Mesna improves outcomes of sulfur mustard inhalation toxicity in an acute rat model


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Running Title Page

Running title: Protection from sulfur mustard toxicity by mesna

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Non-standard Abbreviations:
Cq: quantification cycle
DTNB: 5,5'-dithiobis(2-nitrobenzoic acid)
HCO₃⁻: bicarbonate
hpe: hours post-exposure
HPLC-MS/MS: high performance liquid chromatography tandem mass spectrometry
MES: mercaptoethanesulfate
mesna: sodium 2-mercaptoethane sulfonate
MPO: myeloperoxidase
NAC: N-acetylcysteine
NM: nitrogen mustard
ROS: reactive oxygen species
RT-qPCR: reverse transcription quantitative PCR
SM: sulfur mustard
SpO₂: peripheral oxygen saturation
tPA: tissue plasminogen activator

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Abstract

Inhalation of high levels of sulfur mustard (SM), a potent vesicating and alkylating agent used in chemical warfare, results in acutely lethal pulmonary damage. Sodium 2-mercaptoethane sulfonate (mesna) is an organosulfur compound that is currently FDA-approved for decreasing the toxicity of mustard-derived chemotherapeutic alkylating agents like ifosfamide and cyclophosphamide. The nucleophilic thiol of mesna is a suitable reactant for the neutralization of the electrophilic group of toxic mustard intermediates. In a rat model of SM inhalation, treatment with mesna (3 doses: 300 mg/kg intraperitoneally 20 min, 4 h, and 8 h post-exposure) afforded 74% survival at 48 h, compared to 0% survival at less than 17 h in the untreated and vehicle-treated control groups. Protection from cardiopulmonary failure by mesna was demonstrated by improved peripheral oxygen saturation and increased heart rate through 48 h. Additionally, mesna normalized arterial pH and pACO₂. Airway fibrin cast formation was decreased by more than 66% in the mesna-treated group at 9 h after exposure compared to the vehicle group. Finally, analysis of mixtures of a mustard agent and mesna by a DTNB assay and HPLC-MS/MS demonstrate a direct reaction between the compounds. This study provides evidence that mesna is an efficacious, inexpensive, FDA-approved candidate antidote for SM exposure.
Significance Statement

Despite the use of SM as a chemical weapon for over 100 years, an ideal drug candidate for treatment after real-world exposure situations has not yet been identified. Utilizing a uniformly lethal animal model, the results of the present study demonstrate that mesna is a promising candidate for repurposing as an antidote, decreasing airways obstruction and improving pulmonary gas exchange, tissue oxygen delivery and survival following high level SM inhalation exposure, and warrants further consideration.
Introduction

Exposure to mustard agents, including the chemical weapon sulfur mustard (SM; bis[2-chloroethyl] sulfide; “mustard gas”), results in extensive cellular damage and death through mechanisms involving crosslinking of critical biomolecules, including proteins and nucleic acids, DNA damage, oxidative stress, and disruptions in metabolism (Papirmeister, 1991; Naghii, 2002; Ghabili et al., 2011). SM attacks epithelial tissues, the lungs being a primary target. Acute respiratory symptoms from exposure during combat/conflict include discomfort resulting from inflammation of the upper airways, cough, rhinorrhea, and, after exposure to high concentrations, dyspnea; importantly, the majority of fatalities that occur within hours, days, or a few weeks after exposure to SM are due to injuries to the respiratory tract (Papirmeister, 1991; Ghabili et al., 2010). Currently, there are no effective antidotes for SM inhalation.

Mustard agents form toxic intermediates, especially in an aqueous milieu, such as the airway’s epithelial lining fluid. These electrophilic intermediates attack electron-rich regions, such as sulfhydryl groups (Naghii, 2002). We and others have reported previously that thiol compounds, such as N-acetylcysteine (NAC) and glutathione (GSH), as well as thiopurines and thiosulfates, may directly interact with mustards or decrease their cytotoxicity indirectly (Callaway and Pearce, 1958; Gross et al., 1993; Rappeneau et al., 2000a; Rappeneau et al., 2000b; Liu et al., 2010; Tewari-Singh et al., 2011; Stenger et al., 2017; Sawyer, 2020). Moreover, these agents appear to diminish the toxicity of SM and SM analogs in porcine, rat, and mouse models (Callaway and Pearce, 1958; Kumar et al., 2001; McClintock et al., 2006; Jugg et al., 2013; Gupta et al., 2021). In prior studies, we also found that intratracheally-delivered tissue plasminogen activator (tPA) clears fibrin casts from the airways after mustard agent inhalation exposure, substantially decreasing morbidity and mortality in an acute rat model (Veress et al., 2013; Veress et al., 2015). Despite the demonstration of some degree of efficacy, none of these compounds are currently FDA-approved for clinical use in the treatment of inhalational exposure to SM.
Sodium 2-mercaptoethane sulfonate (mesna; brand names Mesnex and Uromitexan) is a water-soluble thiol compound currently utilized as a chemoprotective agent that reacts with and detoxifies metabolites of chemotherapeutic drugs such as cyclophosphamide and ifosfamide (Shaw and Graham, 1987; Schoenike and Dana, 1990; Dechant et al., 1991). Cyclophosphamide and ifosfamide are nitrogen mustard derivatives and alkylating agents that are metabolized in the liver to active metabolites. Mesna can interact with these activated forms, and/or with further breakdown products thereof (e.g., acrolein) to limit toxicity from the off-target effects of these chemotherapy drugs, thereby preventing dose-limiting side-effects. Due to the chemical similarity between the activated cyclophosphamide/ifosfamide intermediates and reactive mustard intermediates, there is potential for the therapeutic use of mesna to treat individuals exposed to SM.

