Published in final edited form as:

Free Radic Biol Med. 2011 March 1; 50(5): 602–608. doi:10.1016/j.freeradbiomed.2010.12.001.

AEOL10150: A Novel Therapeutic for Rescue Treatment Following Toxic Gas Lung Injury¹

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Abstract

Background—New therapeutics designed as rescue treatments following toxic gas injury such as chlorine (Cl₂) are an emerging area of interest. We tested the effects of the metalloporphyrin catalytic antioxidant AEOL10150, a compound that scavenges peroxynitrite, inhibits lipid peroxidation, and has SOD and catalase-like activities, on Cl₂-induced airway injury.

Methods—Balb/C mice received 100 ppm Cl₂ gas for five minutes. Four groups were studied; Cl₂ only, Cl₂ followed by AEOL10150 one and nine hours after exposure, AEOL10150 only, and control. Twenty-four hours following Cl₂ gas exposure airway responsiveness to aerosolized methacholine (6.25–50 mg/mL) was measured using a small animal ventilator. Bronchoalveolar lavage (BAL) was performed to assess airway inflammation and protein. Whole lung tissue was assayed for 4-hydroxynonenal. In separate groups, lungs were collected at 72 hours following Cl₂ injury to evaluate epithelial cell proliferation.

Results—Mice exposed to Cl₂ showed a significantly higher airway resistance than control, Cl₂/AEOL10150, or AEOL10150-only treated animals in response to methacholine challenge. Eosinophils, neutrophils and macrophages were elevated in BAL of Cl₂-exposed mice. AEOL10150 attenuated the increases in neutrophils and macrophages. AEOL10150 prevented Cl₂-induced increase in BAL fluid protein. Chlorine induced an increase in number of proliferating airway epithelial cells, an effect AEOL10150 attenuated. 4-hydroxynonenal levels in the lung were increased following Cl₂ and this effect was prevented with AEOL10150.

Conclusion—AEOL10150 is an effective rescue treatment for Cl₂-induced airway hyperresponsiveness, airway inflammation, injury-induced airway epithelial cell regeneration and oxidative stress.

¹Supported by the CounterACT Program, National Institutes of Health Office of the Director, and the National Institute of Environmental Health Sciences, Grant Number U54 ES015678 (BJD, CWW, JGM and TM). Dr. Day is a consultant for and holds equity in Aeolus Pharmaceuticals which is developing metalloporphyrin catalytic antioxidants as potential therapeutic agents.

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INTRODUCTION

Chlorine (Cl_2) is a highly reactive oxidant gas that is used in the bleaching of paper, in the production of hydrocarbon solvents, in the disinfection of swimming pools and as a chemical weapon. [1–3] Five-year cumulative data between 1988–1992 from the American Association of Poison Controls Centers' National Data Collection System reported 27,788 exposures to Cl_2 in the United States.[1] Acute human exposures have occurred as a result of industrial accidents or during wartime that have led to long term respiratory dysfunction and even death.[2] Residual effects following acute Cl_2 damage can persist for years and include decreased vital capacity, reduced diffusing capacity, and lowered total lung capacity with a trend towards higher airway resistance.[4, 5] There are no effective pharmacological rescue treatments currently available.

There have been several experimental and case studies performed in both animal and human models characterizing the effects of Cl_2 gas exposure on the respiratory system.[3–7]. Following initial exposure, injury is generally characterized by an influx of inflammatory cells into the airways, specifically neutrophils, lymphocytes, eosinophils and macrophages. In addition, epithelial apoptosis and necrosis and airway hyperresponsiveness can occur.[7, 8] Epithelial cell damage has been observed in rodents exposed to Cl_2 gas including denudation of the epithelium, followed by repopulation of the epithelial cell layers.[8]

