory standards of care.

AEOL10150 administration for pharmacokinetic (PK) analysis: Rats received AEOL10150 dissolved in sterile water-for-injection or dissolved following four sterile formulations: 1) an emulsion composed of 4:2:1 of sesame oil: AEOL10150: KolliphorP188. 2) 2g carboxymethyl cellulose sodium (Sigma Aldrich #C5678) dissolved in 100 ml sterile water-for-injection (2% CMC). 3) 3g polyethylene glycol (PEG) 4000 (Sigma Aldrich #25322-68-3, average molecular mass: 3500-4500) dissolved in 100 ml sterile water-for-injection (3% PEG 4000). 4) 4g PEG 4000 dissolved in 100 ml sterile water-for-injection (4% PEG 4000) administrated by subcutaneous (s.c.) route. Animals were monitored and evaluated for clinical signs of drug-related toxicity, such as lethargy and piloerection following AEOL10150 treatment.
carefully. The rats were sacrificed at 1h, 3h, 6h, 12h and 24h after AEOL10150 administration and the brain and plasma samples were collected and stored at -80°C until assay.

**DFP and AEOL10150 administration:** A standard protocol of DFP administration was conducted according to previously methods (Liang et al., 2018). Atropine methyl nitrate (2 mg/kg, i.m) and 2-pam (25 mg/kg, i.m.) were injected 1min post DFP (4.0 mg/kg s.c.) treatment. SE typically starts at 5-10 min after DFP administration and midazolam (2 mg/kg, i.m.) is administered 20 min after SE initiation. The protocol has been validated that it reproduces the pathological and electrographic features in humans and was recommended by the NIH CounterAct program. The treatment with atropine, 2-pam and midazolam has been defined as “standard therapy” for DFP poisoning. Rats were administered AEOL10150 or vehicle 15 min after DFP-induced SE onset. DFP was replaced by saline in control rats. All rats including control groups were treated with atropine, 2-pam and midazolam as “standard therapy” as well. Therefore, the rats were categorized as DFP + vehicle, DFP + AEOL10150 and saline + vehicle or AEOL10150 groups. The rats with DFP treatment were visually monitored during SE and behavioral seizures and evaluated using the Racine scale (Racine, 1972) with a modification (Hellier and Dudek, 2005). The seizure activities were scored on the following scale: P1- motionless staring, occasional “wet-dog shakes”; P2- some head and facial movements, unilateral forelimb clonus, frequent “wet dog shakes”; P3- salivation, continuous bilateral forelimb clonus; P4- generalized limbic seizures with rearing and loss of postural control, P5- jumping, persistent generalized seizures with tonic limbic extension and death. The rats with the appearance of P3 or higher seizures...
scale followed by a period of continuous seizure activity were defined as SE initiation. The oxidative/nitrative indices markers were measured 24 hours post SE. The pathology and behavior test were performed 7 d post SE and rats were treated with AEOL10150 for 5 days daily post SE.

**Determination of AEOL10150 concentrations by HPLC.** AEOL10150 concentrations were measured by a HPLC with a spectrophotometric detector set at 466 nm (LaChrom Elite System; Hitachi) and a YMC-Pack ODS-A column (4.6 × 150 mm, 3 μm) following the method previously described (Kachadorian et al., 2002). Brain samples were sonicated in 0.1 N perchloric acid (20 % w/v, 200 mg/ml) and plasma samples were given the same volume of 0.2 N perchloric acid and mixed well followed by centrifugation at 13,000 g for 10 min 4°C. The resulting AEOL10150 containing supernatant was filtered through a 0.22-μm filter and a 20-µl aliquot was injected into the HPLC. AEOL10150 retention time is approximately 1.45 min and quantitated using a standard curve of 5-2000 pmol (R² = 0.99).

**Determination of oxidative/nitrative indices markers.** Tyrosine, 3-nitrotyrosine (3-NT), reduced glutathione (GSH) and glutathione disulfide (GSSG) were determined with an ESA 5600 CoulArray HPLC (ESA Inc., Chelmsford, MA) furnished with electrochemical detection using a prior method (Liang et al., 2007) with small modifications. Frozen brain samples were sonicated in 0.1 N perchloric acid (10% w/v, 100 mg/ml) and centrifuged at 13,000g for 10 min at 4 °C and a 20-µl aliquot of the supernatant was injected into the HPLC. The potentials of the electrochemical cells were set at 200/300/400/500/600/700/780/840 mV vs. Pd. Mobile phase consisted of 1% methanol and 50 mM NaH₂PO₄, pH 3.0 with a flow rate of 0.6 ml/min and analytes
separation was achieved with YMC ODS-1 column (150 X 4.6 mm 5 µM, Waters Co., Milford, MA). The retention times for GSH, GSSG, tyrosine and 3-NT were ~10.5 min, ~18.5 min, ~20 min and ~42.5 min respectively.

**Neuronal death and microglia activation staining.** Neuropathology analysis was performed at 7 day post DFP-induced SE. A portion of rat brain of ~4 mm in thickness containing amygdala, piriform cortex and hippocampus was fixed with 10% buffered formalin, paraffin-embedded and cut into 15 µm sections coronally. Fluoro-Jade B (FJB; Histo-Chem Inc.) staining, as a neuronal death marker, was performed following a previously described method (Schmued and Hopkins, 2000). Briefly, the sections were incubated with 0.06% potassium permanganate for 10 min after rehydration then immersed for 15 min in staining solution (0.004% FJB in 0.1% acetic acid). The potassium permanganate solution and the FJB staining solution were prepared fresh. To identify microglial activation, sections were immunostained with rabbit anti-ionized calcium adaptor molecule-1 (iba-1, 1:500, Wako, Japan) followed by incubation with goat anti-rabbit conjugated with FITC (1:200, Sigma Aldrich). A Nikon Eclipse TE2000-U microscope used to capture the images and Nikon NIS-Elements Advanced image analysis software (version 4.6) was used to quantify the fluorescent cell number and density in a given area of piriform cortex, hippocampus and amygdala. The average fluorescent positive cells (for FJB staining) or density (for iba-1 staining) within the given area derived from four sections of each animal (n=6 per group) were expressed as average positive cells or density/high power (HP, 10 x axis).

**Behavioral studies:** A standard Y-maze test used to evaluate hippocampal-dependent spatial memory of the rats followed previously described methods (Vorhees
and Williams, 2006; Pearson et al., 2017). The Y-maze apparatus consists of three identical arms placed at 120° on a central triangular platform. A rat was positioned in one end of an arm of the Y-maze from various positions that were randomized across trials. It was permitted to investigate the maze freely for 8 min. Entry into an arm was recorded when the rats moved all 4 paws into the arm. Each rat was given 4 trials per day and a total of 3 days. Total arm entries, their sequence and their latency was recorded and computed by an automated software for behavioral analysis (Topscan, Clever Sys, Inc., Reston, VA). The rats with nature learning and memory remembered the previously visited arm and desired to explore a novel environment. Therefore, a successful alternation was considered if the rats explored the three arms in a consecutive series (i.e. A-B-C, B-C-A or C-A-B), while unsuccessful alternation completion occurred when the rat returned to the arm that most was recently explored (i.e. A-B-A) or last explored (i.e. A-A). The rat with impaired hippocampal-dependent spatial memory cannot remember which arm he just visited and thus shows decreased alternations. Percentage of alternation was calculated as follows: (the number of actual alternations ÷ the maximum possible alternations) × 100. The maximum possible alternations was calculated as the total number of arm entries subtracted by 2.

