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**AUTHOR(S)**
Anderson, DR, Taylor, SL, Fetterer, DP, Holmes, WW

**PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDT-T
3100 Ricketts Point Road

**SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDZ-I
3100 Ricketts Point Road

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Evaluation of protease inhibitors and an antioxidant for treatment of sulfur mustard–induced toxic lung injury

Dana R. Andersona,*, Stephanie L. Taylorb, David P. Fetterera, Wesley W. Holmesa

a Medical Toxicology Branch, Analytical Toxicology Division, U.S. Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010, USA
b University of Delaware School of Nursing, McDowell Hall, 25 North College Avenue, Newark, DE 19716, USA

ABSTRACT

Sulfur mustard (SM)-induced lung injury has been associated with protease activation, oxidative injury and inflammatory response culminating in tissue necrosis. The protease inhibitors aprotinin and ilomastat and the antioxidant trolox were evaluated for efficacy in ameliorating SM-induced lung injury. Anesthetized spontaneously breathing rats (N = 6–8/group) were intratracheally intubated and exposed to 1.4 mg/kg SM (0.35 mg SM in 0.1 ml of ethanol) or ethanol alone by vapor inhalation for 50 min. At 1 min before the exposure rats were treated with one of the following: intravenous aprotinin, 4.4 mg/kg; intraperitoneal (ip) ilomastat, 25 mg/kg; or ip trolox, 500 μg/kg. Aprotinin-treated animals received supplemental 2.2 mg/kg doses at 1 min and 6 h post-exposure (PE). A whole body plethysmograph system was used to monitor pulmonary function (PF) parameters for 1 h before exposure (baseline), and from 5–6 and 23–24 h post-exposure. SM inhalation caused significant increases in several PF parameters, including tidal volume, peak inspiratory flow, peak expiratory flow, end expiratory pause and enhanced pause. Consistent with the reported development of SM-induced pathology, these changes were minimal at the 5–6 h time and significant at the 23–24 h timepoint. At the later time it is known from previous work that airways are becoming obstructed with loose cellular debris, damaged cells and exudate, which contributed to the changes in PF parameters. Treatment with aprotinin or ilomastat eliminated these PF changes, yielding results comparable with controls for each of these parameters. Lung lavage fluid analysis showed that SM caused a significant increase in total protein (TP) and in the cytokines IL-1β and IL-13. Aprotinin treatment prevented the increases in TP and IL-1β production, ilomastat prevented the increased production of IL-13, and trolox treatment did not significantly prevent the SM-related increases in TP, IL-1β or IL-13. Histopathologic examination of lung tissue 24 h post-exposure showed minimal alveolar effects caused by SM, while damage to bronchiolar regions was much more severe due to the highly reactive nature of SM. While aprotinin and ilomastat both alleviated the PF perturbations, surprisingly only aprotinin reduced the observed pathology, both grossly and histologically. These early results indicate that treatment with aprotinin and to a lesser extent ilomastat reduces some of the direct inflammatory response and damage associated with SM-induced lung injury.

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1. Introduction

Sulfur mustard (2,2-dichlorodiethyl sulfide, SM) is a chemical warfare agent that remains a threat to the tactical strategies of the United States Army and the health of the US soldier. More than lethality, SM causes debilitating effects that can leave an exposed individual incapacitated for days to months, depending on the dose. This not only removes the individual from vital duties, but also places a strain on the medical system. Due to the availability, ease of production and storage, persistence and stability, SM is considered a potential terrorist agent that poses a threat to the civilian population as well. Despite decades of research, no completely satisfactory explanation of the toxic action of SM exists, and as a result, the development of an effective antidote has not been realized (Papirmeister et al., 1991).

Respiratory damage resulting from SM inhalation exposure is dose-dependent and latent in onset. Inhalation of low to moderate levels of SM primarily affects the upper respiratory tract...
with alveolar damage occurring only with high dose exposures (Papirmeister et al., 1991). In severe exposures, SM may enter the circulatory system and affect other organ systems, such as the bone marrow and lymphatic tissue. The associated suppression of the immune system leaves an exposed individual susceptible to infection. The combined effect of SM-induced respiratory lesions and infections secondary to SM-related immunosuppression such as bronchopneumonia has been documented as the leading cause of mortality in WWI (Papirmeister et al., 1991) and the Iran-Iraq war (Willems, 1989). Long-term effects of SM inhalation exposure include the development of chronic obstructive pulmonary disease (mustard lung), bronchiectasis, asthma, and fibrosis (Emad and Rezaian, 1997).

Although not fully understood, SM-induced cytoxicity is attributed to its alkylating properties. The lipophilic characteristics of SM allow it to readily cross cellular membranes and react with many biologically important molecules, such as DNA, RNA, proteins and other membrane components. The downstream effects of SM-induced DNA alkylation have been postulated to include an increase in protease induction and activation (Papirmeister et al., 1991). Several investigators have provided supporting evidence for the role for proteases in the pathogenesis of SM-induced damage. These studies cover a wide range of protease classes including serine, cysteine, and matrix metalloproteases (Woessner et al., 1990; Cowan et al., 1991, 1997; Casillas et al., 2000; Guignabert et al., 2005; Shakarjian et al., 2006). Increased proteolytic activity has been reported with the exposure model used herein (Cowen et al., 1997).

In addition to DNA alkylation, SM may react with and deplete reduced glutathione (GSH) levels. GSH is a molecule that is involved in the detoxification of endogenous and exogenous sources of reduced oxygen radicals (Papirmeister et al., 1991). Free oxygen radicals react with membrane phospholipids, creating lipid peroxides that attack adjacent lipids. This chain reaction can lead to alterations in membrane fluidity, loss of membrane protein function, disruption of membrane integrity and ultimately cell death. Anderson et al. (1993) reported that SM may affect lungs by free radical-mediated mediated membrane and tissue damage. This study identified the generation of an ascorbyl radical at 1 and 24 h post-exposure (PE) and thiobarbituric acid reactive products (TBARs) 24 h PE in bronchoalveolar lavage fluid (BALF), which indicated that lipid peroxidation and membrane damage had occurred in response to SM inhalation.

Epithelial and other cell type damage seen in SM toxicity is associated with the release of extracellular proteases and oxidative radicals that are responsible for the majority of all inflammatory, coagulative, and tissue remodeling processes plus cellular membrane destruction (Cowen et al., 1997; Putnam and Royston, 2003; Papirmeister et al., 1991). Under normal or non-diseased physiological states there is a balance between protease inhibitors and proteases as well as the oxidized and reduced molecules. However, in acute tissue injury these balances can be offset, resulting in an environment dominated by proteases and free radicals. Moreover, Guignabert et al. (2005) showed that levels of tissue inhibitors of metalloproteases (TIMPs) were not sufficient to repress increased gelatinase activity in SM-induced respiratory lesions in guinea pigs.

Protease inhibitors and free radical scavengers have been previously suggested and tested as potential treatments for SM-induced injury with some success in both in vitro and in vivo models (Lindsay et al., 1996; Cowan et al., 1997; Putnam and Royston, 2003). Therefore, the aim of this study was to further test the efficacy of supplemental administration of other protease inhibitors and an antioxidant compound, including the broad spectrum protease inhibitors aprotinin and ilomastat and the antioxidant trolox against SM-induced pulmonary toxicity.

2. Methods
2.1. Animals

Male rats (Crl:CD SD BR), weighing 230–260 g, were purchased from Charles River Laboratory and maintained in an animal care facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. In conducting the research described in this report, the investigators adhered to the “Guide for Care and Use of Laboratory Animals,” revised 1996.