During in vitro model experiments utilizing the exposure of cultured cells to mustard compounds, mesna treatment provided protection from the toxic effects of mustards, including DNA damage and cell death (Jost et al., 2017; Jost et al., 2019). These findings were similar to what was previously observed with NAC (Rappeneau et al., 2000a). Mesna-mediated protection from mustards was observed in experiments involving the pretreatment of cells with mesna before mustard exposure (Jost et al., 2017; Jost et al., 2019), and the degree of NAC-mediated protection was greatest when cells were treated concomitantly with mustard exposure (Rappeneau et al., 2000a). Consequently, it is plausible that the direct interaction of mustards with these compounds is at least partly responsible for the protective effects.

Mesna exhibits antioxidant properties, due to the ability of its thiol group to scavenge reactive oxygen species (ROS), and has demonstrated a protective effect in numerous animal models of disease/injury involving oxidative stress and damage (Shusterman et al., 2003; El-Medany et al., 2005; Sener et al., 2005; Ypsilantis et al., 2008; Keeney et al., 2018; Hagar et al., 2020; Abd El-Baset et al., 2021). It can also inhibit the activity of molecules involved in ROS production, such as myeloperoxidase (MPO) (Jeelani et al., 2017). In addition, mesna has been
found to have significant anti-inflammatory effects on upstream and downstream targets (Shusterman et al., 2003; Ypsilantis et al., 2008; Hagar et al., 2020; Abd El-Baset et al., 2021). Therefore, mesna treatment may provide benefit by acting upon the severe inflammation and oxidative stress that occur as a result of exposure to mustards.

While preliminary studies have described potential therapeutics to treat SM inhalation exposures, an ideal candidate for use in real-world casualty situations has not yet been identified. Based on the biochemical evidence for the neutralization of mustard compounds by mesna, the relative success of thiols in the treatment of cells and animals exposed to mustards, and the detoxifying, antioxidant, and anti-inflammatory capabilities of mesna, we sought to test the efficacy of mesna in an acutely lethal animal model of high-dose SM inhalation injury and further characterize the interaction between the reactive groups of mesna and mustard. Herein, we demonstrate the efficacy of mesna in decreasing acute lung injury and protecting from mortality in a rat model of SM inhalation and describe the direct interaction between mesna and mustard.
Materials and Methods

Chemicals

For rat inhalation exposures, sulfur mustard (SM, 99.4% purity by NMR) was synthesized at the University of Colorado Denver – Anschutz Medical Campus (UCD-AMC) and pharmaceutical mesna was utilized (Baxter Healthcare Corporation, NDC 10019-953-01). The pharmaceutical formulation of mesna consists of 100 mg/mL mesna, 0.25 mg/mL disodium EDTA, 10.4 mg/mL benzyl alcohol, and NaOH for pH adjustment to 7.5-8.5 in sterile water for injection (SWFI) and has an osmolarity of 1000-1500 mOsmol/L. Details regarding the corresponding vehicle control can be found in the Supplemental Materials and Methods. Chemicals utilized for in vitro studies are listed in the Supplemental Materials and Methods.

Study animals and sulfur mustard inhalation exposures

All animal procedures were approved by the UCD-AMC Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (8 to 9 weeks old, weighing 250-275 g, obtained from Charles River Laboratories) were housed in an AAALAC International accredited facility and provided food and water ad libitum. Rats were given at least 7 days to acclimate to the UCD-AMC housing facility prior to study.

On the morning of exposure, animals were randomized into treatment groups, and then exposed to 4.2 mg/kg SM vapor for 50 min using a previously described model (Anderson et al., 1996; Veress et al., 2015). Naive control animals were housed in the same location as SM-exposed animals but were not subject to anesthesia/intubation/vapor generators.

