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Review Article

N-Acetylcysteine as a treatment for sulphur mustard poisoning

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ABSTRACT

In the long and intensive search for effective treatments to counteract the toxicity of the chemical warfare (CW) agent sulphur mustard (H; bis(2-chloroethyl) sulphide), the most auspicious and consistent results have been obtained with the drug *N*-acetylcysteine (NAC), particularly with respect to its therapeutic use against the effects of inhaled H. It is a synthetic cysteine derivative that has been used in a wide variety of clinical applications for decades and a wealth of information exists on its safety and protective properties against a broad range of toxicants and disease states. Its primary mechanism of action is as a pro-drug for the synthesis of the antioxidant glutathione (GSH), particularly in those circumstances where oxidative stress has exhausted intracellular GSH stores. It impacts a number of pathways either directly or through its GSH-related antioxidant and anti-inflammatory properties, which make it a prime candidate as a potential treatment for the wide range of deleterious cellular effects that H is acknowledged to cause in exposed individuals. This report reviews the available literature on the protection afforded by NAC against the toxicity of H in a variety of model systems, including its efficacy in treating the long-term chronic lung effects of H that have been demonstrated in Iranian veterans exposed during the Iran-Iraq War (1980–1988). Although there is overwhelming evidence supporting this drug as a potential medical countermeasure against this CW agent, there is a requirement for carefully controlled clinical trials to determine the safety, efficacy and optimal NAC dosage regimens for the treatment of inhaled H.

1. Introduction

The vesicant sulphur mustard (H; bis(2-chloroethyl) sulphide, Figs. 1 and 2) was first used as a chemical warfare (CW) agent during the First World War. Although only introduced in July of 1917 by the Germans against British troops at Ypres, Belgium, by the end of the war both sides of the conflict had used it extensively and the majority of British (77.5%), and American (75.0%) chemical casualties were attributed to this agent [pp. 129 [1]]. It has been used subsequently in a number of smaller conflicts [2–4], culminating in the Iran/Iraq War (1980–1988) where over 100,000 Iranian and Iraqi-Kurdish civilians and soldiers were exposed to this chemical agent [5]. Almost thirty years later, evidence of H use by non-state actors during the Syrian Civil War has also been reported [6–10].

The primary target organs of H exposure include the skin, eyes, mucous membranes and respiratory tract (Fig. 3), and the clinical and histopathological features of acute exposure have been well studied and reviewed [2–4,11–18]. Skin lesions (Fig. 4) can be produced by either vapour or liquid H exposure, with the injury severity dependent on dose, temperature, humidity, skin moisture and regional skin thickness. At low exposures, erythema is produced after a highly variable latent period. At higher exposures, this latent period becomes shorter and the injury progresses to small vesicles that may consolidate to larger blisters. At very high liquid doses, necrosis of the skin may occur with no blistering, or a ring of blisters surrounding a necrotic zone may be produced [3,4]. Due to the antimitotic nature of H, these lesions are very slow to heal, often with hyper- and/or hypopigmentation of the skin (Fig. 4). Symptoms produced by H vapour or aerosol inhalation are concentration-dependent and include pain or discomfort after a variable latent period of 2–16 h. Larger exposures cause damage to the larynx and upper airways causing cough after shorter time periods. Very large vapour exposures cause extensive damage to the terminal airways, resulting in severe cough, dyspnea, haemorrhage into the alveoli and purulent sputum. Respiratory infections are common and are a major cause of death due to H exposure [3,4,13,16].

The above two routes of exposure represent the major sources of H distribution into the systemic circulation, either directly, or in the case of skin exposure, also indirectly and more slowly through H depots formed in the lipid components of the skin [19,20]. Dermal application of ^{14}C labeled butyl 2-chloroethyl sulfide (BCS) to rats resulted in the distribution of this H simulant to several different organs, including brain within 1 h [21], while chemical analysis of tissues from an Iranian fatality due to H exposure, detected significant quantities of H in lipid-rich organs including (in mg H/kg tissue wet weight) fat (15.1), brain (10.7), skin (8.4), kidney

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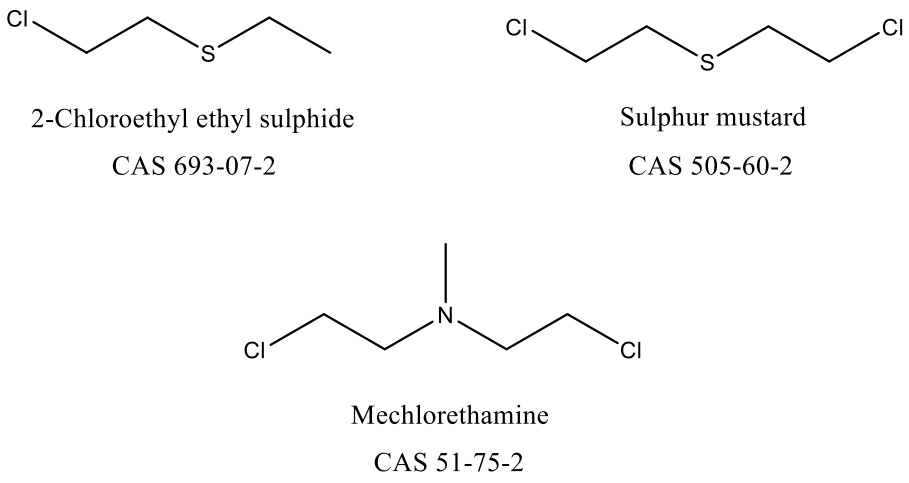
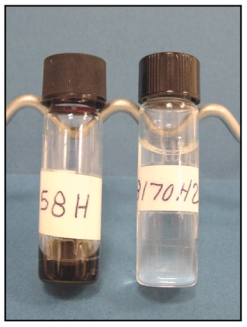


Fig. 1. Chemical structures of sulphur mustard (H), 2-chloroethyl ethyl sulphide (CEES) and mechlorethamine (HN-2).

Sulphur mustard (H)

- bis(2-chloroethyl) sulphide, mustard gas, yperite, yellow cross liquid
- Oily liquid
- MW = 159.1, MP = 14.4°C
- Garlic-like odor



N-Acetylcysteine (NAC)

- Acetylcysteine, N-acetyl-L-cysteine
- White powder
- MW = 163.20, MP = 109-110°C

Fig. 2. Sulphur mustard (H) and *N*-acetylcysteine (NAC) properties. Laboratory-grade H is distilled to a high purity and is a clear oily liquid, compared to the darker munitions-grade H removed from an old artillery shell. *N*-acetylcysteine can be purchased from chemical companies or as over-the-counter capsule products.

(5.6), muscle (3.9), liver (2.4), spleen (1.5), cerebrospinal fluid (1.9), blood (1.1) and lung (0.8) [20]. This re-distribution of H, especially at higher exposures is responsible for the systemic intoxication that involve the cardiovascular, central nervous and immune systems (Fig. 3) [3,22–24]. It also likely plays a role in the findings described in recent Iranian reports that have documented the persistent long-term and chronic effects of H exposure during the Iran-Iraq war that include ocular, dermatological, respiratory, gastrointestinal, haematological, neurological, neuromuscular, immunological and endocrine disruptions (Fig. 3) [25–30].

Although the progression of the injury is well characterized, the mechanism of toxic action of H remains largely unknown. Sulphur mustard is a highly reactive bifunctional alkylating agent, and as such is able to react rapidly with a broad range of cellular constituents and molecular targets. This has resulted in numerous hypotheses being proposed and it has been well recognized as causing the production of reactive nitrogen and oxygen species [31–35], as well as disturbing an array of molecular pathways including; intracellular calcium, anion and pH regulation, poly (ADP-ribose polymerase (PARP) activation, DNA damage and repair, apoptosis induction, inflammation, membrane bound receptors, signalling molecules and extracellular matrices [2,3,11,14,15,36–42]. However, the pathways that ultimately lead to toxicity remain elusive and investigators continue their efforts to define the progression of biochemical/molecular events from exposure to the ultimate expression of overt toxicity.

The oxidative stress and inflammatory processes produced by H in laboratory test models and exposed individuals, have provoked numerous studies investigating the efficacy of the well-known antioxidant *N*-acetylcysteine (NAC; *N*-acetyl-L-cysteine, Figs. 2 and 5) against it and it has been suggested that NAC may be a lead candidate for the treatment of this CW agent [5,43]. Its primary mechanism of action is mediated through its ability relative to cysteine to more rapidly pass through biological membranes. Upon subsequent cleavage of the acetyl group to yield free reduced cysteine, it is incorporated into the reactive oxygen species (ROS)-scavenging tripeptide glutathione (GSH; Fig. 5), most especially in those cases where oxidative stress drives cellular GSH depletion (Fig. 6). In addition, NAC is also known to have limited direct antioxidant activity under physiological conditions that is thought to confer modest protective effects, as well as chemical activities not related to GSH that are less understood [44–48]. While the reactive oxygen scavenging activity of NAC has been broadly exploited in clinical settings, notably with its use as an antidote against acetaminophen (paracetamol)-induced hepatoxicity [49–51], it has become clear that NAC also impacts other pathways either directly or through its GSH-related antioxidant properties, including inflammatory, cell cycle, DNA damage and repair, apoptosis, carcinogenesis and tumour progression, mutagenesis, gene expression, signal transduction, immune modulation, cytoskeleton and mitochondrial function [47,52]. Of interest also are the recent findings that NAC impacts neurotransmission not only through its antioxidant activities, but also through elevation of extracellular glutamate levels via the cystine/glutamate antiporter and subsequent actions on both *N*-methyl-D-aspartate (NMDA) and 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors [46,47,53]. Studies directed at diseases of the nervous system have concluded that there is positive evidence of NAC efficacy in treating a variety of pathologies. However, it has also been noted that the relatively poor membrane permeability of NAC, particularly in penetrating the blood brain barrier may reduce its value as a treatment. Replacement of NAC's carboxyl group with an amide [54] increases its lipophilicity and allows the resultant *N*-acetylcysteine amide (NACA, Fig. 5) to more easily cross cell membranes, facilitating transport into the blood brain barrier, mitochondria and other cellular constituents. This would decrease the doses required and perhaps also alleviate some of

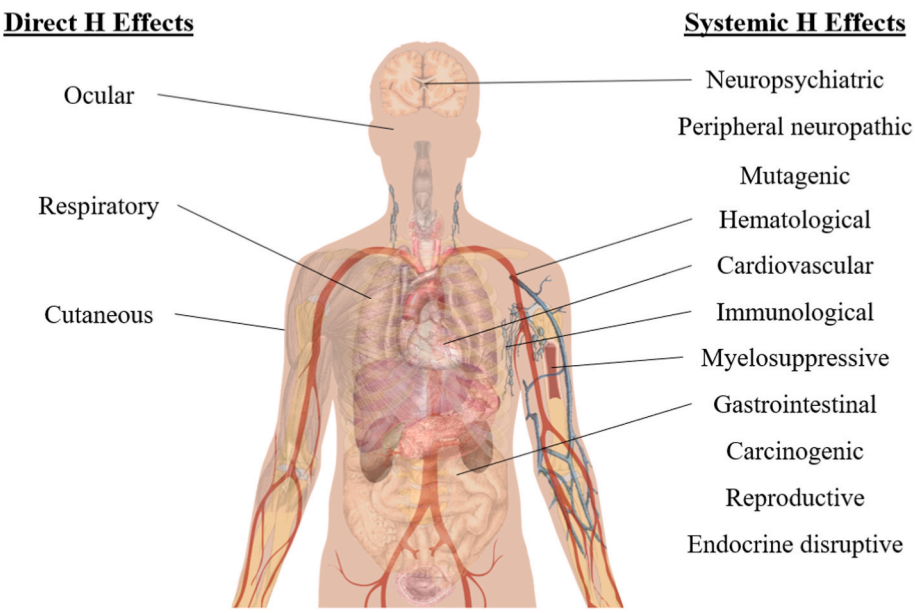


Fig. 3. Direct and indirect effects of sulphur mustard (H) exposure. Dermal or inhalation exposure of an individual to H results in rapid entry of the agent into the circulatory system. This is followed by re-distribution and accumulation of H into lipid-rich tissues, resulting in systemic toxicity involving a broad range of organs and tissues.

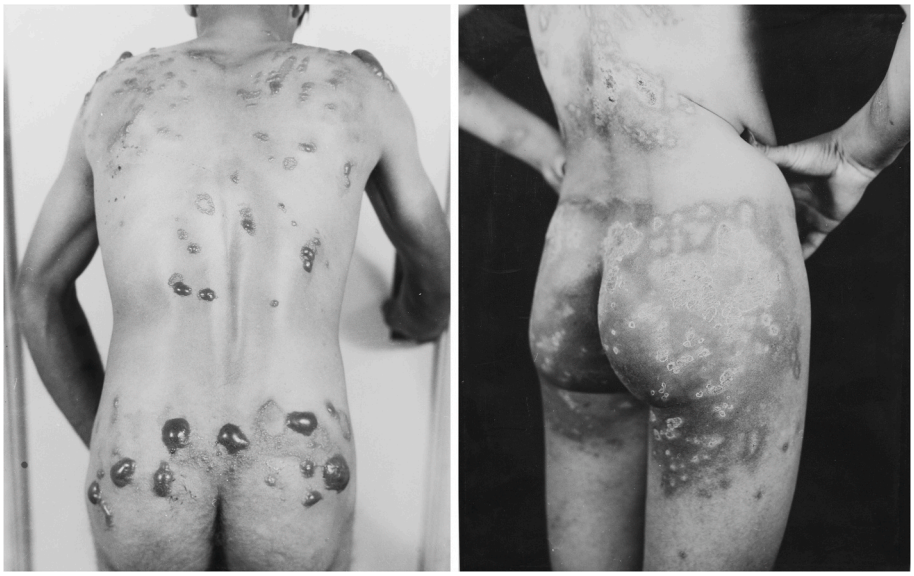


Fig. 4. Effect of sulphur mustard (H) exposure on human skin. The left panel shows blister formation on the back and buttocks one day after H exposure through clothing. Note that vesication occurs predominantly in areas of increased skin temperature and moisture. The right panel shows hyper- and hypopigmentation in the same individual eleven days after H exposure. © Copyright Her Majesty The Queen in Right of Canada as Represented by the Minister of National Defence, 2020.

the side effects noted with NAC. Although no human trials have been conducted with this drug, its reported superior bioavailability and antioxidant activities relative to NAC, show great promise [55–58].

This report consolidates and reviews evidence from both *in vitro* (Table 1) and *in vivo* (Table 2) test systems, as well as from clinical trials of H-exposed patients (Table 3), that support NAC as a strong candidate for the safe treatment of H exposure in humans, particularly as a therapeutic against the long-term effects of inhaled H. The data bases PubMed, Defense Technology Information Center (DTIC) and Clinical Trials.gov were searched up to August 2020. The bibliographies of the reports located were then examined for additional relevant publications. Governmental defence reports were not cited if open literature publications were located duplicating the work.

2. NAC and sulphur mustard

2.1. Sulphur mustard tissue culture studies; non-pulmonary (Table 1)

Building on the finding that H forms GSH conjugates [59–61], and the well known fact that thiols were protective against the toxicity of alkylating agents, Walker and Smith [62] reported in 1969 that thiol pre-treatment of mouse L-cells was protective against H. More than two decades later Smith and Gross [63] noted that NAC increased cellular GSH levels *in vitro* that would react with H and decrease its subsequent toxicity, a finding that was consistent with the increased efficacy of the nitrogen mustard cyclophosphamide against tumours with low GSH levels [64]. Follow-up studies using human peripheral blood lymphocytes showed that a 24 h pre-incubation with 10 mM NAC inhibited the H-induced activation of proteases [65], while a 48 h pre-treatment followed by washout significantly attenuated the toxicity of low H concentrations, but not of high concentrations in these same cells [66]. They concluded that increasing intracellular GSH levels by providing substrates such as NAC for its synthesis may provide a pathway for designing prophylactic strategies to protect against H toxicity. Relatively low NAC concentrations (100 μM) were utilized in studies to compare the efficacy of NAC alone [67] or in combination with other drugs against H toxicity [68]. A 30 or 60 min NAC pre-treatment followed by washout, of 180 μM H-exposed human skin fibroblast HF2FF cells resulted in significant elevations in cellular viability, GSH levels and catalase activity compared to that found in H-only treated cultures.

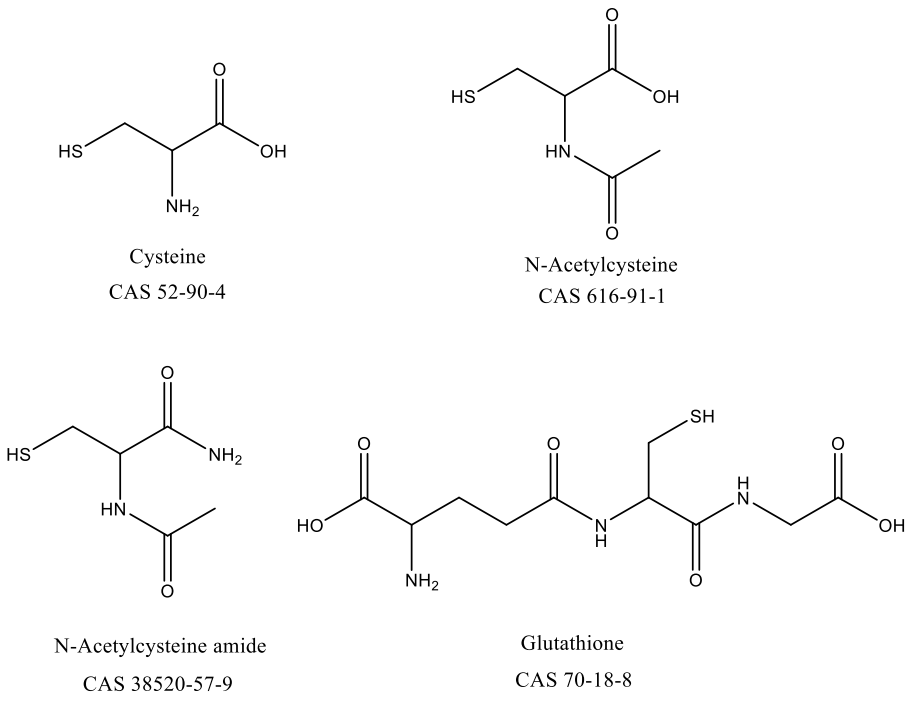


Fig. 5. Chemical structures of L-cysteine, *N*-acetylcysteine (NAC), *N*-acetylcysteine amide (NACA) and glutathione (GSH).

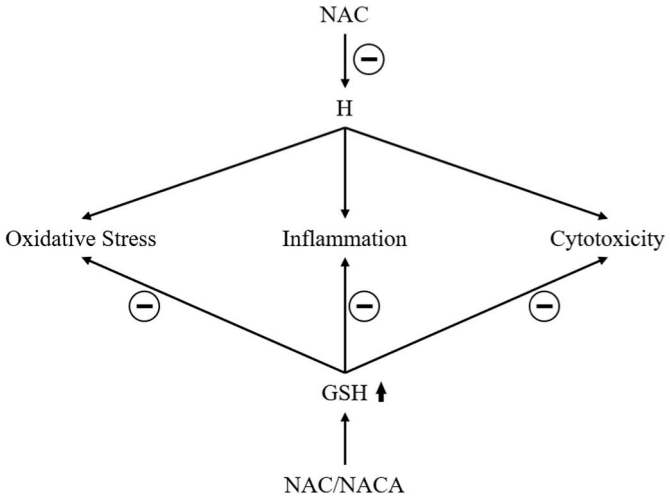


Fig. 6. Effect of *N*-acetylcysteine (NAC) on sulphur mustard (H)-induced effects. Sulphur mustard causes oxidative stress, inflammation and cytotoxicity in a broad range of tissues. Although NAC has been shown to interact directly with H, this role is believed to be minor in preventing its effects. The major role for NAC or its amide derivative NACA, is as pro-drugs that act to up-regulate glutathione (GSH) levels, especially when they are depleted. The elevated GSH levels then ameliorate H-induced oxidative stress, inflammation and/or cytotoxicity.

Further increases in these endpoints were obtained by the inclusion of 2-oxo-thiazolidine-4-carboxylate and acetaminophen. The authors attributed the protection to drug-related increases in GSH levels and catalase activities [68]. In an *in vitro* model of wound healing, mouse embryoid bodies were utilized to examine the effect of ROS scavengers such as α-linolenic acid (15 ng/ml) and NAC (20 mM) on H-induced toxicity and decreased endothelial tube formation [69]. Both compounds exhibited protective effects, but only transiently. Work examining the effect of NAC co-administration with H in a human keratinocyte cell line (HaCaT) showed that 1–10 mM NAC pre-treatment (with no washout) protected against both H-induced apoptotic and necrotic cell death, but ameliorated inflammatory cytokine release only at low H concentrations. One hour NAC pre-treatments followed by removal before H exposure were only slightly protective, while 1 h post-treatments were ineffective, leading the authors to suggest that most of the protective effects noted in their studies were due to the direct scavenging of H [70]. