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Treatment with the catalytic metalloporphyrin AEOL 10150 reduces inflammation and oxidative stress due to inhalation of sulfur mustard analog 2-chloroethyl ethyl sulfide (CEES)

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Abstract

Sulfur mustard (bis-2-(chloroethyl) sulfide, SM) is a highly reactive vesicating and alkylating chemical warfare agent. A SM analog, 2-chloroethyl ethyl sulfide (CEES) has been utilized to elucidate mechanisms of toxicity and as a screen for therapeutics. Previous studies with SM and CEES have demonstrated a role for oxidative stress as well as decreased injury with antioxidant treatment. We tested whether post-treatment with the metalloporphyrin catalytic antioxidant AEOL 10150 would improve outcome in CEES-induced lung injury. Anesthetized rats inhaled 5% CEES for 15 minutes via a nose-only inhalation system. At one and nine hours following CEES exposure, rats were given AEOL 10150 (5 mg/kg, SC). At 18 hours post exposure BALF lactate dehydrogenase activity, protein, IgM, red blood cells and neutrophils were elevated following CEES exposure, and decreased by AEOL 10150 treatment. Lung myeloperoxidase activity was increased after CEES inhalation and was ameliorated by AEOL 10150. Lung oxidative stress markers 8-OHdG and 4-HNE were elevated after CEES exposure and significantly decreased by AEOL 10150 treatment. These findings demonstrate that CEES inhalation increased lung injury, inflammation, and oxidative stress, and AEOL 10150 was an effective rescue agent. Further investigation utilizing catalytic antioxidants as treatment for SM inhalation injury is warranted.

Introduction

Sulfur mustard (2, 2'-dichloro diethyl sulfide, mustard gas, SM) has been used as a chemical weapon throughout the 20th century from its initial use in World War I to more recent uses in the Iran-Iraq War and Iraqi-Kurdish conflicts of the late 1980s. SM continues to be a threat to both civilian and military populations due to its ease of synthesis and large worldwide stockpiles. SM is a potent vesicating and alkylating agent that exerts toxic effects on the skin, eyes, and respiratory tract [1,2]. Respiratory symptoms following SM exposure include sneezing, coughing, and increased mucus discharge with a latency of several hours [2,3].

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Respiratory tract injury results in inflammation, edema, pseudomembrane formation, as well as apoptosis and necrosis of airway epithelium [1]. While external injury can be treated by decontaminating with dilute bleach or soap and water solutions, internal injury is not as readily managed by decontamination. Supportive care is currently the only option for inhalation injury. Thus, it is crucial to elucidate therapeutics capable of minimizing lung damage.

SM is a bifunctional alkylating agent, whereas 2-chloroethyl ethyl sulfide (CEES, half mustard) is a monofunctional analog of SM, lacking one of two terminal chlorine molecules. CEES is commonly utilized to examine mechanisms of SM injury as well as to screen therapeutics. Both compounds readily alkylate DNA, proteins, and nucleic acids. Loss of glutathione (GSH) as a result of SM/CEES alkylation has been reported and can contribute to oxidative stress [4,5]. Pretreatment with GSH, NAC, or NAC in combination with mixed tocopherols has been shown to improve outcomes with CEES-induced inhalation damage in laboratory animals [6–8]. Treatment with superoxide dismutase (SOD) or catalase has also proven beneficial in CEES-induced lung injury [6,7]. These data support a role for oxidative stress in CEES injury.

Catalytic metalloporphyrins are a novel class of small molecular weight antioxidants. One such compound is Mn(III) tetrakis (*N,N'*-diethylimidazolium-2-yl) porphyrin (AEOL 10150), which possesses high SOD activity as well as catalase-like activity [9]. AEOL 10150 also has the capacity to scavenge peroxynitrite and lipid peroxides [9–14]. Recently, an *in vitro* model of CEES injury in lung epithelial cell lines and primary cells demonstrated AEOL 10150 efficacy in reducing cytotoxicity and mitochondrial dysfunction when given 1 hour following CEES exposure [15]. Catalytic metalloporphyrin antioxidants also have shown efficacy in other *in vivo* models of lung injury in which oxidative stress has been implicated, including bleomycin-induced lung fibrosis, radiation-induced lung injury, and in hemorrhage-induced lung injury ('shock lung') [16–18]. Therefore, we examined whether AEOL 10150 would be beneficial in an *in vivo* model of inhaled CEES-induced acute lung injury.

The focus of these studies was to a) characterize lung injury, inflammation, and oxidative stress following inhalation of CEES; and b) to determine whether the catalytic antioxidant AEOL 10150 improved outcomes when given as a rescue treatment.

Methods

Reagents

2-chloroethyl ethyl sulfide was obtained from TCI America (Portland, OR). AEOL 10150 was generously supplied by Aeolus Pharmaceuticals (Laguna Niguel, CA). All other chemicals, of the highest grade available, were obtained from Sigma (St Louis, MO) unless otherwise specified.

Animals—Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 275–350 g were used. Animals were provided with food and water *ad libitum*. All procedures employed were approved by the Animal Care and Use Committee at National Jewish Health. Animals were randomly assigned to one of four groups: control (ethanol-exposed, PBS-treated), 10150 (ethanol-exposed, AEOL 10150-treated), CEES (5% CEES (in ethanol)-exposed, PBS treated), or CEES + 10150 (5% CEES (in ethanol)-exposed, AEOL 10150-treated).

Pulse Oximetry

The MouseOx pulse oximeter (Starr Life Sciences, Oakmont PA), with a rat infrared sensor collar clip, was used to measure arterial hemoglobin oxygen saturation rats before CEES inhalation and at 18 hours post inhalation, immediately prior to euthanasia. Rats were shaved around the neck to expose the skin, and the sensor of the pulse oximeter collar clip was placed

over the carotid artery on either side. Rats were allowed to acclimate to the collar briefly. A total of 5 readings using MouseOx Version 5.1 software were obtained (Starr Life Sciences, Oakmont PA).

CEES Exposure

Rats were anesthetized using a cocktail of ketamine (75 mg/kg), xylazine (7.5 mg/kg) and acepromazine (1.5 mg/kg). Animals were loaded into polycarbonate tubes and placed in a Jaeger nose-only inhalation system (CH Technologies, NJ). Compressed air was delivered at a rate of 12 L/min to 12 ports during loading. For exposure, dilution air was decreased to 6 L/min and aerosolized air added at 6 L/min. A syringe pump (Razel Scientific, St. Albans, VT) connected to the bioaerosol nebulizing generator (BANG) delivered 5% CEES in ethanol at a rate of 12.7cc/hour. Delivery to each port was 13.9 mg over the course of 15 minutes at a concentration of 5% CEES. Animals were exposed for 15 minutes, removed from the system, and allowed to recover in their cage.

Treatment protocol

Rats were randomly assigned to control (ethanol inhalation with PBS treatment), 10150 (ethanol inhalation with 10150 treatment), CEES (CEES inhalation with PBS treatment), or CEES+10150 (CEES inhalation with 10150 treatment). The one dose treatment protocol involved injection of AEOL 10150 (5 mg/kg, SC) or PBS vehicle (1 ml/kg) at one or nine hours after inhalation exposure with 6 animals per group. The two dose protocol animals received injections at both 1 and 9 hours after inhalation (6 animals each in control and 10150 groups, 16 animals each in CEES and CEES+1-15- groups).

Tissue collection

Eighteen hours after exposure, animals were given a lethal dose of sodium pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA). Rats were intubated and two 5ml aliquots each phosphate-buffered saline were gently instilled into the lungs and subsequently aspirated to obtain bronchoalveolar lavage fluid (BALF). Blood was collected by cardiac puncture. Lungs were perfused through the pulmonary artery at a flow rate of 10 ml/min. Following excision from the chest, the lungs were snap-frozen in liquid nitrogen.

Fixed tissue collection

For fixation studies, rats were intubated and fixed intratracheally using 4% paraformaldehyde in phosphate buffered saline for 30 minutes using 20 cm of pressure. Lungs were removed and fixed in 4% paraformaldehyde until dissection.

BALF Differential cell counts

BALF was pooled and centrifuged at $1800 \times g$ for 10 minutes. Supernatant fluid was removed for further biochemical analysis. The pellet was resuspended in 2 ml PBS. The cell pellet was resuspended in 2 ml PBS and utilized for hemacytometer counts. Aliquots were centrifuged in a Cytospin (Shandon Scientific), stained using a modified Wright-Giemsa stain (Protocol Hema 3, Fisher Scientific, Fair Lawn, NJ, USA) and utilized for differential cell counts. The percentages of various cell types were determined by counting 200 cells in a minimum of 3 random fields.

BALF Lactate Dehydrogenase (LDH) Activity Assay

Integrity of the lung epithelium and inflammatory cells within the airways were assessed by measuring release of lactate dehydrogenase (LDH) as previously described [19]. Briefly, to a 96-well plate, 30 μ l of BALF was added to 120 μ l of a 0.24 mM NADH solution prepared in

Tris-NaCl buffer (81 mM Tris, 203 mM NaCl, pH 7.2). The reaction was then initiated by the addition of 20 μ l of a 9.8mM sodium pyruvate solution, also prepared in Tris-NaCl. LDH activity was determined kinetically by measuring NADH consumption at 340 nm using a Spectramax340PC (Microdevices, Sunnydale, CA) over 5 minutes at 30° C, and activity was calculated using an extinction coefficient of 6.2 mM⁻¹.

BALF Protein Concentration

BALF was utilized to measure protein levels with the bicinchoninic acid assay (BCA) (Thermo Scientific, Rockford, IL). Absorbance was measured at 562 nm and protein levels were determined from a standard curve obtained using bovine serum albumin.