The Open Field test was a fast and relatively easy test and one of the most commonly used protocols to evaluate locomotor and anxiety-like behavior in animal research (Seibenhener and Wooten, 2015). A previously described protocol was followed (Stanford, 2007). Open field apparatus consists of a square arena mounted within specially designed soundproof plexiglass shells (50 cm x 50 cm). A rat was placed in the center of the open field arena and allowed to move without restraint for 5
min. The total distance traveled and velocity was measured and quantified by an automated tracking system (Topscan, Clever Sys, Inc., Reston, VA).

**Statistical Analysis:** The pharmacokinetic data for AEOL10150 levels was determined using a one-compartment model (first order input and first order output, PK analyst, model #3, MicroMath, Salt Lake City, UT). Prism 9 software (GraphPad Prism, San Diego, CA) was used for all of statistical analyses. All data was expressed as mean ± S.D. One-way or two-way ANOVA with Tukey's multiple comparisons test was selected depending on the study. Statistical significance was set at P values less than 0.05.
RESULTS:

Determination of AEOL10150 pharmacokinetics in new formulations: The pharmacokinetic profiles of AEOL10150 were determined in four new formulations: emulsion (4:2:1 of sesame oil: AEOL10150: KolliphorP188), 2% CMC, 3% PEG 4000 and 4% PEG 4000. The no observable adverse effect dose for the water-for-injection formulation of AEOL10150 in rats was previously established at 5 mg/kg with subcutaneous administration. We have previously verified that a therapeutic level in the brain of ~100 pmol/g tissue (~100 nM) AEOL10150 required a dosing regimen of six doses per day of 5mg/kg for a total of 30mg/kg. This frequency interfered with behavioral testing. Therefore, to test optimization of new formulations of AEOL10150, the injection dose of 30 mg/kg with subcutaneous administration was initially selected for pilot pharmacokinetic testing. However, the levels of AEOL10150 in the brain failed to consistently reach 100 nM during 24 h post administration (data not shown). Consequently, the dose of 40 mg/kg was chosen for pharmacokinetic analysis. Compared to the standard water-for-injection formulation, the four new formulations achieved eight times (40 mg/kg vs. 5 mg/kg) higher dosing in rats without abnormal symptoms or behaviors. All four new formulations slowed the absorption of AEOL10150 from the injection site as evidenced by the shift in the plasma Time to Maximum Concentration (Tmax) from 3 h in the water formulation to ~5 h in the new formulations. The elimination plasma half-lives (T½) of each of the four new formulations were also significantly extended compared with the water-based injection formulation. The increased dose of AEOL10150 with the new formulations resulted in a 3-10 fold increase in the Maximum Concentration (Cmax) compared with the water formulation.
(Table 1). This optimization produced the therapeutic concentration of AEOL10150 in the brain within 3-6h and this remained elevated for more than 24h with AEOL10150 dissolved in 2% CMC, 3% PEG 4000 and 4% PEG 4000, but not in the emulsion formulation (Figure 2). The brain to plasma ratio of the compound was ~0.7% to 2% at 3~6 h and ~40% at 24 h post administration with all four new formulations, which indicated the bioavailability of the drug in brain was much longer than that in plasma.