Rats were euthanized at 9 h, 24 h, 48 h, or 15 days post-exposure, or earlier if severe distress criteria were met, which was assessed as previously described (Veress et al., 2013; Nick et al., 2020). For studies lasting 15 days, loss of more than 30% of pre-exposure body weight served as an additional criterion for euthanasia prior to end of study. Additional details
Regarding animal monitoring, care, and euthanasia criteria are provided in the Supplemental Materials and Methods.

**Administration of mesna to study animals**

Prior to SM exposure, animals were randomized into four treatment groups: Naive; SM-exposed and given no treatment (SM); SM-exposed and given vehicle (SM + vehicle); and SM-exposed and given mesna (SM + mesna). Mesna (300 mg/kg/dose, undiluted from its pharmaceutical formulation) was administered by intraperitoneal (i.p.) injection. Vehicle control animals received an equivalent volume (3 ml/kg) of the vehicle formulation (see Supplemental Materials and Methods). Treatments were administered at 20 min, 4 h, and 8 h post-SM exposure. In selected studies, treatments were administered at 2 h, 4 h, and 8 h post-exposure.

**Peripheral oxygen saturation and heart rate measurements**

Peripheral oxygen saturation (SpO₂) and heart rate were measured in conscious rats using a MouseOx Plus with a large Rat Collar Sensor (Starr Life Sciences, Oakmont, PA). Baseline measurements were taken one day prior to study. At each timepoint, the average of 3 SpO₂ and 3 heart rate measurements taken within 5 min were utilized.

**Arterial blood gas measurements**

Immediately prior to euthanasia, arterial blood was collected from anesthetized animals, and arterial blood gas analysis performed as previously detailed (Nick et al., 2020).

**Lung fixation and airway cast scoring**

At euthanasia, lungs were inflation-fixed with 4% paraformaldehyde in PBS at 20 cm H₂O for 20 min then removed en bloc. Airways cast scoring of all 5 lobar bronchi was conducted and a composite cast score was calculated using a previously described microdissection
technique and quantification methodology (Veress et al., 2010; Veress et al., 2013; Nick et al., 2020).

**Reverse transcription quantitative real-time PCR (RT-qPCR)**

RT-qPCR was performed on whole lung tissue (perfused blood-free) collected at 9 h post-exposure. Methodology for RT-qPCR analysis is detailed in the Supplemental Materials and Methods.

**In vitro analyses of the mesna-NM reaction**

**DTNB Assay**

Stock solutions of NM (in ethanol) and mesna (in distilled, deionized, and Milli-Q-purified water) were made less than an hour before mixing, and then diluted in reaction buffer (0.1 M Na₂HPO₄, 1 mM EDTA, pH 8.0), mixed together or with reaction buffer alone, and incubated in the dark at room temperature. At the indicated times, 10 microliters of the mixture was removed, added to 100 microliters of reaction buffer and 2 microliters of 10 mM DTNB, and immediately analyzed by measuring the absorbance at 412 nm.

**HPLC-MS/MS analysis of mesna-NM**

Stocks of mesna and NM were prepared in ultrapure water and absolute ethanol, respectively, immediately before use. 1.5 mM mesna and 6 mM NM was then combined in 0.1 M Na₂HPO₄, 1 mM EDTA, pH 8.0 (DTNB reaction buffer) and incubated for 30 min at room temperature, then transferred to -80°C. Samples were then shipped on dry ice to South Dakota State University and stored at -80°C until analysis. The frozen sample solutions (10 ml each; stored in 15 ml centrifuge tubes) of mesna, NM, a mixture of mesna and NM, and solvent blank were placed in separate polypropylene disposable beakers and allowed to thaw at room temperature. The entire volume of the thawed solution was syringe filtered with a 33 mm, 0.2
μm polyethersulfone (PES) syringe filter into another 15 ml centrifuge tube. An aliquot (1 ml) of
the filtered solution was then placed in a 2-ml autosampler vial and capped for HPLC-MS/MS
analysis. The remaining solution was placed in a -80°C freezer until needed. Methodology for
HPLC-MS/MS analysis is detailed in the Supplemental Materials and Methods.

Data analysis and statistics

Statistical analyses were performed using GraphPad Prism versions 8.4.3 and 9.5.0
(GraphPad, San Diego, CA). Details of statistical analyses are provided in the Supplemental
Materials and Methods. Arterial blood gas and cast score data are presented using violin plots
(medium smoothing), with a solid line for the median and dashed lines for the quartiles. All other
summary data are displayed as means with standard deviation (SD). A p-value <0.05 was
considered significant.
Results

Mesna increases survival and improves outcomes in a rat model of sulfur mustard inhalation exposure

In order to examine the efficacy of mesna treatment in vivo, rats were exposed to high-concentration SM using an established vapor inhalation model (Anderson et al., 1996; Veress et al., 2015). The regimen utilized for treatment was based on an established administration scheme for mesna to prevent toxicity from alkylating chemotherapy agents in rats and humans (Menetrey et al., 1999; Kanat et al., 2006; Hensley et al., 2009), and the measurement of a circulating half-life of mesna of 36.3 min in naive rats (Supplemental Figure 1), which was in close agreement with that observed in previous studies (Shaw et al., 1986; Verschraagen et al., 2004). Although mesna is FDA-approved only for oral and intravenous use, neither of those routes were deemed practical for this study, thus mesna was administered intraperitoneally (i.p.) as we have done previously (Stabler et al., 2009).