BALF IgM concentration

BALF IgM concentrations were measured using a standard ELISA protocol (Bethyl Laboratories, Inc., Montgomery, TX). Briefly, MaxiSorp 96-well plates (Nunc, Rochester, NY) were coated with goat anti-rat IgM, blocked with 50 mM Tris, 14 mM NaCl, pH 8.0 with 1% BSA, incubated with rat IgM standards (1000 ng/ml-15.6 ng/ml) or sample BALF, diluted 1:2. IgM was detected using an HRP-conjugated secondary antibody and a tetramethyl benzidine (TMB) (KPL, Gaithersburg, MD). Absorbance was measured at 450 nm. Concentrations were determined from a rat IgM standard curve.

Myeloperoxidase (MPO) Activity Assay

Snap-frozen lung tissue was homogenized (50 mg/ml buffer) in 0.5% hexadecyltrimethylammonium bromide (HTAB) in 80 mM phosphate buffer on ice. Homogenates were centrifuged at 20,000 $\times g$ for 15 minutes, and the supernatant transferred to a new microcentrifuge tube. Centrifugation was repeated until homogenates were clear. The 1 ml reaction cuvette contained 80 mM phosphate buffer, pH 5.4, 0.3 mM H₂O₂, and 1.6 mM tetramethylbenzidine (TMB). The reaction was followed for 3 minutes at 652 nm using a Beckman DU-64 spectrophotometer (Beckman Coulter, Fullerton, CA); an extinction coefficient of 3.9×10^4 M⁻¹ cm⁻¹ was used to calculate the rate in mU activity and normalized to mg of protein using the BCA protein assay (Thermo Scientific, Rockford, IL). To determine the contribution of MPO only, 1 mM reaction cuvettes were incubated with 100 μ M 4,4'-diaminodiphenyl sulfone (dapsone) for 5 minutes prior to adding TMB and H₂O₂. At pH 5.4, dapsone inhibits other peroxidases such as lactoperoxidase (LPO), but not MPO [20].

Lung 8-Hydroxy-2-deoxyguanosine (8-OHdG) Assay

Total DNA from snap-frozen lung was extracted using the Qiagen DNeasy blood and tissue kit per manufacturer's instructions (No. 69504, Qiagen, Valencia, CA). A Nanodrop 1000 spectrophotometer (Thermo Fisher, Pittsburgh, PA) was utilized to measure DNA purity. DNA (6 μ g, purified) was incubated with 4 units of Nuclease P1 (US Biological #N7000, Swampscott, MA) at 60°C for 20 minutes. Samples were then incubated with 4 units of alkaline phosphatase (Sigma, St. Louis, MO) at 37°C for 60 minutes. 8-hydroxy-2-deoxyguanosine (8-OHdG) and 2-deoxyguanosine (2-dG) were analyzed by HPLC coupled with UV and electrochemical detection (CoulArray model 5600; ESA Inc., Chelmsford, MA) respectively. Mobile phase A contained 50 mM sodium acetate, pH 4.0, and mobile phase B consisted of 50 mM sodium acetate with acetonitrile 85:15 (v:v), pH 4.2, with a flow rate of 1 mL/min with a gradient of 100% A for 5 min; 60% A, 40% B for 12 min; 20% A, 80% B for 5 min; and 100% A for 8 min. A C18 reverse phase column was used for analysis (4.6 $\times$ 250 mm, Tosoh Bioscience #K3121, Montgomeryville, PA) with detection of 2-dG by UV at 260 nm and 8-OHdG using electrode potentials of 140, 200, 260, and 320 mV. The retention times for 2-dG and 8-OHdG were 13.0 and 14.1 min, respectively. Concentrations were determined from a

standard curve containing increasing concentrations of 8-OHdG and 2-dG and expressed as a ratio of 8-OHdG/10⁵ 2-dG.

Lung 4-hydroxynonenal (4-HNE) Assay

4-HNE assay was modified from previously reported methods [21,22]. All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Sample preparation was performed using glassware whenever possible. Frozen tissues, or a known amount of 4-HNE standard (Cayman Chemical, Ann Arbor, MI), were placed in 2ml of cold methanol (Thermo Fisher, Waltham, MA) containing 50 µg/ml butylated hydroxytoluene (BHT), with 10 ng d₃-4-HNE (Cayman Chemical, Ann Arbor, MI) internal standard added just prior to homogenization with the Ultra-Turrax T25 (Thermo Fisher, Waltham, MA). An EDTA solution (1 ml of 0.2 M, pH 7) was added. Derivatization was accomplished by the addition of 0.2 ml of 0.1 M HEPES containing 50 mM *O*-(2,3,4,5,6-pentafluoro-benzyl)hydroxylamine hydrochloride (PFBHA-HCl), pH 6.5. The mixture was then vortexed and held at room temperature. After 5 min, 1 ml of hexanes (Thermo Fisher, Waltham, MA) was added, and the samples were shaken vigorously. Brief centrifugation was performed to achieve phase separation and the *O*-pentafluorobenzyl-oxime (PFB-oxime) derivatives were extracted from the upper hexane layer and transferred to a separate tube. This step was repeated with additional 1 ml hexanes and combined. The pooled sample was then dried under a stream of N₂ gas and further derivatized into trimethylsilyl ethers by the addition of 15 µl each of pyridine and *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA). The samples were vortexed and heated to 80 °C for 5 min, then transferred to autosampler vials and analyzed for 4-HNE content by GC/MS.