The molecular properties of Cl_2 are such that it has an extremely high propensity to oxidize. It has been shown to have greater toxicity than nitrogen dioxide (NO_2), oxygen (O_2) or ozone (O_3), a property that may be related, in part, to its high water solubility.[9] The hydration of Cl_2 leads to the production of hydrochloric acid (HCl) and hypochlorous acid (HOCl). It is therefore likely that oxidative injury is also involved in the damage and repair processes.[10, 11] Consistent with this idea, Cl_2 gas is about 30-fold more potent than hydrochloric acid, further emphasizing its oxidant, rather than acidic properties as being the predominant mechanism responsible for its actions.[3, 12] When administered into the airways hydrochloric acid causes airway hyperresponsiveness in mice by mechanisms that have been suggested to relate to epithelial barrier function.[13] Epithelial cells are particularly susceptible to Cl_2 damage and have been implicated as key targets in the damage and repair process. They are among the first cells to encounter Cl_2 in the airway and may be affected by the direct toxicity of Cl_2 or indirectly through its by-products HOCl and HCl . Additionally, epithelial cells are capable of storing, producing, and releasing large quantities of the antioxidant glutathione in response to oxidative stress.[13]

The aim of the current study was to assess the efficacy of a novel catalytic antioxidant in ameliorating airway damage when administered after an acute exposure to inhaled Cl_2 gas. For this purpose, we utilized a catalytic metalloporphyrin that is a member of a novel class of low-molecular-weight antioxidants. The compound, Mn(III) tetrakis (*N,N'*-diethylimidizolium-2-yl) porphyrin, AEOL10150 (AEOL), efficiently scavenges peroxynitrite, inhibits lipid peroxidation, and has SOD and catalase-like activities.[14] Recent studies using the sulfur mustard analog, 2-chloroethyl ethyl sulfide (CEES), a compound that also can induce oxidative stress in the lung, have demonstrated that AEOL is effective in reducing cytotoxicity and mitochondrial dysfunction when given 1 hour after

CEES exposure. *In vivo*, AEOL has shown promising therapeutic properties in protecting the lungs of rats exposed to CEES against inflammatory cell infiltration, reactive oxygen species and DNA damage.[14] In the current study, we used a mouse model to further characterize the effects of Cl₂ gas as well as the ability of AEOL to rescue the lungs from damage caused by Cl₂ inhalation by examining airway function, airway inflammation, bronchial epithelial cell proliferation, and markers of oxidative stress.

METHODS

Animals and protocol

Male Balb/C mice (18–22 g) were purchased from Charles River (Wilmington, Massachusetts) and housed in a conventional animal facility at McGill University. Animals were treated according to guidelines of the Canadian Council for Animal Care and protocols were approved by the Animal Care Committee of McGill University. Animals were provided with water and food *ad libitum* throughout the experiment. Four groups were studied; Cl₂ only (n=10), Cl₂ followed by AEOL10150 (AEOL) (n=10), AEOL only (n=10), or control (n=10). Mice in groups treated with AEOL were given 5 mg/kg intraperitoneally (i.p) one hour and nine hours following Cl₂ exposure. Mice in control or Cl₂ only groups were given 1 ml of phosphate buffered saline intraperitoneally (i.p., PBS, pH 7.4) one hour and nine hours following air or Cl₂ exposure. Mice were studied at 24 hours following initial Cl₂ exposure. In separate groups (n=6/group) mice were studied at 72 hours following initial Cl₂ exposure to evaluate epithelial cell proliferation in the lungs. There was no significant weight loss or behavioral signs of distress at any time point following Cl₂.

AEOL 10150 pharmacokinetics and safety profile

Male C57Bl/6 mice were given a single bolus dose of AEOL intravenously by tail vein injection and blood samples were drawn upon sacrifice by cardiac puncture (2 mice/time point). Blood samples were obtained at 5, 10, 15, 30 minutes and 1, 2, 4, 8, 12 and 24 hours after dosing. Plasma AEOL concentrations were measured using a previously described HPLC analytical method [14]. Pharmacokinetic data fitting was done using WinNonlin non-compartmental model (Pharsight, Cary, NC). (Table 1) AEOL10150 safety assessments were performed in mice, rats, non-human primates and man as described in *FDA IND#67,741*. In mice, AEOL's no observable adverse effect level (NOAEL) was found to be 40 mg/kg/day and the maximum tolerated dose (MTD) was found to be 160 mg/kg/day (data not shown). The structure of AEOL is shown in Figure 1.

Exposure to Cl₂

Mice were restrained and exposed to Cl₂ gas for 5 minutes using a nose-only exposure device. Cl₂ gas was mixed with room air using a standardized calibrator (VICI Metronics, Dynacalibrator®, model 230-28A). The Cl₂ delivery system has two main components, a gas generator, which includes a heated permeation chamber and air flow generator. Dynacal permeation tubes designed specifically for operation with the Dynacalibrator were used and contain the Cl₂. The permeation chamber and air flow generator control the accuracy of the Cl₂ generated to within 1–3% of the desired concentration according to the manufacturer's specifications. Teflon permeation tubes containing Cl₂ in both gas and liquid phases are

contained within the permeation chamber. When the tube is heated the Cl₂ reaches a constant vapor pressure such that it permeates the tube at a constant rate. The desired concentration is delivered at an appropriate flow rate, as specified by the manufacturer. The device is attached to the exposure chamber and allowed to calibrate for 30 minutes until the optimum temperature of 30°C is reached and the Cl₂ flow is constant. Following removal of the animals from the exposure chamber, the chamber was continually flushed with the gas mix to ensure that the desired concentration of Cl₂ was maintained between mouse exposures.

Methacholine Responsiveness

Mice were sedated with xylazine hydrochloride (8 mg/kg,i.p.) and anaesthetized with pentobarbital (30 mg/kg, i.p.). Subsequently, the animals were given tracheotomies using an 18 gauge cannula and connected to a small animal ventilator (FlexiVent, Scireq, Montreal, Canada). Muscle paralysis was induced with pancuronium bromide (0.2 mg/kg i.p.). The mice were ventilated in a quasi-sinusoidal fashion with the following settings: a tidal volume of 10 ml/kg, maximum inflation pressure of 30 cm H₂O, a positive end expiratory pressure (PEEP) of 3 cm H₂O and a frequency of 150/min. Following an equilibration period of 3 minutes of tidal ventilation, two lung inflations to a trans-respiratory pressure of 25 cm H₂O were performed and baseline measurements were taken. The template used was Snapshot-150 v5.2 and mechanics were calculated using the single compartment model. Baseline was established as the average of three perturbations. Following establishment of baseline, methacholine (MCh) was administered using an in-line nebulizer (Aeroneb Lab, standard mist model, Aerogen Ltd, Ireland) and progressively doubling concentrations ranging from 6.25 to 50 mg/ml were administered over 10 seconds synchronously with inspiration. Six measurements were made at each dose of MCh to establish the peak response. The highest value was kept for analysis subject to a coefficient of determination above 0.85. Respiratory system resistance and respiratory system elastance were determined before challenge and after each dose of MCh.

Bronchoalveolar Lavage

Twenty four hours following Cl₂ exposure the mice were euthanized with an overdose of sodium pentobarbital (30 mg/kg, i.p.). The mice were given tracheotomies and 0.5 mL of sterile saline was instilled into the lungs and the fluid recovered was placed in 1.5 mL eppendorf tubes and kept on ice. Fluid recovered from the first wash was centrifuged at 1,500 rpm for 5 minutes at 4°C and the supernatant retained for extracellular glutathione analysis. Three subsequent washes were done with 1mL aliquots of sterile saline to recover lung inflammatory cells and placed in a 15 mL tube for centrifugation. The bronchoalveolar lavage fluid (BAL) was centrifuged at 1500 rpm for 5 minutes at 4°C and total live and dead cells were counted using trypan blue exclusion. Cytospin slides were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA) and stained with Diff Quick (Jorgensen Labs Inc., Loveland, CO). Differential cell counts were determined based on a count of 300 cells/slide. Total protein in the BAL supernatant was quantified using a Bradford colorimetric assay (Bio-Rad, Hercules, CA), and determined by spectrophotometry at 620 nm against a standard curve of bovine serum albumin.

Tissue Preparation for Assessment of Epithelial Regeneration

Mice were allowed to recover for 72 hours in separate groups following Cl₂ exposure before they were euthanized using an overdose of sodium pentobarbital (30 mg/kg, i.p.). The pulmonary circulation was flushed with sterile saline via the right ventricle until the effluent was clear. Following removal lungs were fixed by intratracheal perfusion with 10% buffered formalin at a constant pressure of 25 cm H₂O for a period of 24 hrs. Immunohistochemistry was done following cutting of 5 µm paraffin-embedded sections, which were stained with Ki-67 nuclear staining to determine epithelial cell proliferation.

Immunohistochemistry

Cells undergoing proliferation were detected in tissue sections by immunostaining for Ki-67. Immunohistochemical staining for Ki-67 was performed with the Vectastatin avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA). Following de-paraffination of sections, the slides were immersed in antigen unmasking solution (Vector Laboratories) for 8 minutes. Sections were washed for 5 minutes twice with TRIS buffered saline (TBS; 0.5 M Tris-HCl, 1.5M NaCl, pH 7.6). Lung sections were permeabilized using 0.2% Triton x-100 for 20 minutes. Following washing, non-specific binding sites were saturated with universal blocking solution (Vector Laboratories) for 20 minutes. Primary antibodies against goat Ki-67 (Cayman Chemical, Ann Arbor, MI) and control normal goat serum (Vector Laboratories) used at a dilution of 1:250 were applied to tissue sections for incubation in a humidified chamber at room temperature for one hour. The sections were then washed with TBS twice for 5 minutes. Biotinylated rabbit anti-goat IgG (Vector Laboratories) was applied to the tissue sections at a concentration of 1:50 and incubated at room temperature for 45 minutes. Sections were washed twice with TBS for 5 minutes. Then the slides were incubated with avidin-biotin complex alkaline phosphatase (Vector Laboratories) for 45 minutes. Lastly, Vector Red alkaline phosphatase (Vector Laboratories) was used to develop the sections for 15 minutes. Sections were dehydrated by moving slides through 3 baths of xylene and mounted with Vectamount mounting medium (Vector Laboratories). Lung sections were visualized for positive Ki-67 staining by light microscopy.

Quantitative morphology on airway sections

Numbers of Ki-67-positive cells were quantified following staining. Positive cells were defined as cells that showed strongly positive nuclear staining for Ki-67. Only airways with a major/minor (long axis/short axis) diameter ratio of <2.5 in cross section were selected for analysis. Airways analyzed ranged in size from 0.5–3mm. The number of Ki-67 positive cells in the epithelium was quantified under a light microscope using a 40x objective. The airway basement membrane length was measured by superimposing the image of the airway onto a calibrated digitizing tablet (Jandel Scientific, Chicago, IL), with a microscope equipped with a *camera lucida* projection system (Leica Microsystems, Richmond Hill, ON, Canada). The numbers of proliferating cells corrected for airway size were expressed as Ki-67⁺ cells/mm of basement membrane perimeter (P_{BM}).

Glutathione Measurements

The BAL fluid from control, Cl₂-exposed and AEOL-treated mice were collected for glutathione evaluation by HPLC. BAL samples were collected 24 hours after Cl₂ challenge. Phosphoric acid (60 µL; 1 M) was added to BALF samples to prevent GSH degradation. BAL was centrifuged at 1500 RPM for 5 minutes, and the supernatant was removed for evaluation of extracellular GSH/GSSG. The pellet was reconstituted with 150 µL of PBS, 150 µL of 12 mM CHAPS, and 15 µL of 1M phosphoric acid for analysis of intracellular GSH and GSSG. GSH and GSSG were measured in 50 µl aliquots by RP-HPLC using post-column derivatization as described previously [15]. The mobile phase was a gradient between 0 and 15% acetonitrile containing 0.05% trifluoroacetic acid over 10 min at a flow rate of 1 ml/min. The stationary phase was a column (150 x 4.6 mm) of Ultracarb ODS (5 µm particle size; 150 x 4.6 mm; Phenomenex, Torrance, CA). GSH and GSSG in the column eluate were converted to a fluorescent isoindole derivative by continuously mixing the column eluate with o-phthalaldehyde (370 µM) in 0.2 M tribasic sodium phosphate, pH 12 at 70 °C. Fluorescence was monitored using excitation and emission wavelengths of 336 and 420 nm, respectively. The amounts of GSH and GSSG were determined from a standard curve using the authentic compounds as external standards.

Lung 4-hydroxynonenal (4-HNE) assay

4-HNE assay was measured as described previously.[15] Frozen tissue, or a known amount of 4-HNE standard (Cayman Chemical, Ann Arbor, MI, USA), was placed in 2 ml of cold methanol (Thermo Fisher) containing 50 µg/ml butylated hydroxytoluene, with 10 ng d₃-4-HNE (Cayman Chemical) internal standard added just before homogenization with the Ultra-Turrax T25 (Thermo Fisher). An EDTA solution (1 ml of 0.2 M, pH 7) was added. Derivatization was accomplished by incubation with 0.2 ml of 0.1 M HEPES containing 50 mM O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride, pH 6.5 for 5 min at room temperature. The O-pentafluorobenzyl-oxime derivatives were then extracted with hexane, converted to trimethylsilyl ethers by the addition of 15 µl each of pyridine and N,O bis(trimethylsilyl)trifluoroacetamide and analyzed by gas chromatography/mass spectrometry using a Focus gas chromatograph coupled to a DSQ II mass spectrometer (Thermo Fisher). The stationary phase was a A15-m TR-5MS column (0.25-mm i.d., 0.25-µm film thickness; Thermo Fisher) and the carrier gas was helium (1.0 ml/min). Two microliters 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 and 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 quadrapole at 180°C. Analysis was done by negative-ion chemical ionization using 2.5 ml/min 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 d₃-4-HNE at m/z 153, 286, and 306. Under these conditions, the larger, second peak of the two 4-HNE isomers was used for quantification and exhibited a retention time of 7.18 min, which was just preceded by the elution of d₃-4-HNE at 7.17 min. Quantification was performed using a standard curve generated by graphing the area ratio of 4-HNE to d₃-4-HNE versus concentration.

Statistical Analysis

Differences in responsiveness to methacholine were analyzed by repeated measures ANOVA and a post-hoc Bonferroni test. One-way analysis of variance was used to determine the drug treatment effect on other variables and the post-hoc comparisons were performed using a Dunnett's multiple comparison test for differential cell counts. $P < 0.05$ was considered significant.

RESULTS

Effect of AEOL on MCh responsiveness following Cl₂ challenge

Airway responsiveness to inhaled aerosolized MCh in increasing concentrations (6.25–50 mg/ml) was elevated 24 hours following Cl₂ exposure. Cl₂ significantly increased respiratory system resistance over control and AEOL-treated groups at the two highest doses of MCh (Figure 2A). Respiratory system elastance was increased in the Cl₂ only exposed group compared with control at the two highest doses of MCh (Figure 2B). AEOL did not significantly attenuate the increase in elastance compared to Cl₂ only treated mice (Figure 2B). However, there was also no significant difference between control groups and Cl₂/AEOL-treated mice (Figure 2B).

Changes in bronchoalveolar lavage cells following Cl₂ gas exposure

To assess the airway inflammation induced by Cl₂, bronchoalveolar lavage was performed at 24 hours following Cl₂ exposure. The BAL fluid recovered averaged 75% of the initial volume instilled and did not vary significantly between groups. Total cell counts were significantly increased in the Cl₂-exposed group compared to control groups as well as Cl₂/AEOL (Figure 3). While AEOL was able to reduce total inflammatory cell influx after Cl₂ exposure compared with Cl₂ only exposed mice, there was still a significant difference between Cl₂/AEOL treated groups and control groups. AEOL alone had no effect on total inflammatory cell numbers.

Differential cell counts were performed to examine the pattern of inflammatory cell recruitment to the airways following Cl₂ exposure. Cl₂ exposure significantly increased total numbers of macrophages, neutrophils and eosinophils (Figure 4A–C) above control groups but did not significantly increase lymphocytes or epithelial cells (Figure 4D and E). Treatment with AEOL attenuated the increase in macrophage and neutrophil numbers recruited to the airway following Cl₂ exposure but did not significantly affect eosinophils, lymphocytes or epithelial cell numbers.

Changes in protein level following Cl₂ exposure

Total protein levels were measured in BAL fluid using a Bradford assay 24 hours following Cl₂ exposure to assess cell damage and microvascular leak. Cl₂ caused a 2-fold increase in the total protein present in the BAL compared to control groups. Treatment with AEOL after Cl₂ exposure significantly reduced the amount of protein in the BAL fluid (Figure 5).

Epithelial Cell Proliferation

We have previously reported that epithelial proliferation occurs as early as 48 hrs after high level Cl₂ exposure. [16] We have measured the level of cellular hyperplasia as an indirect method of assessment of epithelial damage. We chose to quantify the level of proliferation of epithelial cells at seventy-two hours following exposure. Ki-67 nuclear staining was performed to determine numbers of proliferating epithelial cells. The total numbers of positive cells were quantified in each airway and corrected for airway size by dividing by the perimeter of the basement membrane of the airway. A minimum of six animals totaling at least 24 airways were studied in each group. Cl₂ induced an increase in the total number of proliferating epithelial cells in the airways (Figure 6A). Treatment with AEOL significantly reduced the number of proliferating epithelial cells compared to Cl₂ only treatment. Figure 6, panels B–D show representative pictures of control (Figure 6B), Cl₂ only (6C) or Cl₂/AEOL (6D). Darker nuclei indicate Ki-67 positive cells.

BAL GSH and GSSG levels at 24 hrs are unaffected by Cl₂

At 24 hrs following Cl₂ and AEOL treatments, extracellular glutathione (GSH) levels were measured in BAL fluid (Figure 7A). There were no significant differences in the levels of either GSH (Figure 7A) or GSSG (Figure 7B) among the four groups.

AEOL protects against Cl₂-induced increases in 4-HNE

Twenty-four hours following Cl₂ exposure, lungs were harvested and lung homogenates analyzed for 4-HNE. Cl₂ induced a significant increase in 4-HNE levels compared to control and AEOL-treated groups (Figure 8). AEOL alone had no effect on 4-HNE levels, but completely blocked the response to Cl₂.

DISCUSSION

In the current study we wished to explore whether rescue treatment with the synthetic metalloporphyrin AEOL could prevent the deleterious effect of Cl₂ on the lungs, including airway hyperresponsiveness to MCh, inflammatory cell influx into the airways, epithelial cell and oxidative damage. Our results showed that although AEOL itself had no effect on the lungs, it was effective in attenuating the effects of Cl₂ on all of the above parameters. BAL glutathione levels were not perturbed at 24 hours after chlorine. However, at the same time, there was clear evidence of oxidative lung damage measured by 4-HNE that was prevented by AEOL. The data show that post-hoc treatment is effective and supports the concept that oxidative damage continues beyond the immediate effects of acid and oxidant damage caused by Cl₂.

Previous studies have shown that exposure to Cl₂ causes increased airway responsiveness (AHR) in human, rat, and mouse models.[3, 16–18] The mechanism of AHR following Cl₂ may be similar to that of ozone in that both forms of injury are associated with oxidant damage to the tissues.[19] In our study, mice received a single dose of Cl₂ and although baseline mechanical parameters were unchanged there was an increase in respiratory system resistance and elastance in response to MCh. Airway epithelial damage most likely contributed to the AHR to inhaled MCh. We noted a marked increase in numbers of

proliferating epithelial cells in Cl₂-exposed mice compared to controls and AEOL-treated groups, an indication of the intensity of the epithelial regeneration necessitated by prior damage by Cl₂ exposure. AEOL attenuation of this effect indicates a protective effect on the airways, making epithelial cell repair less necessary. An alternative interpretation of AEOL's action on the airways is that it is inhibiting epithelial cell repair. However, this is improbable considering AEOL's favorable effects on other study outcomes. We did not, however, see high numbers of shed epithelial cells in the BAL fluid after Cl₂, which suggests that shed cells may already have been removed by phagocytosis or that we may not have sampled BAL fluid early enough to detect the signal. Whether airway hyperresponsiveness is caused by loss of epithelial-derived bronchorelaxant factors or through disruption of barrier function remains to be determined.

Consistent with previous studies, we noted an increase in macrophage, neutrophil and eosinophil populations following Cl₂ exposure.[8] In addressing potential mechanisms associated with chlorine damage, these cells, especially neutrophils, produce reactive oxygen species from activation of endogenous enzymes such as myeloperoxidase (MPO) and NADPH oxidase. Activated neutrophils exacerbate airway damage, causing an increase in microvascular permeability with the escape of proteinaceous fluid into the airways. AEOL was effective at reducing BAL fluid protein, likely reflecting favorable effects on cellular necrosis and microvascular permeability following Cl₂ exposure.[20, 21] Production of reactive oxygen species may contribute to Cl₂-induced AHR. Previous studies report that exposure to ozone induces several genes such as thyroid hormone- β receptor, nitric oxide synthase, and glutathione reductase.[22] Nitric oxide synthase has been implicated as a potential mechanism leading to airway hyperresponsiveness in response to Cl₂; as a nitric oxide synthase inhibitor (1400W) abrogated the Cl₂-induced changes in responsiveness.[11] The increase in eosinophils is a result consistent with previous reports showing oxidative stress induces eosinophilia.[23] Eosinophilia in an oxidative injury model may be of consequence as oxidative stress induces production of 5-oxo-6,8,11,14-eicosatetraenoic acid (5-Oxo-ETE), a potent chemoattractant for granulocytes that function through G-protein coupled receptors suggesting a possible mechanism by which oxidative stress may increase inflammatory cell influx.[24]

Because the effects of Cl₂ exposure could be at least partially mediated by oxidative stress we measured two markers: glutathione and 4-HNE. The glutathione redox system responds rapidly to oxidative stress, resulting in the oxidation of GSH to GSSG. This can lead to an initial reduction in the ratio of GSH to GSSG, but this is often temporary, as there are compensatory changes in the enzymes involved in glutathione biosynthesis and metabolism. While there are studies reporting an increase in glutathione 24 hours following Cl₂ inhalation, these studies used high concentrations of Cl₂ for longer periods of time.[7, 8, 18, 25] In accordance with our findings, a recent study using similar doses of Cl₂ but for longer periods of exposure found that GSH/GSSG levels were reduced at 1 h but had recovered by 24 h following Cl₂ exposure.[26] Additionally, our previous work has shown an increase in BAL fluid GSH levels 10 minutes following chlorine, but not at 1 hour, indicating a rapid response to oxidative stress. [27] The present study was designed to investigate the longer term (24 h) protective effects of AEOL and it is likely that these times were too late to detect changes in GSH and GSSG.

4-HNE is a biologically active aldehyde and a stable end product of lipid peroxidation. The evaluation of lipid peroxidation through the assessment of 4-HNE is a highly sensitive way of measuring oxidative stress. Increases in 4-HNE levels have previously been reported to be associated with endothelial barrier dysfunction and increases in microvascular leak.[28] Importantly, in the present study mouse lung levels of 4-HNE were reduced to baseline by post-Cl₂ treatment with AEOL, suggesting that lipid peroxidation is an important mechanism for Cl₂ induced lung damage

Mn(III) tetrakis (*N,N*'-diethylimidazolium-2-yl) porphyrin (AEOL10150) is a member of a class of catalytic metalloporphyrins characterized as small-molecular-weight antioxidants. [15] AEOL effectively scavenges peroxynitrite and lipid peroxides and has high SOD and catalase-like activity.[29] Additionally, AEOL is also effective as a scavenger of lipid hydroperoxides. It is unique as a potential therapeutic as it reacts with and detoxifies multiple reactive oxygen and nitrogen species in a catalytic rather than in a stoichiometric fashion. As a highly effective superoxide scavenger AEOL may have prevented the neutrophil induced damage to airway epithelial cells by reducing the toxicity of superoxide by-products released from activated neutrophils.