At the dose of SM utilized (4.2 mg/kg), the animals consistently succumbed to the SM-induced injury within 16.5 hours when no intervention was introduced (0% survival for SM and SM + vehicle control groups, Figure 1). In animals given three sequential doses of mesna i.p. at 20 min, 4 h, and 8 h post-SM exposure, 85.7% survival was observed at 24 h post-exposure, and 73.5% of rats survived until 48 h (end of the study) (SM + mesna, Figure 1). In two smaller, subsequent studies utilizing this mesna treatment regimen, animals were monitored for 15 days post-SM exposure. Remarkably, an 18.4% survival proportion was observed for the mesna-treated group on day 15, compared to 0% survival for the SM + vehicle group, which had all succumbed to injury by 16.2 h post-exposure (Supplemental Figure 2). When the initial mesna dose was delayed until 2 h post-SM exposure, 90.0% of animals survived to 24 h, but proportionally fewer animals survived to 48 h compared to the regimen in which mesna was initiated at 20 min post-exposure (45.0% vs. 73.5%, respectively) (Supplemental Figure 3).
After exposure to SM, rapid decreases in tissue oxygen delivery [assessed by peripheral oxygen saturation (SpO₂) measured via pulse oximetry] and heart rate were observed (Figure 2). Mesna-treated animals demonstrated a delay in the decrease in tissue oxygen delivery compared to the SM and SM + vehicle control groups (Figure 2A). While the heart rates of both control and mesna-treated animals declined immediately after exposure (2 h; note that animals have not fully recovered from the effects of anesthesia during this time), the heart rates of mesna-treated animals improved modestly and were better maintained following this initial drop as compared to the continued decrease in heart rate observed in the control animals (Figure 2B).

Upon meeting euthanasia criteria (due to distress or end of study), rats were anesthetized and arterial blood was collected. SM exposure induced a decrease in blood pH that was prevented by mesna treatment (Figure 3A). Arterial partial pressures of CO₂ (pACO₂) and O₂ (pAO₂) were significantly altered in SM-exposed control animals, indicating a decrease in pulmonary gas exchange in these animals (Figure 3B and C). Mesna treatment decreased pACO₂ and increased pAO₂ relative to control animals. Blood concentrations of bicarbonate (HCO₃⁻) were not significantly different between vehicle- and mesna-treated animals (Figure 3D).

**Mesna treatment decreases airway obstruction in SM-exposed rats**

As previously described in rats, rabbits, dogs, swine, and humans, inhalation of SM results in destruction of the respiratory epithelium and the formation of obstructive, fibrin-rich casts within the airways (Papirmeister, 1991; Anderson et al., 1996; Fairhall et al., 2010; Veress et al., 2010; Veress et al., 2013; Veress et al., 2015). In the current investigation, histologic examination of lung tissue from SM-exposed rats (11 to 20 h post-exposure) demonstrated epithelial sloughing and necrosis, along with epithelial and smooth muscle hypertrophy and hyperplasia, septal thickening, and inflammatory cell infiltration (Supplemental Figure 4).
increase in fine fibers was also noted in the periairway and perivascular areas, which may be indicative of collagen deposition as previously reported (Veress et al., 2010). Through the use of morphometric analysis using airway microdissection, obstructive casts were observed and measured in the present study, and the airways became progressively more occluded over time after SM exposure (Figure 4 and Supplemental Figure 4). Treatment with mesna resulted in a 66.4% reduction in the degree of occlusion at 9 hours post-exposure (hpe) (Figure 4A). In animals meeting euthanasia criteria (due to distress or end of study) between 9 and 24 hpe, airway occlusion trended lower for the mesna-treated group, but a statistically significant difference compared to the vehicle-treated group was not observed (Figure 4B). This decrease/delay in airway casts may account for the improvement in the clinical outcomes described above, in particular the normalization of arterial carbon dioxide and pH.