GC/MS analysis was performed using a Focus GC coupled to a DSQ II mass spectrometer and an AS 3000 autosampler (Thermo Fisher, Waltham, MA). A 15 m TR-5MS column (0.25 mm i.d., 0.25 µm film thickness; Thermo Fisher, Waltham, MA) was used with ultra high purity helium as the carrier gas at a constant flow rate of 1.0 ml/min. 2 µl of sample was injected into the 270°C inlet using split mode with an injection ratio of 10 and a split flow of 10 ml/min. The initial oven temperature was 100°C then ramped to 200°C at 15°C/min, followed by an increase in temperature to 300°C at 30°C/min and held for 1 min. The MS transfer line temperature was held constant at 250°C and the quadrupole at 180°C. Analysis was done by negative ion chemical ionization using 2.5 ml/min of methane reagent gas. Ions were detected using SIM mode with a dwell time of 15.0 ms for each fragment of 4-HNE at *m/z* 152, 283, and 303; and d₃-4HNE at *m/z* 153, 286 and 306. Under these conditions, the larger, second peak of the two 4-HNE isomers was used for quantitation, and exhibited a retention time of 7.18 min, which was just preceded by the elution of d₃-4-HNE at 7.17 min. Quantitation was performed using a standard curve generated by graphing the area ratio of 4-HNE to d₃-4-HNE versus concentration.

AEOL 10150 Pharmacokinetics

A total of 15 rats were administered AEOL10150 (SC, 5 mg/kg) at time 0. At 1, 2, 4, 6, and 8 hours post injection, 3 rats per time point were euthanized and blood collected via cardiac puncture using heparin as an anticoagulant. Plasma samples were extracted and deprotonated with perchloric acid (0.09 N) and thereafter centrifuged at 20,000 × *g* for 12 minutes. The resulting supernatant was filtered through a 0.22 µm filter.

AEOL 10150 levels were measured using HPLC with spectrophotometric detection (Elite LaChrom System, Hitachi) using a YMC-Pack ODS-ATM column (4.6×120 mm, 3 µm, 120 Å) (Waters, Milford, MA, USA). Mobile phase A contained 20 mM triethylamine and 20 mM trifluoroacetic acid, and mobile phase B consisted of 100% acetonitrile. Flow rate was 1 mL/min. The AEOL 10150 peak was measured at 446 nm with a retention time of 3.14 min. AEOL 10150 concentrations were determined from a standard curve that was linear over the

concentrations reported. Recovery of 10150 from plasma samples was determined to be greater than 99%.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare the means using Prism software (GraphPad, La Jolla, CA). Post hoc analysis was performed using Tukey's Multiple Comparison Test. A p value of < 0.05 was considered significant. Data were expressed as mean $\pm$ SEM.

Results

General observations, pulse oximetry, and survival

Eighteen hours following CEES exposure, rats were noted to be mildly lethargic with variable tachypnea. Arterial hemoglobin oxygen saturation (SpO₂) was used as an estimation of the oxygen saturation levels. Using each animal as its own control, there was no significant change in SpO₂ when comparing baseline (mean 93.2 ± 0.8 SEM, $n=6$) readings to those 18 hours after CEES exposure (mean 92.8 ± 0.7 SEM, $n=6$) indicating lack of hypoxemia. These numbers were comparable to those of adult male rats at Denver altitude without anesthesia or exposure to aerosols of ethanol or CEES.

Of the 18 rats that were exposed to 5% CEES, 89% (16 of 18) survived for 18 hours. All 18 rats that received AEOL 10150 survived for that duration. This difference was not statistically significant (Chi-square analysis).

Estimation of rat inhaled dose

Guyton's formula was used to estimate respiratory minute volume (mean animal weight, 321 g) at 159.3 ml/min. By this approach, the estimated inhaled dose (ID) of CEES was 2.2 mg over the 15 minute exposure.

CEES inhalation results in minimal airway injury 4 hours after exposure

CEES inhalation resulted in minimal airway injury at 4 hours after exposure. As shown in Table 1, a slight, non-significant elevation in BALF protein concentration was noted at 4 hours after CEES inhalation as compared to controls. Also, BALF PMNs at 4 hours post CEES inhalation were not significantly elevated as compared to controls. BALF protein concentrations and BALF PMNs at 18 hours are also shown for comparison.

AEOL 10150 decreased CEES-induced lung injury

LDH is a soluble cytosolic enzyme that is released following loss of membrane integrity in injured cells. CEES exposure resulted in significant elevation of BALF LDH as compared to either the control or AEOL 10150 groups (Figure 1). When rats received AEOL 10150 at both one and nine hours after CEES exposure, LDH levels were significantly attenuated as compared to the CEES group ($p < 0.001$).

AEOL 10150 decreased CEES-induced lung protein and IgM levels

The presence of increased protein in the BALF can indicate cellular injury and/or increased endothelial/epithelial permeability. Protein levels in BALF were significantly increased after 18 hours as a result of CEES exposure (Figure 2A). AEOL 10150 treatment decreased CEES-induced protein levels in the BALF ($p < 0.001$). The presence of very high molecular weight molecules such as IgM (900 kD) that are normally present in serum but excluded from the BALF indicates increased vascular permeability. Figure 2B demonstrates that levels of IgM in the BALF were significantly increased as a result of CEES exposure and were significantly

decreased when AEOL 10150 treatment followed CEES. Combined, these data demonstrate AEOL 10150 treatment following CEES exposure decreased CEES-mediated lung vascular and epithelial permeability.

AEOL 10150 treatment reduced CEES-mediated lung hemorrhage and inflammation

CEES exposure resulted in significant airway hemorrhage as indicated by increased numbers of RBC in the BALF (Figure 3A). This CEES-induced hemorrhage was ameliorated by AEOL 10150 treatment ($p < 0.05$). CEES exposure also caused increased influx of polymorphonuclear neutrophils (PMN) in the BALF (Figure 3B). CEES-induced PMN in the BALF were significantly decreased following AEOL 10150 treatment ($p < 0.05$) as compared to CEES-exposed, PBS-treated animals. Although there was a decrease in numbers of macrophages in the BALF with CEES exposure, this difference, relative to the ethanol-exposed rats, did not reach significance (Figure 3C).

MPO is a glycoprotein expressed in all cells of the myeloid lineage but is most abundant in the azurophilic granules of PMNs. MPO released by activated PMNs measured in whole lung homogenate demonstrates tissue accumulation and is a useful complement to the measurement of PMN in the BALF. Lung MPO activities were significantly increased as a result of CEES inhalation indicating an increase in tissue PMN accumulation (Figure 4). When AEOL 10150 treatment followed CEES exposure, tissue accumulation of PMN was significantly decreased as compared to CEES exposure with PBS treatment ($p < 0.05$).

AEOL 10150 prevented CEES-induced oxidative stress

Oxidative stress occurs when oxidant production exceeds antioxidant defense. One biomarker of oxidative damage is DNA oxidation, which is often quantitated by the formation and accumulation of the relatively stable marker 8OHdG. The ratio of 8OHdG to 2dG significantly increased 18 hours after exposure in the CEES-exposed rats as compared to controls (Figure 5A). The ratio of 8OHdG to 2dG was significantly decreased when CEES exposure was followed with 10150 treatment as compared to CEES exposure with PBS treatment ($p < 0.05$).

Another marker of oxidative damage is the formation of lipid peroxidation products including 4-hydroxynonenal (4-HNE). 4-HNE is an unsaturated aldehyde and a major product formed during lipid peroxidation [23]. 4-HNE levels in the lung 18 hours after CEES exposure were significantly increased compared to EtOH-exposed controls (Figure 5B). When AEOL 10150 treatment followed CEES exposure, lipid peroxidation was significantly inhibited ($p < 0.05$).

Changes in lung histopathology following CEES inhalation

Lung histopathologic changes due to CEES (5%) inhalation tended to be patchy and mild. Central airways showed occasional small non-obstructing protein exudates with associated cellular material or debris that tended to be located in the dependent lobes. These changes were inconsistent, but the lesions observed in rats treated with AEOL 10150 tended to be smaller and occurred less frequently. In surveys of lung sections from both groups, airway epithelium showed minimal damage observable by light microscopy. Distal airways (eg alveoli) showed virtually no evidence of edema, exudates, or hyaline membranes. Figure 6 shows the spectrum of airway lesions present in saline- versus AEOL 10150-treated animals after 5% CEES inhalation. Hematoxylin and eosin (H&E) stained lung transverse sections at 100 $\times$ total magnification, with an inset panel at 400 $\times$ total magnification, for each treatment are shown. Figure 6A shows a control airway; 6B shows a 10150 airway; 6C demonstrates an example of severe damage following CEES inhalation with extensive proteinaceous exudate as well as areas of epithelial loss (note inset); 6D demonstrates a moderately damaged airway following CEES inhalation. There are areas of epithelial loss as well as protein exudates and RBCs present in the airway shows a mildly affected CEES-exposed airway (Figure 6E). Although there are

RBCs present within the airway, epithelium is intact and there is no proteinaceous exudate. Figure 6F represents a severe CEES+10150 airway. There are areas of epithelial loss (inset) as well as protein exudate within the airway. Figure 7G represents a moderately afflicted CEES +10150 airway with some epithelial compromise near the site of the protein exudate, as shown in the inset. Figure 6H shows a mildly affected CEES+10150 rat lung that appears unaffected by CEES exposure.

Single doses of AEOL 10150 did not protect against CEES-induced lung injury

Assessment of AEOL 10150 protection window was assessed with just a single dose one hour after CEES exposure and a single dose 9 hours after CEES exposure. No protection was afforded from CEES-induced increases in protein, IgM, or PMNs in the BALF when animals only received one dose of AEOL 10150 at either one or nine hours after CEES inhalation (Table 2).

AEOL 10150 plasma pharmacokinetics

Since a single dose of AEOL 10150 was not effective against CEES inhalation, we further investigated the pharmacokinetics of AEOL 10150. Figure 7 demonstrates that following a single subcutaneous dose of AEOL 10150 at 5 mg/kg, plasma AEOL 10150 levels at 1 hour were $3.90 \mu\text{M} \pm 0.174$ and declined to $0.25 \mu\text{M} \pm 0.047$ by 8 hours post injection.

Discussion

The present report demonstrates that lung injury, inflammation, and oxidative damage related to inhalation of CEES were ameliorated by AEOL 10150 treatment. Specifically, increased BALF LDH following CEES inhalation demonstrated increased cellular injury. Protein and IgM levels in BALF were increased following CEES exposure. All of these indicators of lung injury were diminished by AEOL 10150 treatment. CEES inhalation increased PMN in the BALF and lung tissue. Treatment with AEOL 10150 decreased PMN infiltration. CEES inhalation also increased oxidative stress, which was also diminished by AEOL 10150 treatment. Finally, we demonstrated in histopathologic studies the presence of variable proteinaceous exudate and epithelial loss following CEES inhalation. These changes also appeared to be diminished by AEOL 10150 treatment, consistent with the findings of BALF protein and IgM.

Real world SM exposures can vary dramatically due to ambient temperatures, proximity, and air flow [2,24]. Injury can vary from mild to lethal, although human mortality due to SM exposure in military settings is normally low at 2–3% [2]. In battlefield conditions initial aerosolization is usually via explosives, and accurate measurements of concentrations delivered are not available. Following human exposures, a symptom-free interval of several hours to days occurs in inverse correlation with the absorbed dose [25]. This delay is also mirrored in rat toxicokinetic studies showing SM is eliminated in a two-compartment model with a quick distribution ($t_{1/2\alpha}=5.56$ min), and a long terminal half life ($t_{1/2\beta}=3.59$ hr)[26]. As mortality is low and injury is delayed, these initial studies investigated a concentration of CEES that resulted in delayed lung injury with minimal mortality.

SM produces dose-dependent damage to the respiratory tract, beginning with the upper airways and descending to the lower airways as the exposure level increases [27]. Previous studies of lung injury have utilized intratracheal (IT) delivery of CEES or SM [6,8,28–30]. While this does allow for direct delivery to the airways, it also results in focal injury that differs from an actual inhalation exposure. SM has little effect on lung parenchyma, although distal airways involvement is noted in severe exposure cases [31].

Lung injury, and specifically central airways injury, is a hallmark of SM inhalation injury. In the absence of inflammatory cell infiltration in the BALF, an increase in LDH would be indicative of epithelial damage. Our data shows increases in BALF RBCs and PMNs, which when lysed also could also contribute to elevated BALF LDH activities. Thus, care must be taken in interpreting these data as exclusively representing epithelial damage. Previous studies have demonstrated LDH can be oxidatively inactivated [32]. This could lead to underestimation of LDH activity. Paradoxically, when antioxidant treatment is present, LDH levels could be even more elevated as compared to animals receiving vehicle. This was not the case in this study, as the vehicle-treated, CEES-exposed group had increased BALF LDH activities that were decreased by treatment with AEOL 10150.

Increases in BALF protein and IgM were evident following CEES exposure. Increased protein could be indicative of cellular necrosis, vascular leak of plasma contents, or both. However, the presence of the high molecular weight immunoglobulin IgM in the BALF was strongly indicative of increased permeability of vascular endothelium and movement of fluid into airways. Luminal entry of plasma is often associated with epithelial damage and sloughing [33,34]. However, it has also been shown that plasma exudates can move between epithelial cells into the airway lumen even when the epithelial barrier is visibly intact [35,36]. At the light microscopic level, airway epithelium appeared mostly intact in both diluent- and AEOL 10150-treated groups. Occasionally, we saw minor epithelial damage in some airways while others appears undamaged at the light microscopic level. It is therefore possible that both loss of epithelium and leak between epithelial cells play a role in allowing increased plasma components to enter the airway lining fluid. BALF changes indicative of this type of lung injury were decreased by AEOL 10150 treatment.

Acute inflammation is typified by increases in PMNs at the site of injury. While PMN are beneficial in phagocytosis of bacteria and possibly even damaged cells, their production of large quantities of ROS can damage local tissue. In fact, in previous studies, depletion of PMN prior to CEES exposure reduced lung injury indices [6]. While we cannot assume that injury is due exclusively to ROS production by PMN, the current study shows that AEOL 10150 treatment reduced PMN infiltration and, very possibly, PMN activation. This reduction in PMNs entry into lung should therefore result in reduced ROS production. A similar metalloporphyrin antioxidant (MnTBAP) also attenuated PMN increases in a model of paraquat-induced lung injury [37]. Another catalytic metalloporphyrin reduced inflammation in an asthma model as well as a hyperoxia-induced model of bronchopulmonary dysplasia [38,39]. Inflammatory cells contribute to increased ROS production, but are not the only source in SM or CEES exposure. Studies of CEES *in vitro* have demonstrated diminished mitochondrial membrane potential and subsequent ROS production [15]. AEOL 10150 protected lung epithelial cells from mitochondrial damage in that model. Besides direct scavenging of ROS, potential mechanisms by which AEOL 10150 could improve indices of injury and disease are still not clear.

One outcome of oxidative stress is an increase in oxidation of macromolecules. In this study, CEES inhalation resulted in increased lung DNA oxidation, as measured by 8-OHdG. Whether DNA oxidation was due directly to CEES, its metabolites, or ROS formed as a result of CEES is not clear. CEES exposure is also known to cause lipid peroxidation [8,28]. 4-HNE is a biologically active aldehyde and a stable end product of lipid peroxidation. While often viewed as simply a marker of injury, 4-HNE has biological functions as well. Increased 4-HNE levels have been linked to endothelial barrier dysfunction resulting in increased permeability [40, 41]. Thus, lipid peroxidation may be a mechanism by which CEES-induced permeability occurred. AEOL 10150 may have reduced lung injury, in part, by limiting this process.

Microscopic examination of fixed airways following CEES inhalation showed the presence of proteinaceous exudate in varying levels of the conducting airways. By contrast, this material was not detected in alveolar spaces, as detected by light microscopy, consistent with previous reports of SM exposure. Following CEES exposure, the exudates were not consistently noted in all airways. Sectioning in consistent regions of the lung did not always yield protein exudates and these exudates were present in varying concentrations, even within the same animal. A few sloughed airway epithelial cells were noted in BALF, but different animals were used for fixation and lavage, so we could not determine correlation. Material in the exudates may not have been freely soluble and accessible by lavage. In addition, it is possible that some of these exudates could have been lost during fixation, potentially underestimating damage. There are parallels between airways injury by CEES and following burn or smoke inhalation, the latter of which results in plasma leak from the bronchial circulation [42,43]. This provides an interesting potential avenue for future mechanistic investigation.

Despite extensive studies of SM/CEES exposure over more than a century, the mechanisms underlying airway injury have not been fully elucidated. Injury from SM was previously believed to be due to direct alkylation of macromolecules. In fact, the major *in vivo* metabolites are GSH conjugates [44]. Depletion of this important endogenous antioxidant can allow increases in oxidants to go unchecked. Antioxidants have shown efficacy in a number of models of SM/CEES exposure, although the mechanism of protection is not clear. In addition, recent studies have shown that sulfur and nitrogen mustards can react with cellular reductases, altering electron transfer and increasing free radical production [45]. The efficacy of AEOL 10150 is often attributed to its ability to directly scavenge oxidants. AEOL 10150 and similar metalloporphyrin compounds also have the capacity to redox cycle with cellular reductase domains in enzymes including cytochrome P450 reductase, NADH oxidase, and nitric oxide synthases [9,46]. Although redox cycling is typically considered deleterious, AEOL 10150 counteracts such effects with its antioxidant capacity.

The two dose protocol for AEOL 10150 treatment was based on success with previous studies of radiation-induced lung injury [17,47]. Having initially found two doses of the compound to be effective in this short term model, we later investigated whether one dose, either at 1 hour or 9 hours post exposure would be effective. As the data demonstrate, one dose was not effective at the tested concentration. Investigation of pharmacokinetics of this compound indicates that by 8 hours post injection, blood levels of AEOL 10150 are minimal but still detectable. Given the 8 hour difference between doses in our two dose protocol, it appears that, in order to be effective, there must be at least a minimal level of drug in the plasma. *In vitro* studies have shown an increase in mitochondrial ROS peaking at 12 hours after CEES exposure, which was ameliorated by AEOL 10150 treatment beginning one hour after exposure [15]. This demonstration of delayed injury seen with *in vitro* CEES exposure may provide some basis for the requirement of a second dose of the drug.

The present results build on current literature that has demonstrated that oxidative stress plays a role in the pathogenesis of CEES-induced lung injury. Herein, we demonstrated that substantial rescue of CEES-induced lung injury, inflammation, and oxidative stress was possible with AEOL 10150. Further studies are needed to determine whether AEOL 10150 also can afford protection against lung injury due to inhalation of sulfur mustard.

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Figure 1.

Effect of treatment with AEOL 10150 on CEES-induced BALF LDH activity. Rats were either exposed to EtOH or 5% CEES for 15 minutes and then given PBS (1 ml/kg, SC) or AEOL 10150 (5 mg/kg, SC) treatment 1 and 9 hours after inhalation exposure. BALF was harvested 18 hours after exposure and lung injury was assessed by measuring changes in LDH levels. Columns with different letters are significantly different from one another. Asterisks denote a significant difference between CEES inhalation and CEES inhalation with AEOL 10150 treatment ($p < 0.05$). Data are shown as mean $\pm$ S.E.M., $n=5$ (control, 10150) and $n=9$ (CEES, CEES+10150).

Figure 2.

Effect of AEOL 10150 on CEES-induced increases in markers of acute lung edema. Rats were either exposed to EtOH or 5% CEES for 15 minutes and then given either PBS (1 ml/kg, SC) or AEOL 10150 (5 mg/kg, SC) at 1 and 9 hours after CEES exposure. At 18 hours post exposure, rats were lavaged and levels of BALF protein (A) and IgM (B) were determined by a BCA assay and ELISA assay, respectively. Columns with different letters are significantly different from one another. Asterisks denote a significant difference between CEES inhalation and CEES inhalation with AEOL 10150 treatment ($p < 0.05$). Data are shown as mean $\pm$ S.E.M., protein $n=6$ (control, 10150) and $n=16$ (CEES, CEES+10150), IgM $n=6$ for all groups.

Figure 3.

Effect of AEOL 10150 on CEES-induced increases in numbers of hemorrhage and inflammation. BALF was centrifuged, and cells were resuspended in PBS. Rats were either exposed to EtOH or 5% CEES for 15 minutes and then given PBS (1 ml/kg, SC) or AEOL 10150 (5 mg/kg, SC) at 1 and 9 hours after CEES exposure. At 18 hours after CEES exposure, rats were lavaged and BALF was centrifuged with resulting cell pellets resuspended in PBS. (A) Absolute numbers of BALF RBC, (B) absolute numbers of BALF PMN, and (C) absolute numbers of BALF macrophages are shown. Columns with different letters are significantly different from one another. Asterisks denote a significant difference between CEES inhalation

and CEES inhalation with AEOL 10150 treatment ($p < 0.05$). Data are mean $\pm$ S.E.M., $n=6$ (control, 10150) and $n=13$ (CEES, CEES+10150).

Figure 4.

Effect of AEOL 10150 on CEES-induced increases in lung tissue MPO activity. Rats were either exposed to EtOH or 5% CEES for 15 minutes and then given PBS (1 ml/kg, SC) or AEOL 10150 (5 mg/kg, SC) at 1 and 9 hours after CEES exposure. Snap-frozen lung tissue was homogenized and MPO activity measured. Columns with different letters are significantly different from one another. An asterisk denote a significant difference between CEES inhalation and CEES inhalation with AEOL 10150 treatment ($p < 0.05$). Data are shown as mean $\pm$ S.E.M., $n=6$ for all groups.

Figure 5.

The effect of AEOL 10150 on CEES-induced lung oxidative stress. Rats were either exposed to EtOH or 5% CEES for 15 minutes and then given PBS (1 ml/kg, SC) or AEOL 10150 (5 mg/kg, SC) at 1 and 9 hours after CEES exposure. (A) Lung 2-dG and 8-OHdG levels were measured using HPLC with UV and electrochemical detection, respectively. Standard curves of 8-OHdG and 2-dG were used to determine concentrations. Data was expressed as the ratio of 8-OHdG/ 10^5 2-dG. (B) 4-HNE was measured using GC/MS analysis. Concentration of 4-HNE was determined using a standard curve generated by graphing the area ratio of 4-HNE to d_3 -4-HNE. An asterisk denote a significant difference between CEES inhalation and CEES inhalation with AEOL 10150 treatment ($p < 0.05$). 8-OHdG data were shown as mean $\pm$ S.E.M.,

$n=12$. 4-HNE data presented as mean $\pm$ S.E.M., $n=11$ (control and CEES), $n=5$ for (10150 and CEES+10150).

Figure 6.

Representative light photomicrographs of the lung following EtOH or CEES inhalation and subsequent PBS or AEOL 10150 treatment. Tissues were stained with hematoxylin & eosin; low magnification (100× total) views with inset at 400× total magnification are shown. Lungs from (A) control (ethanol-exposed) and (B) AEOL10150-treated rat lungs demonstrate normal airways following ethanol inhalation. (C) Shows severe damage, (D) shows moderate damage, and (E) shows minimal damage following CEES inhalation. (F) shows severe damage, (G) shows moderate damage and (H) shows minimal damage following CEES inhalation with AEOL 10150 treatment.

Figure 7.

Plasma concentrations of AEOL10150 following a single SC injection at 5 mg/mg. A total of 15 rats were injected with AEOL 10150 and 3 rats per time point were sacrificed at 1,2,4,6, and 8 hour time points to measure AEOL 10150 levels.

Table 1

Comparison of EtOH, 4 hr CEES and 18 hr CEES exposures

	BALF Protein mg/ml	BALF IgM ng/ml	total PMN $\times 10^5$
control	0.176 ± 0.011	8.85 ± 1.07	0.005 ± 0.0034
CEES 4 hrs	0.2875 ± 0.023	42.36 ± 22.33	0.244 ± 0.083
CEES 18 hrs	1.833 ± 0.2618	333.9 ± 48.34	6.436 ± 1.668

Table 2

Single Dose 10150 Treatment, 1 or 9 hours post inhalation

	CEES	CEES+10150
<i>SC, 1 hour post inhalation</i>		
Protein, mg/ml	1.976 ± 0.4619	1.437 ± 0.2881
Total PMN × 10 ⁵	1.288 ± 0.646	0.921 ± 0.264
<i>SC, 9 hours post inhalation</i>		
Protein, mg/ml	1.186 ± 0.3965	1.550 ± 0.2791
Total PMN × 10 ⁵	0.876 ± 0.300	1.012 ± 0.318