In summary, we have shown that AEOL is an effective rescue treatment against Cl₂-induced markers of airway injury including airway hyperresponsiveness, airway inflammation, oxidative stress and epithelial proliferation. O'Neill *et al* have shown that AEOL is also effective in preventing the effects of other chemical toxins such as half mustard (2-chloroethyl ethyl sulfide), [15] suggesting that AEOL may be effective as a therapeutic agent against a variety of reactive toxic chemical agents.

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AEOL 10150

Figure 1. Structure of AEOL10150

Figure 1 depicts the skeletal formula for compound, Mn(III) tetrakis (*N,N'*-diethylimidazolium-2-yl) porphyrin, AEOL10150 (AEOL).

Figure 2. AEOL reduces airway responsiveness to methacholine following Cl₂ challenge
 Airway responsiveness was determined by *flexiVent* twenty-four hours following Cl₂ exposure. Panel A shows total respiratory system resistance following MCh in control, Cl₂ only, Cl₂/AEOL and AEOL only treated mice. A two-way ANOVA showed a significant decrease between mice post-treated with AEOL when compared to animals receiving Cl₂ only at the two highest doses of MCh. There was no difference between AEOL treated mice or control groups. Panel B shows the effects of Cl₂ exposure and AEOL treatment on total respiratory system elastance. There were differences only in control vs Cl₂ only mice at the two highest doses of MCh. AEOL treated groups were not significantly different than control or Cl₂ only treated animals. (n=10 per group; **p<0.01, ***p<0.001)

Figure 3. Effects of Cl_2 exposure on the numbers of cells in BAL fluid

Mice were sacrificed 24 hours following Cl_2 exposure. Cl_2 led to a significant increase in total leukocytes compared to controls and AEOL treated groups. AEOL only was not different than control; however, Cl_2 /AEOL treated mice had more total cells in their BAL compared to AEOL only mice. (n=10 per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 4. Cellular composition of BAL fluid following Cl_2 exposure

Differential cell counts were done 24 hours following Cl_2 exposure. Macrophages, neutrophils and eosinophils were significantly elevated in Cl_2 groups compared to control (A–C). AEOL attenuated this increase in macrophages and neutrophils (A and B). There was no difference between control and AEOL treated groups (A–E). There were no differences in any of the other cell types assessed (D and E). (n=10 per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 5. Effects of Cl₂ exposure and AEOL treatment on BAL fluid protein

Protein levels in BAL fluid were assessed by Bradford assay. There was a two-fold increase in total protein at 24 hours after Cl₂ exposure compared to control groups. Post-treatment with AEOL following Cl₂ also significantly limited the increase in protein content in the BAL. (n=10 per group; * p < 0.05).

Figure 6. Epithelial cell proliferation following Cl_2 exposure

Actively proliferating cells were labeled using Ki-67 staining 72 hours following Cl_2 exposure. Cl_2 caused a significant increase in numbers of proliferating epithelial cells in the airways compared to control. AEOL treatment attenuated this effect; however, Cl_2 /AEOL groups were significantly increased compared to controls (panel A). Panels B–D show representative examples of airway sections stained with Ki-67 at 250x magnification. Dark nuclei indicate a positive cell against the methyl green counter stain. (n=6 per group; * p < 0.05., ***p < 0.001)

Figure 7. Effects of Cl₂ exposure and AEOL treatment on glutathione levels in BAL fluid
(A) 24 hours following Cl₂ exposure, GSH levels in the BAL cell fraction show no increase in any of the treatment groups. (B) GSSG levels show no change in any groups 24 hours following Cl₂ exposure. (n=10 per group)

Figure 8. 4-HNE levels in total lung homogenates

Lungs were collected 24 hours following Cl_2 exposure and snap frozen. Total lung homogenates were assessed for 4-HNE levels. Cl_2 induced a significant increase in 4-HNE production compared to controls and AEOL treatment groups. There were no differences between any of the control and AEOL treatment groups. (n=6 per group; * p < 0.05)

Table 1

Mouse pharmacokinetics of AEOL 10150*

	Plasma half-life (hours)	Cmax (ng/ml)	Tmax (minutes)	Clearance (ml/min/kg)
10150 (2 mg/kg. iv)	6.6	4.046	10	14

* Values calculated from curve fit data from 2 mice/time point