**Novel formulations of AEOL10150 attenuate DFP-induced ROS/RNS in the brain:** The concentrations of GSH, GSSG and the GSH/GSSG ratio are widely used as major intracellular biomarkers of oxidative stress, particularly in brain (Valko et al., 2007). 3-NT is a biomarker for protein nitration, a posttranslational modification specific to the tyrosine residues by the highly reactive nitrogen species peroxynitrite (ONOO⁻) (Szabo et al., 2007). Due to their favorable pharmacodynamics profiles, AEOL10150 40 mg/kg in 2% CMC and in 4% PEG 4000 were selected for their ability to alter DFP-induced changes in GSH/GSSG and 3-NT levels in the hippocampus and piriform cortex. Rats were administered AEOL10150 (40mg/kg dissolved in sterile 2% CMC or 4% PEG 4000) via s.c. route 15 min after the onset of DFP-induced SE. A subgroup of rats received a second dose of AEOL10150 12h after the onset of SE and were referred to as “DFP + AEOL10150 q12h group” while the group receiving only a single dose is referred to as “DFP + AEOL10150 q24h group”. Some rats in the saline group were also treated with a vehicle or AEOL10150 in the respective vehicle. Rats were sacrificed 24h post-SE for the measurement of ROS/RNS indices. The concentrations of reduced GSH decreased by ~25 to ~30%, the levels of GSSG increased by ~160% to ~180%, and the levels of 3-NT increased by ~225% to ~265% in the hippocampus.
Novel AEOL10150 formulation attenuate neuronal death: To determine if the new AEOL10150 formulations attenuated neuronal death resulting from DFP exposure, FJB staining, a marker of neuronal death (Schmued and Hopkins, 2000) was quantified in four brain regions 7 days following DFP exposure alone or with AEOL10150 40 mg/kg s.c. in 4% PEG 4000 daily treatments for 5 days (Figure 5). FJB positive staining
was minimal in any observed brain areas in rats treated with saline + vehicle or AEOL10150 alone (not shown). The average positive cell number with FJB stain from 4 sections/rat of 6 rats in the DFP + vehicle group was 789.0 ± 51.5; 855.5 ± 78.25; 342.5 ± 42.2; 303.2 ± 37.9 (mean ± S.D.) in the piriform cortex, amygdala, CA3 and CA1 region of the hippocampus 7 days post-SE, respectively. The average FJB positive cells number with AEOL10150 40 mg/kg in 4% PEG-4000 s.c. treatment was significantly reduced by 23.6%, 39.5% 35.4% and 38.6% in these same brain regions (piriform cortex: p=0.0072, amygdala: p=0.0088, CA3: p=0.0478, CA1: p=0.0314) respectively (Figure 5).

**Novel AEOL10150 formulation attenuates microglial activation:** To determine the effect of AEOL10150 on neuroinflammation, microglial activation was assessed 7 days post-SE by Iba1 immunofluorescence. Average Iba-1 fluorescence density was significantly increased by 703%, 594%, 299% and 492% in the piriform cortex, amygdala, hilus and hippocampal CA1 region respectively in the DFP + vehicle group compared with the saline control group (piriform cortex: p=0.0001, amygdala: p=0.0001, hilus: p=0.0001, CA1: p=0.0001). The DFP-induced increases in the average Iba1 immunofluorescence density were significantly attenuated by 41.7%, 40.8%, 24.5% and 33.6% with AEOL10150 40 mg/kg in 4% PEG 4000 s.c. administration compared with the DFP + vehicle group (piriform cortex: p=0.0068, amygdala: p=0.0010, hilus: p=0.0313, CA1: p=0.0160) respectively (Figure 6).

**Novel AEOL10150 formulation inhibits DFP-induced deficits in spatial memory:** To determine the efficacy of the new formulations of AEOL101050 against DFP-induced cognition deficits, an open field (7 day post SE) and Y-Maze test (10 day
post SE) were performed with vehicle (4% PEG 4000) or AEOL10150 in 4% PEG 4000 (40 mg/kg) s.c. daily for 5 days post-SE. Compared with saline controls, DFP-treated rats were deficient in the open field test as indicated by a significantly prolonged time spent in the center of the field (p=0.0167). AEOL10150 administration did not significantly reduce the time DFP-treated rats spent in the center of field (Figure 7A). No differences in overall locomotion or total arm entries were observed among any of the groups in the Y-Maze test (Figure 7B). DFP-treatment produced a 36.6% decrease in Y-maze alternation (p=0.0001) which assesses deficits in hippocampal-dependent spatial memory. Administration of the new AEOL10150 formulation significantly improved Y-maze alternation by 30.4% (p=0.0001) to a level not significantly different from the saline groups (Figure 7C). In fact, the extent of improvement by AEOL10150 in the Y-maze test was roughly equivalent with its ability to attenuate hippocampal neuronal damage.

