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SULFUR MUSTARD-INDUCED PULMONARY INJURY: THERAPEUTIC APPROACHES TO MITIGATING TOXICITY

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Abstract

Sulfur mustard (SM) is highly toxic to the lung inducing both acute and chronic effects including upper and lower obstructive disease, airway inflammation, and acute respiratory distress syndrome, and with time, tracheobronchial stenosis, bronchitis, and bronchiolitis obliterans. Thus it is essential to identify effective strategies to mitigate the toxicity of SM and related vesicants. Studies in animals and in cell culture models have identified key mechanistic pathways mediating their toxicity, which may be relevant targets for the development of countermeasures. For example, following SM poisoning, DNA damage, apoptosis, and autophagy are observed in the lung, along with increased expression of activated caspases and DNA repair enzymes, biochemical markers of these activities. This is associated with inflammatory cell accumulation in the respiratory tract and increased expression of tumor necrosis factor-α and other pro-inflammatory cytokines, as well as reactive oxygen and nitrogen species. Matrix metalloproteinases are also upregulated in the lung after SM exposure, which are thought to contribute to the detachment of epithelial cells from basement membranes and disruption of the pulmonary epithelial barrier. Findings that production of inflammatory mediators correlates directly with altered lung function suggests that they play a key role in toxicity. In this regard, specific therapeutic interventions currently under investigation include anti-inflammatory agents (e.g., steroids), antioxidants (e.g., tocopherols, melatonin, N-acetylcysteine, nitric oxide synthase inhibitors), protease inhibitors (e.g., doxycycline, aprotinin, ilomastat), surfactant replacement, and bronchodilators. Effective

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treatments may depend on the extent of lung injury and require a multi-faceted pharmacological approach.

Keywords

Sulfur mustard; vesicants; toxicity; lung

The possibility that human populations will be exposed to sulfur mustard (SM) in warfare or as a consequence of a terrorist act is considered significant, in large part because of the ease and low cost of production, stockpiling, and delivery of this vesicant [1–3]. The pulmonary symptoms resulting from exposure often predominate, and are major determinants of mortality and long-term morbidity. Recent reports on the sequelae of disease pathogenesis in human survivors, as well as new insights into the mechanisms of injury in animal models and in lung cell cultures, have led to novel paradigms for treating pulmonary complications resulting from SM intoxication and these are discussed in this review.

1. Acute and Chronic Pulmonary Effects of SM in Humans

The pulmonary effects of exposure of humans to SM are often lethal in the short term, and a source of ongoing symptoms and disability in long term survivors. Exposure to SM is associated with inflammatory and oxidative injury, resulting in both upper and lower respiratory tract damage and pulmonary symptoms [4,5]. Upper airway involvement presents as acute pharyngitis and laryngitis, and edema and hyperemia of the mucosa. Lower airway pathology is characterized by shortness of breath and productive cough. Spirometric studies reveal patterns of obstructive injury (53%), restrictive injury (2%), or both (19%) [6]. Severe lower respiratory disease manifests as acute respiratory distress syndrome (ARDS), with high mortality. Although the acute symptoms of SM intoxication are often non-specific and transient, exposure frequently leads to the development of a characteristic pattern of chronic disease of both the upper and lower respiratory tract. At 1–3 weeks post exposure, bronchoscopy reveals inflammation of the trachea with signs of necrosis that is sometimes severe. At this time, chest X-rays are normal, indicating that the onset of chronic disease is delayed, possibly allowing a window for the initiation of therapeutic interventions [2]. At 10 year follow-up, exposed individuals have been diagnosed with asthma (11%), bronchitis (59%), bronchiectasis (9%), airway narrowing due to scarring (10%), and pulmonary fibrosis (12%), including chronic obstructive pulmonary disease (COPD), at rates greatly exceeding background incidence [7]. At 15 years, 24% of those referred for severe respiratory disorders have been reported to have tracheobronchial stenosis, ranging from diffuse involvement to isolated glottic or subglottic stenosis [8]. After 17–19 years, decreased FEV1 and hyper-responsiveness to methacholine challenge is observed, consistent with the development of reactive airway disease [9]. At 20 years after exposure, progressive lung deterioration is apparent, with bronchiolitis obliterans appearing as the main pathologic feature of significant SM exposure [10].

Associated long-term markers and sequelae of SM poisoning have recently been described in the Sardasht-Iran Study, and in other large cohorts of individuals exposed during the Iran-Iraq conflict of the late 1980's [11,12]. Even 20 years after SM exposure, there is evidence of systemic and pulmonary inflammatory effects in survivors including alterations in serum levels of cell adhesion molecules (e.g., selectins), as well as interleukin (IL)-8 and IL-6 [11,12]. Moreover, serum levels of inflammatory markers are directly related to pulmonary symptoms. For example, elevations in serum IL-8 levels in SM-exposed survivors are correlated with incidence and severity of wheezing [11]. C-reactive protein, a non-specific marker of systemic inflammation, is also increased in patients with COPD due to SM

poisoning, at levels comparable to the severity of disease [13]. Similarly, serum levels of the pro-apoptotic protein, soluble Fas ligand, are elevated in long-term survivors of SM exposure with persistent abnormalities in pulmonary function [14]. Pulmonary fibrosis following SM exposure is also correlated with increases in inflammatory cytokines and chemokines in bronchoalveolar lavage fluid (BAL), including IL-1α, IL-1β, IL-5, IL-6, IL-8, IL-12, IL-13, tumor necrosis factor (TNF)-α, CCL5, and CCL11 [15,16]. Markers of pulmonary inflammation in individuals exposed to SM are accompanied by evidence of oxidative stress, characterized by an accumulation of lipid peroxidation products such as malondialdehyde (MDA) in the lung and/or BAL, and decreases in antioxidants like superoxide dismutase (SOD) [17,18]. Lung glutathione levels are reduced in survivors 20 years post-exposure, and this is directly linked with altered pulmonary functioning. Recent data also indicate that SM exposure leads to an increased incidence of early-onset lung cancer, which is associated with mutations in the tumor suppressor gene, p53 [19].

2. Mechanisms of Toxicity

2.1 *In Vitro* Studies

Primary lung cells and cell culture models have been used to assess mechanisms of vesicant-induced toxicity. While cytotoxicity is thought to be initiated by DNA alkylation and consequent DNA damage, glutathione depletion and oxidative stress have been shown to be key events contributing to cell death. In co-culture models of bronchial epithelial cells and fibroblasts, vesicant-induced cytotoxicity is characterized by morphologic changes that are similar to *in vivo* pulmonary effects of SM, including decreased cell matrix adhesion, increased mucus production, and loss of ciliary function [20,21]. Loss of cell-cell contact and cellular disorganization and swelling are also observed in cultured lung cells following SM exposure, as well as increases in expression of caspases and Bax, and TUNEL staining [21–26]. These data indicate that SM induces necrosis, as well as apoptosis. This is supported by findings that expression of intact and cleaved poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme important in both of these processes, is upregulated in lung cells following vesicant exposure [27–30]. Recent mechanistic studies in lung cells have suggested that vesicants may also exert cytotoxicity by selectively targeting enzymes involved in regulating cellular homeostasis including thioredoxin reductase [31]. Thioredoxin reductase contains a unique selenocysteine in its active site, and vesicants inhibit enzyme activity by binding to this amino acid. NADPH cytochrome P450 reductase has also been reported to be a target for vesicants in lung epithelial cells [32]. Inhibition of NADPH cytochrome P450 reductase, which can block cellular metabolism, results in enhanced production of reactive oxygen species, a process that can cause oxidative stress and toxicity.

Treatment of isolated lung epithelial cells or macrophages with SM and related vesicants results in production of inflammatory mediators, which are thought to contribute to oxidative stress and cytotoxicity. For example, in airway epithelial cells, SM up regulates inducible nitric oxide synthase (iNOS) and stimulates the production of reactive nitrogen species [33]. SM also stimulates the production of the proinflammatory cytokines, IL-6 and IL-8, as well as matrix metalloproteinases by these cells [21,34]. Similarly, in human monocytes, the half mustard analog, 2-chloroethylethylsulfide (CEES) induces the release of TNFα [35], and in bronchial and small airway epithelial cells, production of reactive oxygen species [36]. Related bifunctional vesicants, mechlorethamine or its phenylalanine derivative, melphalon, generically referred to as nitrogen mustards, also induce the secretion of proinflammatory cytokines, chemokines and growth factors including TNFα, IL-1, IL-6, IL-8, IL-15, RANTES, macrophage chemotactic protein (MCP)-1, IP-10, and granulocyte monocyte-colony stimulating factor from differentiated human respiratory epithelial cells [23,37].

While it is important to avoid drawing definitive conclusions regarding *in vivo* mechanisms of vesicant-induced pulmonary toxicity, *in vitro* studies have suggested potential therapeutic approaches to investigate for use in mitigating human toxicity. In this regard, glutathione derivatives, N-acetylcysteine (NAC), macrolide antibiotics, antioxidant metalloporphyrins, melatonin and protease inhibitors, which have been shown to protect lung epithelial cells in culture from vesicant-induced cytotoxicity, as well as inhibitors of apoptosis and inflammation [28,33,38–40], are currently being assessed as countermeasures in various *in vivo* experimental models.

2.2 Animal Models

In order to develop efficacious therapeutics for SM-induced pulmonary toxicity, it is essential to elucidate its disease pathogenesis *in vivo*. Most data on mechanisms of injury are based on animal models using SM or bifunctional vesicants such as nitrogen mustard, or the half mustard, CEES. In these studies, vesicants are typically administered to the animals by inhalation or intratracheal (IT) instillation. However, it appears that the lung is also a remote target of toxicity following SM exposure by the percutaneous (PC), subcutaneous (SC), intraperitoneal (IP), and oral routes of administration. Greater pulmonary toxicity is observed after PC or IP exposure relative to SC or oral dosing, and is characterized by bronchiolar occlusion, along with lung inflammation, edema, congestion, and hemorrhage [41]. Biochemical evidence of oxidative stress, including increased levels of oxidized glutathione and lipid peroxidation products, as well as glutathione S-transferases, are also observed in the lung within 1–24 hr after SC or IP administration of half mustard [42–44]. Mechanisms mediating the distal toxicity of vesicants have not been established. SM has a half-life of only 30–60 min in blood; thus, its extrapulmonary actions are unlikely to be direct [45]. Mishra et al. [46] have shown that SM induces a rapid (30 min – 12 hr) immune response in the skin, characterized by infiltration of both CD4+ and CD8+ T-cells. This results in the generation of inflammatory cytokines, which may mediate the peripheral effects of SM and related vesicants.

Direct actions of SM and its analogs on the respiratory tract have been characterized in various animal models following inhalation or IT exposure. Histopathological changes are noted in the upper airways, including detachment of tracheal and bronchial epithelial cells from the basement membrane, deposits of fibrin containing cellular debris in the airway lumen, and edema of the submucosal lining within 6 hr of exposure of rodents to SM [47–53]. This is followed by tracheal epithelium blistering, columnar cell shedding, vacuolar degeneration and detachment of ciliated and epithelial cells from basement membranes, along with inflammatory cell accumulation in the submucosa [48,51]. By 14 days post SM exposure, tracheal epithelium is disorganized, with decreased cell density [49]. Similar results have been described in the respiratory tract following exposure of animals to CEES or nitrogen mustard [54,55]. Occlusive fibrin-rich bronchial casts are seen within 18 hr after inhalation of CEES, preceded by extravasation of fibrin, IgM, and other proteins into the bronchial and alveolar spaces [56]. These findings demonstrate that bronchial vascular injury plays a key role in the acute pathologic response to vesicants in the lung. Subsequent lower airway effects of mustards include thickening of alveolar septal walls and perivascular edema, suggesting alterations in the integrity of the alveolar epithelial lining [47,51,52,57]; these changes are evident within 24 hr and persist up to 6 weeks. With time following vesicant exposure, fibrin and collagen deposition increase in the lung leading to a collapse of alveolar structures, and the appearance of honeycombing [54,57]. Additionally, lung parenchymal congestion and hemorrhage are evident, as well as injury to the spleen, liver, and kidneys. Increases in urinary uric acid, a product of DNA degradation, are also detected following exposure of mice to inhaled SM [58].

DNA damage leads to activation of repair enzymes, such as PARP-1, which are key in maintaining survival and genomic integrity under conditions of genotoxic and oxidative stress [29]. PARP-1 is known to be a target for proteolytic degradation by the pro-apoptotic enzyme, caspase-3. Following SM inhalation by rodents, expression of both intact and cleaved PARP-1 increases in the lung [52], confirming that SM induces DNA damage, and that this is linked to apoptosis [30]. Apoptosis of lung epithelial cells after exposure to SM is associated with activation of caspase-3, caspase-8, and caspase-9, suggesting the importance of the death receptor pathway in mediating this effect [59]. Reports of increased expression of activated cleaved caspases in the lung of vesicant-treated animals, and morphologic changes in bronchial epithelial cells that are characteristic of apoptosis, including cell shrinkage and chromatin condensation [52,55,60] are consistent with this idea. The observation that these effects are prominent in epithelium that is detached from the basement membrane suggests cell detachment-dependent apoptosis or anoikis, a process also noted in the skin following SM exposure [22,52]. Recent studies suggest that SM-induced toxicity is associated with autophagy [52]. This is a tightly regulated catabolic process involving intracellular self-degradation; it is considered an alternative form of non-apoptotic cell death and has been implicated in the pathogenesis of chronic lung diseases [61,62]. Markers of autophagy have been noted in the lungs of patients in the early stages of the development of COPD [61]. The fact that there is evidence of autophagy in lungs of animals shortly after exposure to SM suggests that this cytotoxic mechanism may be important in the pathogenesis of chronic lung diseases in exposed individuals. This is supported by findings that exposure to cigarette smoke, which is also a causative agent for COPD, induces autophagy in lung epithelial cells [61].

Functional changes in the lung have also been described following exposure of animals to vesicants. Within 5 hr of administration of SM to rodents, respiratory system resistance and microvascular permeability are markedly increased, and by 24 hr, alterations in tidal volume, respiratory frequency, peak inspiratory and expiratory pressure, and airway hyperreactivity are observed [47,63]. After 14 days, airway hyperreactivity to substance P and histamine are noted, consistent with asthma-like symptoms [49,64]. Similar alterations in pulmonary mechanics have been described in rodent models of CEES- or nitrogen mustard-induced pulmonary intoxication [55,65–67]. CEES induces desensitization of beta-2 adrenergic receptors in the lung, possibly contributing to bronchospasm [68]. In guinea pigs, SM administration results in increased BAL surface tension, indicating altered lung surfactant [47]. Treatment of animals with CEES has also been reported to result in suppression of cholinephosphotransferase, an enzyme that is essential in the generation of phosphatidylcholine in the lung [69]. Increased accumulation of ceramides in the lung following exposure to vesicants may also contribute to impaired phosphatidylcholine synthesis. This can lead to decreased generation of pulmonary surfactant, resulting in atelectasis and lung injury.

Structural and functional alterations in the respiratory tract following exposure of animals to SM or related vesicants are accompanied by inflammatory cell accumulation in the airways and lung [50,52,55,65,66,70,71]. The majority of these cells are neutrophils and macrophages, supporting the idea that phagocytic leukocytes and inflammatory mediators they release are important in the pathogenic response to inhaled vesicants [54,72]. One notable macrophage-derived mediator is the proinflammatory cytokine TNFα, which is rapidly generated in pulmonary tissues in response to oxidative stress and injury [73]. Although it has been suggested that initiation of the TNFα cascade is a major pathway in vesicant-induced lung injury [55,60,74,75], the precise role of this cytokine in mustard gas-induced toxicity is unknown. TNFα is unique among proinflammatory cytokines in that it has the capacity to directly induce necrosis and apoptosis, which may be important in its cytotoxic actions [73]. TNFα also promotes oxidative metabolism in phagocytic leukocytes

resulting in increased production of cytotoxic reactive oxygen and nitrogen species, and it stimulates the synthesis of proteases such as matrix metalloproteinase-9 (MMP-9), which are important in epithelial cell detachment from the basement membrane [50,76]. TNFα generation by alveolar macrophages is also associated with an accumulation of ceramides in the lung, which are thought to contribute to apoptosis and, as indicated above, impaired surfactant production [74].

As observed in humans, a number of other inflammatory cytokines and chemokines, besides TNFα, are upregulated in the lungs of animals following exposure to vesicants. These include IL-1, IL-6, IL-8, IL-13, MCP-1 (CCL-2) and interferon (IFN)-γ, as well as connective tissue growth factor [46,55,63,65,66,77], and evidence from other models of lung injury and disease pathology suggest that they are important in the acute and long term pulmonary effects of SM poisoning. Reactive oxygen and nitrogen species and proinflammatory prostaglandins released by phagocytic leukocytes may also contribute to vesicant-induced toxicity. This is supported by findings that iNOS, the enzyme mediating the production of nitric oxide by macrophages and epithelial cells, and the eicosanoid generating enzyme, cyclooxygenase (COX)-2, are rapidly up regulated in these cells following vesicant exposure [28,52,55,66,78]. Moreover, animals lacking iNOS, or treated with an iNOS inhibitor or peroxynitrite scavenger, are protected from vesicant-induced lung injury [67,79].

3. Therapeutics

3.1 General Symptomatic Approaches

Treatment of pulmonary SM intoxication in humans has primarily been supportive, including interventions such as humidification of inhaled air to provide symptomatic relief from upper respiratory symptoms. Early tracheostomy and continuous positive airway pressure have been used with some success, as well as bronchoscopy to help remove pseudomembranes and necrotic debris from the airways [5]. Bronchoconstriction is a prominent component in both the acute and chronic sequelae following SM poisoning. SM induces early asthma-like symptoms in animals; symptoms and mortality are reduced by intratracheal administration of salbutamol, a β2 agonist [47]. Similarly, human subjects with chronic bronchiolitis and reactive airway disease as a consequence of SM exposure are responsive, or even hyperresponsive, to inhaled bronchodilators [80,81]. A recent report has suggested that the use of helium-oxygen mixtures with non-invasive ventilation decreases airway resistance and work of breathing in subjects with chronic dyspnea following sulfur mustard exposure [82]. As mechanistic data accumulate on SM-induced pulmonary injury, more specific pharmacologic approaches are being tested in animal models which may, in the future, prove efficacious in humans. These include anti-inflammatory agents, antioxidants, protease inhibitors and surfactants (Table 1).

3.2 Anti-inflammatory Agents

As described above, inflammatory cell accumulation in the respiratory tract is a prominent histologic feature of mustard gas-induced pulmonary injury. The well documented role of inflammatory cells in the pathogenesis of lung diseases such as bronchitis, asthma and COPD, which are all long term consequences of SM poisoning, has prompted investigations on the use of anti-inflammatory agents to mitigate pulmonary injury induced by vesicants. *In vitro*, macrolide antibiotics (e.g., erythromycin, azithromycin, roxithromycin) suppress vesicant-induced expression of pro-inflammatory cytokines and nitric oxide synthase in human airway epithelial cells and monocytes [33,78,83]. These agents also restored chemotactic and phagocytic activity of monocytes after SM exposure, which may contribute to improved clearance of apoptotic material in the injured airway [78]. In rodents,

administration of betamethasone, a moderately potent anti-inflammatory glucocorticoid, from day 7 to day 14 following SM exposure, decreases airway injury, as assessed by increases in epithelial cell density, and proliferation [48]. Treatment of animals with betamethasone for 7 days after SM also abolishes hyperresponsiveness to substance P, presumably by increasing the activity of neutral endopeptidase in airway smooth muscle [64]. Similarly, dexamethasone, a more potent glucocorticoid analog, administered 1 hr after exposure of mice to nitrogen mustard, reduces airway inflammation, lymphocyte activity, and collagen deposition [65]. Inhaled corticosteroids also improve pulmonary function in patients with chronic bronchiolitis as a result of SM inhalation, and this effect is synergistic with inhaled β-2 agonist bronchodilators [80]. The specific inflammatory cell type and mediator involved in the pathogenic response to vesicants has not been established. Neutrophil depletion has been reported to markedly attenuate lung injury, edema, and hemorrhage after exposure of rats to CEES [54]. These data, together with findings that dexamethasone blocks SM-induced activation and proliferation of alveolar macrophages [72], provide support for an involvement of phagocytic leukocytes in the pathogenic response to vesicants.

Newer therapeutic approaches for treating pulmonary diseases have focused on specific pro- and anti-inflammatory mediators to ameliorate vesicant-induced lung injury. For example, IFNγ, in combination with low dose prednisolone, results in improvement in lung function in patients with chronic bronchitis due to mustard gas poisoning [84]. Recent observations that TNF receptor-1 knockout mice are protected from CEES-induced injury and altered lung functioning suggest that targeting TNFα may also prove effective in treating patients exposed to SM [55]. Upstream signaling pathways are also promising targets for future drug development. Mechanistic studies have demonstrated activation of NF-κB and AP-1 in the lung within 1–2 hr of exposure to CEES [59,74,75,77]. These ubiquitous transcription factors regulate the activity of a number of inflammatory genes implicated in pulmonary toxicity, including iNOS, COX-2, and TNFα. Mitogen activated protein kinase signaling is also up regulated in the lung following mustard exposure [75]. Pharmacologic antagonists against one or more of these signaling molecules may prove useful in mitigating vesicant-induced pulmonary toxicity.

3.3 Antioxidants

SM intoxication is associated with oxidative stress, caused by an imbalance between production of oxidants and antioxidants in the lung and respiratory tract, and this is thought to be a primary event triggering the inflammatory cascade and tissue injury [27]. Markers of oxidative stress, including malondialdehyde, 8-hydroxydeoxyguanosine, 4-hydroxynonenal, and heme oxygenase-1, are increased in the respiratory system after exposure of animals to SM or related vesicants [52,85–87]. This occurs concomitantly with decreases in lung glutathione and SOD activity [70,88]. Consequently, antioxidant therapies have been investigated as a means of ameliorating lung injury due to vesicants with some success. Antioxidants such as Trolox, a water-soluble analog of α-tocopherol (vitamin E), as well as quercetin, have been reported to reduce markers of oxidative damage induced by SM [87]. Tocopherols, delivered via liposomes, also block nitrogen mustard-induced inflammatory cell and cytokine accumulation in the lung and suppress the generation of collagen, a key component of oxidative-inflammatory injury leading to chronic lung disease [65]. Liposomes containing tocopherols, alone or in combination with NAC, which stimulates glutathione synthesis and scavenges free radicals, also suppress CEES-induced lung injury [85,89,90]. A similar decrease in CEES-induced lung injury, as well as inflammatory cell accumulation is observed after IT administration of liposomes containing catalase and/or SOD [54,57,77]. Recent studies have also shown that a catalytic antioxidant that possesses

both SOD and catalase activity reduces inflammatory and oxidative stress following inhalation of CEES by rats [86].

NAC is also being evaluated as a potential countermeasure against SM-induced pulmonary toxicity. Oral administration of NAC has been reported to exert protective effects when administered 3–30 days prior to exposure to CEES [60]. Intravenous NAC, simultaneously or as late as 60–90 min after CEES administration, also significantly reduces acute lung injury [54]. Analogous protection by NAC has been described against SM-induced injury [71]. NAC-containing liposomes, administered IT immediately after CEES, reduce both the lung permeability index and pro-inflammatory mediators in BAL to control levels [57,89]. Several studies have addressed mechanisms mediating the protective effects of NAC in the lung. NAC does not appear to alter SM-induced activation of protein kinases, but rather to down regulate the activity of the AP-1 transcription factor, contributing to reduced infiltration of inflammatory cells into alveolar spaces [90]. The idea that CEES-induced pulmonary toxicity and NAC cytoprotection are mediated by inflammatory mechanisms is supported by the observation that the salutary effects of NAC are synergistic with neutrophil or complement depletion, resulting in an 80% reduction in CEES-induced lung injury [54,57]. NAC has also been shown to ameliorate the symptoms of chronic lung injury, including cough and dyspnea, in human survivors many years after SM exposure, an effect likely related to its combined anti-inflammatory and antioxidant activities [91].

The specific cytotoxic oxidants involved in SM-induced lung injury are unknown. Accumulating evidence suggests that reactive nitrogen species are important in the pathogenic process. Nitric oxide is generated in the lung by macrophages and epithelial cells via an inducible form of the enzyme, nitric oxide synthase [92]. Once generated, nitric oxide readily reacts with superoxide anion forming peroxynitrite, a relatively long-lived cytotoxic oxidant. Nitric oxide and peroxynitrite oxidize and covalently modify membrane lipids, thiols, proteins and DNA, inducing cytotoxicity and perpetuating inflammation. Expression of iNOS is upregulated in lung macrophages and epithelial cells following exposure of rodents to vesicants including SM, nitrogen mustard and CEES [52,55,66,67,79,93]. Ebselen (a peroxynitrite scavenger) and melatonin (a potent antioxidant that scavenges both reactive oxygen and nitrogen species) ameliorate lung injury and oxidative stress induced by nitrogen mustard in rodents, suggesting a potential therapeutic target for treating mustard gas poisoning [79,93]. This is supported by recent studies demonstrating that transgenic mice with a targeted disruption of the gene for iNOS are protected from CEES-induced pulmonary toxicity and altered lung functioning [67].

3.4 Protease Inhibitors

MMPs are zinc-dependent endopeptidases that degrade extracellular matrix proteins, contributing to inflammatory cell recruitment, tissue injury, and fibrosis [76,94,95]. MMPs, including MMP-9, increase in the respiratory tract within 6–24 hr of exposure to SM [50,52]. Particularly high expression levels are noted in bronchiolar epithelium and alveolar macrophages. A similar expression pattern of MMP-9 has been described in the respiratory tract after exposure of rodents to CEES or nitrogen mustard [55,66,67]. The effects of SM on MMP-9 expression are dose-dependent and persist for at least 24 hr. At this time, 92 kDa gelatinase activity is detectable at sites of intraepithelial cleavages, associated with disruption of alveolar epithelial integrity and increased BAL albumin content [52]. These findings suggest a role for inflammatory and epithelial cell-derived MMPs in epithelial barrier disruption. Interestingly, MMP-9 protein and 92 kDa gelatinase activity are also detectable in BAL within 6 hr of SM exposure and persist for at least 7 days [50–52], suggesting that biologically active MMPs are secreted during the pathogenic response to vesicants. In contrast to the stimulatory effects of SM on MMP-9/92 kDa gelatinase, expression of tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2), is unaffected by

exposure to SM, indicating that vesicant-induced lung injury is due to an imbalance between proteases and antiproteases [51]. These findings have led to investigations of anti-protease therapy for the treatment of SM-induced pulmonary toxicity, with promising results. When administered immediately prior to SM, the serine protease inhibitor, aprotinin, or the broad spectrum MMP inhibitor, ilomastat, ameliorate vesicant-induced decreases in pulmonary function parameters [63]. Aprotinin also prevents SM-induced increases in total BAL protein and lung histopathology, as well as IL-1α and IL-13 production, suggesting that anti-inflammatory activity may also contribute to its protective effects.

Doxycycline is a semi-synthetic tetracycline that has been reported to exhibit non-specific MMP inhibitory activity, and it appears to exert significant protective effects against SM-induced lung toxicity [96]. Treatment of guinea pigs with doxycycline 3 hr prior to SM results in reduced activity of gelatinases (MMP-2 and MMP-9) and decreased evidence of lung inflammation and injury, including cellularity and protein levels in BAL [51]. In addition to inhibiting MMPs, doxycycline and related tetracyclines have been reported to attenuate iNOS expression and nitric oxide production, to reduce inflammatory cytokine release, and to scavenge reactive oxygen species [97–99]. It is likely that these diverse anti-inflammatory actions enhance its efficacy as a therapeutic against SM poisoning.

3.5 Surfactant Therapy

Defective secretion of pulmonary surfactant by alveolar type II cells and surfactant dysfunction have been implicated as causative factors in ARDS, an inflammatory outcome of SM exposure in the lower airway [49,100]. CEES administration to guinea pigs significantly decreases expression of cholinephosphotransferase, a key enzyme involved in surfactant biosynthesis, and resultant increases in ceramides, which are thought to contribute to apoptosis and surfactant dysfunction [69,74]. Recent studies have also demonstrated that expression of surfactant protein D, which possesses anti-inflammatory activity, is markedly reduced in lung epithelium following SM treatment of rats [52,55]. Curosurf is a naturally-derived surfactant used to treat neonatal respiratory distress syndrome. IT administration of Curosurf one hour following SM exposure has been reported to reduce SM-induced mortality in guinea pigs although not as effectively as the bronchodilator, salbutamol [47]. These data suggest that surfactants, in combination with bronchodilators or anti-inflammatory agents, may be useful in mitigating vesicant-induced lung injury, but this remains to be investigated.

4 Conclusions and Future Directions

Respiratory toxicity due to exposure to SM, including long-term effects like COPD and fibrosis, is a significant health concern even decades after exposure. Thus, it is essential to identify efficacious treatments for both acute and chronic diseases induced by this vesicant. Studies on SM and its analogs in animals suggest that individual or combination therapies using anti-inflammatory, anti-oxidant, and anti-protease agents may be effective in ameliorating the toxicity of SM in humans. However, use of these countermeasures is limited due to their relatively non-specific actions. Consequently, there remains a pressing need to identify more specific therapeutics, and effective strategies for delivering these agents to target organs. Of particular concern is the lung, since impaired functioning and inflammation due to SM poisoning may hamper the delivery of therapeutics by standard inhalation approaches. Since clinical trials on SM exposure cannot be performed, insights into the optimal approaches for mitigating its toxicity will most likely be gained by evaluating strategies currently utilized to treat human diseases with similar pathologies. For example, lung injury in ARDS patients is reduced by conventional mechanical ventilation, sedation, and decreased threshold for blood cell transfusion. Other promising therapeutic approaches to ARDS in adults and/or children include endotracheal surfactant, high-

frequency oscillatory ventilation, noninvasive ventilation, extracorporeal membrane oxygenation, corticosteroids, and restrictive fluid management. Prone positioning, bronchodilators, inhaled nitric oxide, and high-flow nasal cannula may also be useful [101]. For chronic injury associated with COPD, inhaled anticholinergic and β-agonist bronchodilators are useful in ameliorating symptoms, and corticosteroids have some limited utility in reducing inflammation associated with severe disease [102]. In order to devise a uniform and rational approach to treating SM toxicity that minimizes untoward effects, it is essential to integrate the knowledge gained from cell culture and animal studies, human exposure, and current clinical management of acute and chronic lung injury.

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ABBREVIATIONS

ARDS	acute respiratory distress syndrome
CEES	2-chloroethylethylsulfide
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
iNOS	inducible nitric oxide synthase
IL	interleukin
IT	intratracheal
MMP	matrix metalloproteinase
NAC	N-acetylcysteine
PARP	poly (ADP-ribose) polymerase
SM	sulfur mustard
TIMP	tissue inhibitors of metalloproteinases
TNF	tumor necrosis factor

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Table 1

Examples of Therapeutic Strategies for Mitigating SM-induced Lung Injury

Target	Therapeutics	References
Inflammation	Anti-inflammatory agents (macrolide antibiotics, glucocorticoids)	33,48,64,65,72,78,80,83,84
Oxidative stress	Antioxidants (tocopherols, NAC, catalase, SOD, catalytic metalloporphyrins, melatonin)	28,40,54,57,60,65,71,77,85,86,87,89,90,91,93
Proteases	Protease inhibitors (doxycyline, ilomastat, aprotinin)	40,51,63
Pulmonary surfactants	Surfactants (Curosurf)	47