SM exposures induces changes in whole lung transcripts that are not significantly impacted by mesna treatment

RNA was isolated from whole lung homogenates of SM-exposed animals obtained at 9 hpe, and transcripts for targets of interests were quantified using reverse transcription quantitative PCR (RT-qPCR). To examine for changes in pathways of interest and relevance to SM-induced acute lung injury (Dillman et al., 2005; Tewari-Singh et al., 2012; Jowsey and Blain, 2014; Jugg et al., 2016; Tahmasbpour et al., 2016; White et al., 2016; Borna et al., 2019), an initial gene expression study on a small group of animals (n=3-4 per treatment group) was performed using PrimePCR Arrays (Supplemental Figures 5-8). Targets of potential importance were investigated in a larger, subsequent study (n=7-8 animals per treatment group) (Supplemental Table 1, Figure 5 and Supplemental Figure 9). Transcripts with expression levels that were significantly altered after SM exposure were identified (Figure 5). These included transcripts for genes with roles in coagulation (e.g., F3 and Plat), hypoxia response (e.g., Nos2 and Hmox1), oxidative stress (e.g., Sod2 and Nfe2l2), and anti-apoptosis/TNF/NFκB signaling.
(e.g., Nfkbib and Tnfrsf1a). Interestingly, although the expression levels of several targets tended towards being different between mesna- and vehicle-treated animals (e.g., F3, Hmox2, IL-6, Nfkbib), no transcript levels in SM-exposed animals were significantly impacted by treatment with mesna.

**Reaction of the mesna thiol with mustard compounds**

For in vitro studies examining the reaction of mesna with a mustard compound, nitrogen mustard (NM; mechlorethamine; mustine) was utilized in place of SM due to safety, security, and infrastructure required for handling and transporting SM. Nitrogen mustard is structurally similar to SM, mimics SM-induced toxicity and injury, and is a suitable substitute for examining the reaction of mesna with the reactive group of a mustard.

Examination of the kinetics of the reaction of NM with mesna was performed by monitoring the loss of the thiol of mesna using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman’s Reagent) in the presence of increasing concentrations of NM. The reaction of a thiol with DTNB yields spectrophotometrically detectable TNB₂⁻, allowing the measurement of free thiols in solution. Reaction mixtures of various concentrations of NM with a fixed concentration of mesna were sampled over a 4 h time course for measurement of thiol content (Figure 6A). In the absence of NM, the thiol of mesna reacted with DTNB to form a colored product; in the presence of NM, there was a reduction in the formation of colored product, which is interpreted as a loss of the free thiol of mesna due to the reaction of this group with NM. Nitrogen mustard alone had no observable effect on DTNB (Supplemental Figure 10).

Using a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) approach [modified from Donkor et al. (Donkor et al., 2022)], the formation of a reaction product between NM and mesna via interaction of the thiol group of mesna with NM was identified (Figure 6B). HPLC-MS/MS analysis of aqueous solutions of NM and mesna showed an abundant 242 m/z ion, corresponding to the 1:1 reaction of a mercaptoethanesulfate (MES)
and NM with subsequent hydrolysis to form the product shown in Figure 6B. This product was further evaluated by performing a product ion scan of the precursor 242 m/z ion. The fragmentation of this ion produced abundant fragments which were assigned to the NM-MES product and included portions of both NM and MES. For example, the signal at 198 m/z corresponds to the NM-MES product following fragmentation of the ethyl hydroxylate moiety.
Discussion

Mesna is an effective rescue countermeasure for severe SM inhalation in an acute rat model. Treatment with mesna delayed airway fibrin cast formation, improved oxygenation, and improved alveolar ventilation, cardiopulmonary function, and survival in a uniformly lethal model. Arterial blood gas findings, especially pH and pACO₂, indicate that alveolar ventilation was improved with mesna treatment, and this, combined with the delay in cast formation in the larger airways, suggests that airways obstruction may have been diminished by mesna treatment. Because it is widely and safely utilized in cancer medicine and rheumatology, FDA-approved, and inexpensive, mesna may be a useful adjunctive therapy for casualties of high concentration SM inhalation exposure.

While several RNA transcripts impacted by SM exposure demonstrated a trend towards a difference with mesna treatment, there were no SM-related changes in gene expression that were significantly altered by mesna therapy. One important caveat of the RT-qPCR data is that all analyses were performed on samples obtained from animals 9 hours after exposure as, after this time, control animals begin meeting euthanasia criteria; this short time period may not be sufficient for the detection of all transcript changes resulting from SM exposure, and this may especially not be long enough to detect mesna-mediated impacts. Additionally, these analyses were performed on whole lung tissue, which dramatically decreases the ability to detect transcriptional changes in specific cell types/subsets. Inflammatory cells, particularly neutrophils and macrophages/monocyte-derived macrophages, infiltrate the lungs and airways after SM inhalation (Calvet et al., 1996; Calvet et al., 1999; Anderson et al., 2000; Malaviya et al., 2010; Veress et al., 2010; Jugg et al., 2013; Xiaoji et al., 2016; Supplemental Figure 4). These cells (as well as airway epithelial cells) have been shown to produce numerous inflammatory cytokines, chemokines, and other pro-inflammatory mediators in response to mustard agents (reviewed in Shakarjian et al., 2010; Weinberger et al., 2011; Khazdair et al., 2015). Differences
in inflammatory cell recruitment (numbers and/or cell subtypes) could, in part, account for the heterogeneity of the inflammatory response transcripts that was observed. Future work to clarify the mechanisms of protection by mesna may require more sensitive analyses, such as single-cell RNA sequencing. Furthermore, it is plausible that mesna might not affect transcript levels but might affect protein expression and/or function at the protein level. Previous studies have described the ability of mesna to regulate the enzymatic activity of MPO (Jeelani et al., 2017), inhibit the activity of NF-κB in the presence of oxidative damage (Ypsilantis et al., 2008), and normalize SOD2 activity and protein levels of TNFα and IL-1β in an acute pancreatitis rat model (Hagar et al., 2020). Interestingly, mice that are genetically deficient in MPO are resistant to mustard toxicity in exposed skin (Jain et al., 2014).

The results obtained using a DTNB assay and HPLC-MS/MS demonstrate a direct interaction between mesna and a mustard. This observation is not unexpected, as previous interactions between mesna and mustard derivatives (e.g., cyclophosphamide, ifosfamide) as well as similar alkylating agents (e.g., cisplatin) have been described both in vitro (Oprea et al., 2001) and in vivo (Manz et al., 1985). Although mesna is also able to decrease urotoxicity of cyclophosphamide/ifosfamide by neutralizing acrolein (metabolite of these compounds) (Scheef et al., 1979; Brock et al., 1981), acrolein is not a metabolite of sulfur mustard or cisplatin. However, as acrolein can be generated endogenously in the presence of inflammation and oxidative stress (Stevens and Maier, 2008), acrolein produced from host molecules may be a target for mesna in the SM inhalation model. Interestingly, while mesna administration decreases the off-target (i.e., non-cancerous cell) effects of activated alkylating agents and their metabolites, it does not decrease in vivo anti-tumor efficacy of cyclophosphamide/ifosfamide (Scheef et al., 1979), and the same is true for cisplatin, as long as mesna is given after cisplatin in a separate injection (Dorr and Lagel, 1989). Although mesna predominantly accumulates in
the kidney/urinary tract after administration, low levels of mesna can be detected in other tissues, including the lungs (Shaw et al., 1986; Verschraagen et al., 2004), and the observations made in our study suggest it is having effects in the airways. Given that, in the present study, the highly reactive mustard compound was delivered to the airways and mesna was delivered i.p., it is possible that mesna is acting upon less reactive intermediates, metabolites, or other endogenous compounds involved in response to oxidative stress and defense against reactive chemicals and chemical imbalances. For example, relationships between GSH levels and the cellular metabolism of SM, cyclophosphamide, and cisplatin have been reported (Ono and Shrieve, 1987; Mistry et al., 1991; Gross et al., 1993; Pendyala et al., 1997; Townsend et al., 2003). Further, mesna has been shown to impact levels of GSH and its precursor, cysteine (Stofer-Vogel et al., 1993; Souid et al., 2001; Stabler et al., 2009; Li et al., 2013; Abd El-Baset et al., 2021). As mesna treatment can increase intracellular cysteine levels (Stofer-Vogel et al., 1993), resulting in increased intracellular GSH, this pathway may be involved in the mechanism of mesna efficacy in our model. Unlike tPA or matrix proteases, mesna is not thought to have an ability to break down airway casts. Rather, we believe that neutralization of mustard or mustard intermediates/metabolites and decreasing of mustard-induced epithelial damage may be responsible for the delay/slight decrease in airway cast formation. As casts were still observed in mesna-treated animals, future studies will explore the impact of combination therapy with tPA, as we speculate that treatment with tPA (or other effective fibrinolytics) might provide additive, if not synergistic, beneficial effects in this model.

Importantly, in the model utilized in the current study, mesna treatments were given subsequent to SM inhalation (beginning 20 min or 2 h after the 50-minute SM exposure was terminated). Other thiol compounds like NAC, GSH, and thiosulfate often have required administration to cells or in vivo prior to SM exposure in order to be maximally effective (Callaway and Pearce, 1958; Rappeneau et al., 2000a; Jost et al., 2017; Jost et al., 2019).
During our experiments, drug delivery was performed after the inhalation event to simulate scenarios more relevant to real-world conditions (e.g., military conflicts and accidental exposures) for antidote administration. Also relevant to real-world conditions is the route of drug delivery. Because it is less palatable due to its bad taste, mesna was administered to animals parenterally in this study, using the i.p. route, as we and others have done previously (Shusterman et al., 2003; El-Medany et al., 2005; Sener et al., 2005; Stabler et al., 2009; Jeelani et al., 2017; Keeney et al., 2018; Hagar et al., 2020; Abd El-Baset et al., 2021). For field use of mesna, an alternative mode of administration such as intramuscular or subcutaneous would likely be preferable, although it is currently FDA-approved for use orally and intravenously. Several studies have tested the efficacies of therapeutics delivered via intubation under general anesthesia. In previous work performed by our group, tPA delivered in this manner demonstrated substantial efficacy in an SM-exposure model similar to that utilized in the present report, but the treatment regimen consisted of repeat intratracheal administration every 4 h for 48 h (Veress et al., 2015). Mesna usage could offer a practical therapeutic option or adjunctive therapy that avoids this disadvantage. However, although intubation may not be ideal for rapid and immediate drug delivery after real-world exposure (which may be incredibly important for decreasing mustard-induced injury), it is possible that candidate therapeutic compounds, including mesna, could be formulated for delivery to the airways by a hand-held inhaler. In the future, it may be desirable to evaluate alternate routes of mesna delivery, including direct delivery to the site of exposure.

While testing various routes of drug delivery is one potential avenue for the optimization of mesna treatment, other factors that may significantly impact the efficacy of mesna in our SM exposure model include the amount of drug given and the timing(s) of drug delivery. When delivered intraperitoneally in rats, the LD$_{50}$ of mesna is 1529 (male) and 1251 (female) mg/kg (Brock et al., 1982); given the severe damage and intense physiological stress induced by SM
exposure in our inhalation model, approaching the LD$_{50}$ values may result in additional stress as opposed to clinical benefit. However, it is possible that doses higher than those given in this study (300 mg/kg/dose) may provide increased efficacy. As mesna has a short half-life (Shaw et al., 1986; Verschraagen et al., 2004; Supplemental Figure 1), administering lower doses of mesna repeatedly at intervals may be a more effective approach than a single high-dose bolus. Delivering mesna at shorter or longer intervals, administering the initial dose at an earlier or later time post-exposure, and/or continuing the dosing beyond the three administrations given in our study may also provide additional clinical benefit. While dosing optimization was not explored in the current study, the treatment regimen employed resulted in the extended survival (to at least 15 days) of 18.4% of the animals exposed to SM. Therefore, the use of mesna therapy for SM exposure is promising and, with dosing optimization, more dramatic improvements in outcome and survival may be obtained.

As mentioned above, previous studies have evaluated the efficacy of various compounds for the treatment of SM exposure in animal models. However, some of these models may be less relevant than others with regards to real-world exposures for humans; for instance, results obtained from models utilizing systemically-delivered SM may not be pertinent to human exposure during conflict/combat (although they may be informative regarding mustard-derived chemotherapeutics used clinically). It is also important to note that additional differences in experimental models may strongly influence the measured effectiveness of therapies. Because of this, it is difficult to compare the therapeutic potential of a drug tested in a lethal exposure model with that of a drug tested in a sublethal model. Furthermore, it is not possible to conclude whether the therapy-mediated improvements in outcomes reported for studies following animals over a short time course post-exposure will be maintained over a longer time period. In order to more accurately compare the efficacy of various candidate therapies, it will be necessary to test these using the same model of SM exposure. We suggest
that both lethal and sublethal models have direct relevance to real-world exposures, and that longer-term studies may be more valuable than shorter-term studies for developing a therapy for real-world application. However, short-term studies may be valuable for determining mechanisms of drug actions and identifying pathways for targeted therapies.

In addition to the efficacy observed in the SM exposure model utilized in this study, mesna has a number of strengths as a therapeutic. The clinical utilization of mesna has a strong safety record over the past 40-50 years due to its repeated use in fragile patients receiving ifosfamide or cyclophosphamide for severe oncologic and rheumatologic diagnoses. It is also easily administered to the patient. Furthermore, mesna is inexpensive and, due to its chemical stability in water, it is easily stored and prepared for use. Therefore, mesna may be an ideal candidate countermeasure against SM exposure.

Further studies are required in order to fully elucidate the mechanisms involved in mesna-mediated protection from SM inhalation, including the identification and measurement of SM reaction products in the presence of mesna in vivo. Additionally, future experiments will explore the impact of timing and route of delivery on the efficacy of mesna-mediated protection. Overall, we have demonstrated the excellent potential in the therapeutic use of mesna for the treatment of SM inhalation exposures.
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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
Authorship Contributions

*Participated in research design:* Nick H., Veress L., Logue B., Bratcher P., White C.

*Conducted experiments:* Nick H., Johnson C., Stewart A., Christeson S., Bloomquist L., Appel A., Donkor A., Bratcher P.

*Contributed new reagents or analytic tools:* Logue B.


*Wrote or contributed to the writing of the manuscript:* Nick H., Bratcher P., White C.
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Footnotes

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H.J.N., B.A.L., P.E.B., and C.W.W. have a patent pending for the use of mesna in treatment of subjects exposed to toxic inhaled chemicals. The remaining authors have no conflicts of interest to declare.
Figure Legends

Figure 1. Survival proportions of SM-exposed rats treated with mesna or vehicle. At 20 min, 4 h and 8 h after SM inhalation exposure (denoted by arrows), mesna (red line) or vehicle (blue line) was administered i.p. and survival was monitored for 48 h. Survival of animals exposed to SM that received no treatment is shown by the black line. Data are from 3 independent experiments. p<0.0001 for mesna to vehicle comparison (Mantel-Cox log-rank test).

Figure 2. Effect of mesna treatment on peripheral oxygen saturation (SpO2) and heart rate in SM-exposed rats. (A) SpO2 and (B) heart rate (beats per minute, bpm) were monitored at regular intervals in conscious animals using a rodent pulse oximeter. At the beginning of the study, animal numbers were as follows: n=21 SM; n=14 SM + vehicle; n=21 SM + mesna. Timepoints of treatment administration are denoted by arrows. Data are expressed as mean+SD from 3 independent experiments. a: Significant difference between vehicle and mesna groups at 5 hours post-exposure (hpe) and from 7-13 hpe (Kruskal-Wallis with Dunn’s multiple comparisons tests). b: Significant difference between vehicle and mesna groups from 5-13 hpe (5-9 hpe: ANOVA with Tukey’s multiple comparisons tests; 10-13 hpe: Kruskal-Wallis with Dunn’s multiple comparisons tests).

Figure 3. Effect of mesna treatment on arterial blood gas measurements. Arterial blood was collected from anesthetized rats immediately prior to euthanasia (<48 hpe). (A) blood pH, (B) partial pressure of carbon dioxide (pACO2), (C) partial pressure of oxygen (pAO2), and (D) bicarbonate (HCO3–). n=16 naive, n=9 SM, n=10 SM + vehicle, and n=10 SM + mesna. Solid lines specify the median, and dashed lines specify the quartiles for each data set. Data are from 3 independent experiments. ns: not significant, ****p<0.0001, **p=0.0049, *p=0.0382 (one-way ANOVA with Tukey’s multiple comparisons test).
**Figure 4.** Assessment of airway occlusion following SM inhalation and treatment with mesna. Airway obstruction was evaluated by microdissection. A score of 7 indicates 100% occlusion of all five lobar bronchi. (A) Extent of airway obstruction in animals terminated at 9 hpe. n=5 per group. (B) Extent of airway obstruction in animals euthanized between 9 and 24 hpe. n=16 SM; n=14 SM + vehicle; n=7 SM + mesna. Solid lines specify the median, and dashed lines specify the quartiles for each data set. Data are from 2 independent experiments. *p=0.0286 (Mann-Whitney U test); ns: not significant (p=0.18) (Kruskal-Wallis with Dunn’s multiple comparisons test).

**Figure 5.** Relative gene expression changes measured in the lung at 9 hpe. Expression of genes involved in (A) coagulation, (B) hypoxia response, (C) oxidative stress, and (D) anti-apoptosis/TNF/NFkB signaling pathways was examined in whole lung tissue by RT-qPCR. Target gene quantification cycle (Cq) was normalized to Rpl13a and quantified relative to the mean expression of the naive control group. n=7 naive, n=8 SM + vehicle, and n=8 SM + mesna. Means±SD are shown. ns: not significant, *p<0.05, **p<0.01, ***p<0.001 compared to the naive control group (Kruskal-Wallis with Dunn’s multiple comparisons test).

**Figure 6.** Direct interaction between mesna and nitrogen mustard (NM). (A) The formation of a colored product through the reaction of the thiol group of mesna with DTNB was measured in reaction mixtures with NM after incubation across a dose and time course. Mesna concentration was held constant at 3 mM. Data are means±SD from 3 independent experiments. (B) Proposed reaction between mesna and NM. Product ion scan mass spectrometric analysis (negative ion mode) of the NM-mesna reaction product (242 m/z) was performed, and chemical structures were assigned to the abundant fragments.
Figure 1

A Kaplan-Meier survival curve showing the percent survival over time for three different groups: SM (n=21), SM + vehicle (n=14), and SM + mesna (n=21). The curve for SM + mesna remains significantly higher than the other two groups for the duration of the observation period, with p<0.0001 indicating a statistically significant difference.
Figure 2
Figure 3
Figure 4

A

B
Figure 5