**Novel AEOL10150 formulation attenuates DFP-induced mortality:** Mortality associated with DFP treatment was observed for 7-day post SE in rats. In this study, 5 of 16 observed rats met euthanasia criteria (2 in the 24h time point, 2 in the 48h time point and 1 in the 72h time point, respectively) post-SE. This resulted in a mortality rate of ~ 31.3% in the DFP + vehicle group. By contrast, no animal deaths were observed in 16 rats administered DFP + AEOL10150 daily for 5 days (Table 2). This result suggests that reformulated of AEOL10150 markedly reduces mortality associated with DFP administration.
DISCUSSION:

In this study, we established the pharmacokinetic profiles of AEOL10150 in several new formulations, including emulsion (4:2:1 of sesame oil: AEOL10150: KolliphorP188), 2% CMC, 3% PEG 4000 and 4% PEG 4000. The standard water-for-injection AEOL10150 formulation required 6 daily injections to attenuate oxidative stress biomarkers in the brain which interfered with behavioral testing. Optimization of the formulation of AEOL10150 decreased the daily injections (1-2 vs 6) and allowed us to better target cognitive defects that are delayed consequences of nerve agent exposure. We have previously validated a therapeutic level of ~100nM AEOL10150 in brain tissues was required to attenuate neuronal damage in a number of SE models including kainate (Rowley et al., 2015), pilocarpine (Pearson et al., 2015; McElroy et al., 2017), DFP (Liang et al., 2018) and soman (Liang et al., 2019). AEOL10150 in 2% CMC, 3% PEG 4000 and 4% PEG 4000 but not with the emulsion allowed the administration of the drug at a much higher dose (40mg/kg), once a day to achieve and maintain therapeutic levels for 24 h in the brain without the previous dose limitation seen in the water-based-injection formulation. At this dose, AEOL10150 in two formulations (2% CMC and 4% PEG-4000) significantly protected against DFP-induced oxidative stress, and in one formulation (4% PEG-4000) significantly protected against DFP-induced neuronal damage, microglial activation, delayed memory impairment and mortality.

Numerous studies including work from our laboratory have reported increased ROS/RNS in multiple brain regions after exposure to nerve agents (Pazdernik et al., 2001; Liang et al., 2018; Vanova et al., 2018; Liang et al., 2019; Guignet et al., 2020; Putra et al., 2020). Optimization of dosing intervals and preparations of AEOL10150
resulted in two options: once daily in 40 mg/kg in 2% CMC or 4% PEG 4000 formulations. Both formulations were sufficient to attenuate DFP-induced ROS/RNS indices in the hippocampus and piriform cortex 24 h post SE when treatment was initiated 15 min after onset of SE. By contrast, the previous water-based-injection formulation of AEOI10150, which necessitated a 4h dosing interval, was effective in attenuating ROS/RNS only when administered 5 min after the onset of DFP-induced SE (Liang et al., 2018). Therefore, optimization of the formulations resulted in a longer post-DFP therapeutic window likely due to delivery of a higher dose that achieved more rapid therapeutic brain levels.

Several groups have shown that DFP administration in rats produces profound neuronal damage using FJB staining in the hippocampus, piriform cortex, amygdala and thalamus but not cerebellum (Li et al., 2011; Liang et al., 2018; Enderlin et al., 2020; Guignet et al., 2020; Spampanato et al., 2020). In this study, we observed neuronal damage in the piriform cortex, amygdala, CA3 and CA1 of hippocampus area of rats 7 days after DFP administration but slightly lower than the magnitude we observed previously 1 day after DFP (Liang et al., 2018). We also assessed microglial activation using Iba1 staining. Microglia, the resident immune cells of the brain, play important roles in physiological conditions and respond to infection and injury in various diseases (Wolf et al., 2017). Microglia are rapidly activated in multiple brain regions after SE, release proinflammatory cytokines (Benson et al., 2015; Flannery et al., 2016), which in turn induce neuroinflammation (Vezzani et al., 2011; Benson et al., 2015; Hiragi et al., 2018). In this study, we observed robust microglial activation in the piriform cortex, amygdala as well as the hilus and CA1 regions of the hippocampus 7 days after DFP-
induced SE, but not 1 or 2 days (data not shown). Both neuronal damage and microglial activation were significantly attenuated by AEOL10150 in the 4% PEG 4000 formulation.

The reformulation of AEOL10150 allowed assessment of cognitive impairment, a critical delayed outcome of nerve agent exposure and to determine if the loss of hippocampal neurons had a functional impact. The Y-maze test is useful in evaluating hippocampal-dependent spatial memory in rodent cognition experiments (Morris, 1984; Vorhees and Williams, 2006), particularly since the hippocampal neuronal damage is a common feature of temporal lobe epilepsy that contributes to the associated cognitive dysfunction (Pitkanen et al., 2002; Marissal, 2021). Our study revealed significant impairment of hippocampal-dependent spatial memory 10 days after DFP exposure compared to controls and marked protection by AEOL10150 in 4% PEG 4000 formulation. In addition, AEOL10150 formulation inhibited DFP-induced mortality, consistent with previous results in the pilocarpine model (Pearson-Smith et al., 2017). In summary, the new AEOL10150 formulation allows a once-daily dosing protocol for 5 days decreased delayed and long-term neurologic comorbidity of OP intoxication.

Oxidative stress has been shown to be a pathogenic factor in seizure-induced neuronal death (Pearson-Smith and Patel, 2017; Shekh-Ahmad et al., 2019). Excessive oxidative stress produced post-SE results in oxidative damage to proteins, DNA and lipids as well as excitotoxicity (Reynolds and Hastings, 1995; Patel et al., 1996) in CNS. Neuroinflammation occurs in response to SE and has been shown to play a pathogenic role in the development of epilepsy (Vezzani, 2014; Vezzani et al., 2019). Significant cross-talk occurs between oxidative stress and neuroinflammation (Fabisiak and Patel,
2022). Studies have shown that antioxidant therapy can attenuate markers of neuroinflammation such as pro-inflammatory cytokine production and microglial activation following SE (McElroy et al., 2017; Liang et al., 2018; Liang et al., 2019; Pauletii et al., 2019; Putra et al., 2020). Here we have shown that reformulation of AEOL10150 resulted in therapeutic levels sufficient to exert neuroprotection and attenuation of oxidative stress and inflammatory biomarkers in the brain.

In summary, the new formulation of AEOL10150 allowed higher doses of the drug with improved pharmacodynamics. This dosing paradigm with one or more formulations was sufficient to inhibit DFP-induced oxidative stress, neuroinflammation, neuronal damage, mortality and cognitive impairment. Therapeutic targeting of secondary and delayed effects of nerve agent toxicity is much needed as a countermeasure effort. Given its broad-spectrum effects against multiple threat agents, AEOL10150 could be used without prior knowledge regarding the nature of the toxicant. It may be used immediately at the site of exposure or in the hospital setting as it targets delayed effects from OP exposure.

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Data Availability Statement:
"The authors declare that all the data supporting the findings of this study are contained within the paper”.

Authorship Contributions:
Participated in research design: Patel, Day, Liang and Pearson-Smith
Conducted experiments: Liang and Pearson-Smith
Performed data analysis: Day, Liang and Pearson-Smith
Wrote or contributed to the writing of the manuscript: Patel, Day and Liang

REFERENCES:


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**Conflict-of-interest statement:**

No author has an actual or perceived conflict of interest with the contents of this article.
Table 1

AEOL10150 Plasma pharmacokinetics analysis*

<table>
<thead>
<tr>
<th>PK Parameters</th>
<th>Water formulation (n=3)</th>
<th>Emulsion formulation (n=3)</th>
<th>2% CMC Formulation (n=3)</th>
<th>3% PEG-4000 formulation (n=3)</th>
<th>4% PEG-4000 formulation (n=3)</th>
</tr>
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<tbody>
<tr>
<td>Elim half-life (h)</td>
<td>2.3 ± 0.12</td>
<td>3.5 ± 0.29</td>
<td>4.2 ± 0.17</td>
<td>4.3 ± 0.29</td>
<td>4.0 ± 0.12</td>
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<td>Tmax (h)</td>
<td>3.0 ± 0.12</td>
<td>5.1 ± 0.40</td>
<td>5.0 ± 0.23</td>
<td>4.1 ± 0.35</td>
<td>5.8 ± 0.12</td>
</tr>
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<td>Cmax (ng/ml)</td>
<td>1960 ± 35</td>
<td>5430 ± 1010</td>
<td>17,506 ± 916</td>
<td>10,065 ± 320</td>
<td>18,567 ± 655</td>
</tr>
<tr>
<td>AUC (ng x h/ml)</td>
<td>15,270 ± 595</td>
<td>77,490 ± 17,673</td>
<td>246,418 ± 21,610</td>
<td>123,126 ± 4830</td>
<td>289,833 ± 9,428</td>
</tr>
</tbody>
</table>

* AEOL10150 5mg/kg in water for injection vs. 40mg/kg in emulsion, 2 % CMC, 3 % PEG 4000 or 4% PEG 4000 formulations with subcutaneous administration.

Data are means + S.E.M.

Table 2

The Mortality by DFP alone or with AEOL10150 Treatment

<table>
<thead>
<tr>
<th>Mortality DFP+ vehicle</th>
<th>Mortality DFP+ AEOL10150</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 dead of 16 treated (31.3%)</td>
<td>0 dead of 16 treated (0%)</td>
</tr>
</tbody>
</table>

Mortality in rats 7 days post-DFP SE. Vehicle or AEOL10150 40 mg/kg (in 4% PEG 4000) s.c. 15 min post SE onset and 24h thereafter for 5 days.
Figure 1. Structure of AEOL 10150. AEOL 10150 (manganese (III) meso-tetrakis (N-N-diethylimidazole) porphyrin). Molecular Weight: 1175. AEOL10150 is a catalytic antioxidant with broad-spectrum antioxidant activities to be designed for strong and fast scavenger against hydrogen peroxide, superoxide, lipid peroxide, and peroxynitrite.

Figure 2. Concentrations of AEOL10150 in the plasma (A) and brain (B) at 1, 3, 6, 12 and 24h after different formulations delivered, including water-for-injection(5mg/kg), emulsion (composed of 4:2:1 of sesame oil: AEOL10150: Kolliphor P188), 2% CMC, 3% PEG 4000 and 4% PEG 4000 (40 mg/kg) with subcutaneous administration. Points represent mean + S.D., n=3-6 rats.

Figure 3. The concentrations of reduced glutathione (GSH), glutathione disulfide (GSSG), GSH/GSSG ratio and 3-nitrotyrosine (3-NT) in the hippocampus and piriform cortex of rats 24h after DFP administration. AEOL10150 (40mg/kg in 2% CMC, s.c.) or vehicle was administered 15 min post DFP-induced SE initiation. AEOL10150 was injected again 12h after SE onset in a subgroup of rats designated as “DFP + AEOL10150 q12h”; whereas the group receiving a single dose is designated as “DFP + AEOL10150 q24h”. Bars represent mean + S.D., *p<0.01 vs. saline + vehicle, # p<0.05 vs. DFP + vehicle, one-way, ANOVA with Tukey’s multiple comparisons test, n= 6 per group.

Figure 4. The concentrations of reduced glutathione (GSH), glutathione disulfide (GSSG), GSH/GSSG ratio and 3-nitrotyrosine (3-NT) in the hippocampus and piriform cortex of rats 24h after DFP administration. AEOL10150 (40mg/kg in 4% PEG 4000, s.c.) or vehicle was administered 15 min post DFP-induced SE initiation. AEOL10150 was injected again 12h after SE onset in a subgroup of rats designated as “DFP +
AEOL10150 q12h”; whereas the group receiving a single dose is designated as “DFP + AEOL10150 q24h”. Bars represent mean + S.D., *p<0.01 vs. saline + vehicle, # p<0.05 vs. DFP + vehicle, one-way, ANOVA with Tukey's multiple comparisons test, n= 6 per group.

Figure 5. Representative images of neuronal cell death assessed by Fluoro-Jade B staining of piriform cortex, amygdala, CA3 and CA1 areas of hippocampus in rats 7 days after administration of DFP with vehicle or AEOL10150 40mg/kg in 4% PEG 4000 s.c. daily injections for 5 days post DFP. (A) DFP + vehicle, (B) DFP + AEOL10150, (C) Quantification data of average Fluoro-Jade B staining positive cell number (neuronal death)/HP (high power, 10 × axis). Bars represent mean + S.D., *p<0.05, **p < 0.01 vs. DFP + vehicle, multiple t-test, Two-tailed, n=6-7 per group.

Figure 6. Representative images of ionized calcium adaptor molecule 1 (Iba-1) immunostained microglia activation in the piriform cortex, amygdala, hilus and CA1 area of hippocampus of rats 7 day after DFP injection with vehicle or AEOL10150 40mg/kg in 4% PEG 4000 s.c. daily treatment for 5 days post DFP. (A) Saline + vehicle, (B) Saline + AEOL 10150, (C) DFP + vehicle, (D) DFP + AEOL10150, (E) Quantification data of Iba1 staining (microglia activation) expressed as average density/HP (high power, 10 × axis). Bars represent mean + S.D., **p<0.01 vs. saline + vehicle and saline + AEOL10150, # p<0.05 vs. DFP + vehicle, Two-way, ANOVA with Tukey's multiple comparisons test, n=3-7 per group.

Figure 7. Behavioral testing evaluated 7 days (open field) and 10 day (Y-Maze) after DFP. (A) Duration in the center of the open field test. (B) Total number of arm entries in the Y-Maze. (C) Percent alternation in the Y-Maze. Vehicle or AEOL10150 in 4% PEG
4000 40 mg/kg, s.c. daily for 5 days treatment post DFP. Bars represent mean + S.D.,
*p<0.01 vs. saline + vehicle and saline + AEOL10150, #p<0.05 vs. DFP + vehicle, two-
way, ANOVA with Tukey's multiple comparisons test, n=5-8 per group.
Figure 1

Superoxide $k = 6.78 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
Hydrogen peroxide $k = 2.2 \text{ min}^{-1}$
Lipid peroxide $F_2-IP IC_{50} = 0.1 \mu \text{M}$
Peroxynitrite $k = 1.01 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
Figure 2

A. Plasma
- Water-for-injection
- Emulsion
- 2% CMC

B. Brain
- 3% PEG 4000
- 4% PEG 4000

Time (h)

μM

pmol/g tissue (nM)
Figure 4

**Hippocampus**

A

**GSH**
- Saline + Vehicle

**GSSG**

**GSH/GSSG**

**3-NT**

B

**Piriform Cortex**

**GSH**
- Saline + Vehicle

**GSSG**

**GSH/GSSG**

**3-NT**

*Significant difference compared to control group.

*Significant difference compared to DFP + Vehicle group.

*Significant difference compared to DFP + AEOL10150 q12h group.
Figure 5

(A) Neuronal cell death in the Piriform Cortex, Amygdala, CA3, and CA1 regions.

(B) Comparison of neuronal cell death in DFP + Vehicle and DFP + AEOL10150 conditions.

(C) Graph showing the number of FJB positive cells per HP in each region, with statistical significance marked by asterisks.

Legend:
- ● DFP + Vehicle
- □ DFP + AEOL10150

Statistical significance:
- * p < 0.05
- ** p < 0.01