2.2. Intubation & SM exposure

This exposure model has previously been described, and the SM dose employed has been shown to produce consistent, non-lethal pathology at 24 h post-exposure (Anderson et al., 1996). SM was diluted to 3.5 mg/ml1 in absolute ethanol. Rats were anesthetized with an intramuscularly (im) administered combination of ketamine (80 mg/kg) and xylazine (10 mg/kg), and intubated with a modified glass Pasteur pipette (ca. 5 cm long) using a laryngoscope to visualize the larynx and a piece of PE50 tubing as a guide tube. The length of the glass endotracheal tube was sufficient for distal placement in the trachea at the point of the larynx and the bifurcation of the trachea. Once in place the endotracheal tube was secured by wrapping a piece of porous tape around the tube and the rostrum of the rat. A glass endotracheal tube was necessary to minimize absorption of SM. 100 μl of SM (0.35 mg) in absolute ethanol or ethanol alone (control) was placed in a water jacketed (37 °C) glass vapor generator (custom fabricated by Atmox Glass, Kennett Square, PA), and spontaneously breathing rats were connected to this device and exposed for 50 min. By the end of the exposure period, the SM in ethanol was completely vaporized and inhaled. This passive exposure system includes an inlet one-way respiratory check valve (Hans Rudolph, Inc., Kansas City, MO) to insure that the only source of air for the animal during the exposure was through the vapor generator. Exhaled air passes through a two-way non-rebreathing Rudolph valve and then through a charcoal-filtered bleach trap to decontaminate any exhaled SM. At the conclusion of the 50-min exposure, the rats were disconnected from the vapor generator, the endotracheal tube was removed, and the rats were returned to their cage.

2.3. Treatment

Rats were assigned to one of five treatment groups (N = 6–8): ethanol exposed/no treatment (control); SM-exposed/no treatment; SM-exposed/aprotinin-treated; SM-exposed/ilomastat-treated; SM-exposed/trolox-treated. 1 min prior to SM exposure rats were treated with aprotinin (SIGMA, St. Louis, MO), 4.4 mg/kg, intravenously (iv); ilomastat (QuickMed Technologies, Inc., Gainesville, FL), 25 mg/kg, intraperitoneally (ip); or trolox (SIGMA, St. Louis, MO), 500 μg/kg, ip. To maintain a sufficient dose of circulating aprotinin (half-life 0.7 h; Levy et al., 1994) rats assigned to this treatment group were administered additional 2.2 mg/kg, iv injections at 1 min and 6 h PE. The total aprotinin dose (8.8 mg ≈ 80,000 KIU) is within the clinically relevant dose range of 60,000–120,000 KIU/kg, and was used by Asimakopoulous et al. (2000), as an anti-inflammatory dose in rats. The dose of ilomastat was recommended to be by Dr. Richard Galardy from QuickMed Technologies, as a dose to use with a systemic injection in rats. Due to the slow clearance of ilomastat (half-life 5–6 h), we felt one dose was adequate for these preliminary studies. The dose for trolox was from Kumar et al. (2001), who reported increased survival times using trolox against sulfur mustard inhalation injury. In their study trolox was given once in 24 h, therefore we followed this same schedule. All drugs were prepared in phosphate buffered saline.

2.4. Whole body plethysmography (WBP)

A Buxco unrestrained WBP system with Biosystems XA software (Buxco Research Systems, Wilmington, NC) was used to monitor pulmonary function (PF) parameters in conscious rats. These parameters included tidal volume, peak inspiratory flow, peak expiratory flow, end expiratory pause, and enhanced pause, an indirect measure of bronchoconstriction. Prior to ethanol (vehicle controls) or SM exposure, rats were placed in the WBP chambers for a total of 2 h (1 h for acclimation and 1 h for data collection) to establish a baseline for PF parameters. At the conclusion of each SM exposure, the rats were placed in their original cages for four hours (PE) as described, through the 24-h time point. Data were averaged by group for each 1-h data collection period.

2.5. Bronchoalveolar lavage (BAL)

Twenty-four hour PE rats were deeply anesthetized, a blood sample was drawn from the dorsal vena cava, and then the rats were exsanguinated by clipping the vena cava and descending aorta. Differential blood cell counts were performed using a CELL-DYN 3500 (Abbott Laboratories, Abbott Park, IL). The trachea was isolated, and a 16 ga. gavage needle was introduced into the trachea and secured using 3/0
suture. The chest cavity was opened and the trachea/heart/lung block was removed. PE50 tubing was passed through the gavage needle and tied into the left mainstem bronchus to serve as a catheter for bronchoalveolar lavage. The left lung was lavaged three times with 3-ml aliquots of saline, which was collected via low-pressure vacuum. The left lung was tied off and removed following lavage. The recovered BALF was immediately pooled and centrifuged (10 min at 300 × g), and the supernatant was removed and stored at −80°C until biochemical analyses were performed.

2.6. Histopathology

Immediately following lavage, the trachea and the four right lung lobes were removed and formalin fixed (10% buffered phosphate formalin, Fisher Scientific, Pittsburgh, PA) for hematoxylin and eosin staining and evaluation. All right lung lobes (cranial, medial, caudal, and accessory) were assessed for extent of damage based on the following parameters: bronchial exudate, bronchial epithelial necrosis, bronchial polymorphonuclear (PMN) infiltrates, bronchial associated lymphoid tissue (BALT) necrosis, alveolar exudate, alveolar hemorrhage, alveolar PMN infiltrates, alveolar epithelial necrosis, and perivascular fibrin/edema. Each parameter was given a score of 0 through 3: 0 = normal; 1 = minimal, present in 1–10% of the section; 2 = moderate, present in 10–50%; 3 = severe, present in greater than 50% of the section. Incidence of pathology was noted for the four right lung sections and then recorded as sections showing pathology versus total number of sections.

2.7. Biochemical evaluation

BALF supernatant was analyzed for total protein (TP), IL-1α, and IL-13. TP levels were determined using a Bio-Rad Protein Assay (Hercules, CA) modified for a 96-well plate. Absorbance was read at 595 nm using a SPECTRAMax Plus 384 (Molecular Devices, Sunnyvale, CA), microplate spectrophotometer. The concentrations of cytokines IL-1α and IL-13 were determined using a Linco-plex multi-plexed cytokine assay (Linco, St. Charles, Missouri) and analyzed on a Bio-Plex multiplex analyzer (Bio-Rad, Hercules, CA).

2.8. Data analysis

The data are shown mean ± S.E. for both text and figures. Group comparisons were conducted using one-way ANOVA followed by Tukey’s post-hoc multiple comparison test. Pathology data were analyzed across groups with a Chi square test. If it was significant, all groups were compared to EtOH and also to HD alone using a Fisher’s exact test. Significant results were identified when p < 0.05.

Fig. 1. Pulmonary function parameters measured on the Buxco whole body plethysmography system. SM inhalation caused significant increases (*) relative to control (EtOH) in several parameters at 24 h post-exposure, including tidal volume (A), peak inspiratory flow (B), peak expiratory flow (C), end expiratory pause (D) and enhanced pause (E). Treatment with aprotinin, ilomastat and to a lesser extent trolox ameliorated these effects.
3. Results

3.1. Pulmonary function parameters

SM inhalation caused significant increases in several parameters, including tidal volume, peak inspiratory flow, peak expiratory flow, end expiratory pause and enhanced pause (Fig. 1A–E). Consistent with the reported development of SM-induced pathology, these changes were minimal at the 5- to 6-h timepoint and significant at the 23- to 24-h timepoint. It is known from previous work (Anderson et al., 1996) that by 24 h, airways are becoming obstructed with loose cellular debris, damaged cells and exudate. These physical obstructions may contribute to the changes in PF parameters observed here. Increases in these parameters are consistent with the deep gasping breaths of the SM-exposed animals having to work harder to breathe through partially occluded airways. Treatment with aprotinin or ilomastat eliminated these changes, yielding results comparable with ethanol controls for each of these parameters. The antioxidant trolox generally was not as effective as the two protease inhibitors, reducing only the SM effect on tidal volume (Fig. 1A), end expiratory pause (Fig. 1D) and enhanced pause (Penh, Fig. 1E).

3.2. Biochemical evaluation

We, as well as others, have used BALF to characterize the temporal biochemical changes within the bronchoalveolar region of the lung following a SM exposure, and these changes are quantified to determine the efficacy of tested therapeutic compounds (Anderson et al., 1997; Cowan et al., 1997; Kumar et al., 2001; Guignabert et al., 2005). BAL is a technique that can be extremely useful in the biochemical assessment of pulmonary health (Henderson, 1989). This technique samples the epithelial lining of the lungs, which is the body fluid closest to the site of injury caused by inhaled toxicants, and allows for the evaluation/quantification of the level of cytotoxicity or inflammation induced by a toxicant (Henderson, 2005). Results from the analysis of BALF were less consistent than the PF data. SM caused a significant increase in TP, IL-1α and IL-13. Aprotinin treatment prevented the increases in TP and IL-1α production, ilomastat prevented the increased production of IL-13, and trolox treatment did not significantly prevent the SM-related increases in TP, IL-1α and IL-13 (Fig. 2A–C). Trolox did reduce the depletion by SM of total glutathione levels in BALF; however, due to high variability of this data it was not statistically significant (data not shown).

3.3. Histopathology

Histology data show minimal alveolar effects caused by SM, which is consistent with previous reports using this model (Anderson et al., 1996), while damage to bronchiolar regions is
4. Discussion

In the current study we have evaluated three compounds, aprotinin, ilomastat, and trolox, for efficacy against SM-induced lung injury. These compounds were evaluated with varying degrees of effectiveness by WBP, analysis of BALF, and histology. Our results indicate that aprotinin was the most effective of the three compounds followed by ilomastat, and trolox was the least effective.

Aprotinin is a serine protease inhibitor that functions by irreversibly binding to proteases that contain a serine amino acid residue in their catalytic site inhibiting the actions of trypsin, plasmin, kallikrein, elastase, urokinase, and thrombin in an increasing dose-dependent manner, respectively (Putnam and Royston, 2003). These proteases play important cellular and humoral roles in hemostatic dysfunction and activation of the inflammatory response by activating cytokines, coagulation factors, fibrinolytic, and complement cascades. Prior studies using our model have shown a time-dependent up-regulation of gene transcripts that are activated by serine proteases, such as coagulation factor III and tissue factor (Anderson et al., 2000). Aprotinin (Trasylol®) is an FDA approved drug used in transplant and orthopedic surgery to reduce perioperative bleeding and hemorrhaging. In recent studies using this model, and as described here, we have observed that aprotinin was able to control reactive oxygen species generation (Holmes et al., 2006), eliminate SM-induced gross pulmonary hemorrhaging up to 24 h post-exposure (Fig. 3), and restore lung function parameters to near control levels (Fig. 1). The role of aprotinin in maintaining alveolar/capillary barrier integrity was also evident in reduced TP levels (Fig. 2A) in BALF analysis. Further evidence of this was shown histologically (Fig. 4) where significantly reduced perivascular edema was observed. This also could be due, in part, to the reduced BALT necrosis. Since one role of BALT is to drain excess fluids, plasma protein and particulate matter from pulmonary tissue (Witschi and Nettasheim, 1982), injury to BALT could lead to edema and respiratory insufficiency (Anderson et al., 1996; Baum, 1974). Histologically, protection by aprotinin was most evident in the significantly lower bronchiolar effects (Fig. 4), which indicate less direct injury to airways, and in clearer airways, which was corroborated by the PF data.

Iломastat is a broad spectrum matrix metalloprotease (MMP) inhibitor that exerts its inhibitory effects on MMPs that are characterized by a zinc binding motif in their active site. MMPs are involved in the degradation of the extracellular matrix components, growth factors and receptors that are essential for healing (Schultz et al., 1992). MMP-mediated degradation of the extracellular components results in the activation of chemokines as well as MMP-9, which together produce inflammation and contribute to the pathogenesis of SM toxicity (Sabourin et al., 2002). Guignabert et al. (2005) found that MMP-9 levels increased in BALF of SM-exposed guinea pigs, which could be associated with airway epithelial detachment. Several studies have demonstrated the ability of tissue MMPs and ilomastat to inhibit SM-induced MMP-9 activity, inflammation levels, and airway epithelial lesions in guinea pigs pretreated intratracheally or subcutaneously with doxycycline (Guignabert et al., 2005), microvesicle formation in human skin cells treated with ilomastat (Schultz et al., 1992), and reduced eye opacity and bullae scores in rabbits treated with ilomastat (Schultz et al., 1992). We previously demonstrated increases in pro-inflammatory cytokines and lipid peroxidation in rats following SM inhalation (Anderson et al., 2006). Fig. 1 shows several lung function parameters where ilomastat treatment clearly ameliorated SM-induced changes. Pemberton et al. (2005) showed that aerosolized ilomastat reduced MMP-mediated chronic lung degeneration and the permanent loss of pulmonary function that is associated with emphysema in mice through the inhibition of macrophage and neutrophil recruitment into the lungs. In our biochemical analysis of lavage fluid, ilomastat significantly reduced levels of the pro-inflammatory cytokine IL-13 compared to SM alone (Fig. 2C). IL-13 is a mediator of tissue fibrosis and is linked to MMP-9 levels (Lee et al., 2001), which are inhibited by ilomastat. While Fig. 1 shows efficacy in crucial PF parameters, this was not supported by prevention of lung pathology (Fig. 4).

Trolox is a water soluble vitamin E analog that is capable of penetrating cellular membranes where it can protect the cells from oxidative damage. In the treatment of oxidative stress, the best antioxidant depends on the specific molecules causing the stress and the cellular or extracellular location of these specific molecules (Naghi, 2002). Since lipid peroxidation is considered a component of SM toxicity, an antioxidant that is active in cellular membranes might eliminate or at least ameliorate membrane damage. Bhattacharya et al. (2006) showed that in SM-exposed human peripheral blood lymphocytes pretreatment with trolox significantly reduced cell death, lactate dehydrogenase (LDH) leakage, and peroxide generation. Trolox, ip, has also been reported to increase survival time and protect the lung from oxidative damage following SM challenge in mice (Kumar et al., 2001). Using our model system we have observed a reduction in BALF LDH and γ-glutamyl transferase (GGT) levels and a reduction in peroxide generation following SM inhalation with concurrent administration of trolox (Anderson and Holmes, 2005; Schroth et al., 2006). The use of antioxidants to protect against SM lung injury has been investigated by several others with varied success. McClintock et al. (2006) used intratracheally instilled liposome encapsulated N-acetyl cysteine to reduce 2-chloroethyl-ethyl sulfide (CEES) injury to rat lungs. We previously (Anderson et al., 2000) used N-acetyl cysteine with some success against sulfur mustard-induced lung injury in rats. Wilde and Upshall (1994) evaluated several cysteine esters that provided some protection against SM in an in vitro model. In this study, trolox was generally ineffective against SM-induced changes. We did observe a reduction in SM-induced changes in the lung function parameters tidal volume (Fig. 1A), end
expiratory pause (Fig. 1D) and enhanced pause (Fig. 1E) with trolox, but these were the only positive results noted. The negative results with trolox in this study may be the result of the single relatively low dose of drug given. We hope to address this deficiency in future work.

The doses and routes of drug administration used in this study were chosen based on what was found in the literature (aprotinin and trolox) or manufacturers recommendations (ilomastat). While these provided a starting point for this study, using identical routes of administration would have been desirable. The next step in this work will be to evaluate these compounds using common routes of administration, either iv or inhalation treatment. The compounds administered iv were not as efficacious as the aprotinin, so we will evaluate them using the same route as aprotinin. We also feel that aiming the treatment directly at the site of injury, i.e., via inhalation, may significantly improve lung function over the systemic administration models. As stated previously, Pemberton et al. (2005) demonstrated efficacy with aerosolized ilomastat against emphysema-induced loss of pulmonary function.

In addition to the differing routes of administration, in our future work we will investigate longer-term effects (up to 28 days PE) of SM inhalation. All of our previous work has been aimed at documenting the development of the SM-induced lung injury up to 24 h. Calvet et al. (1994) documented respiratory effects in guinea pigs up to 7 days post-exposure. In addition to measures described in this report, we intend to evaluate the effect of therapies on SM-induced lung fibrosis (in addition to our routine measures), which Emad and Rezaian (1997) indicate is typical of SM inhalation exposure.

Conflict of interest

None.

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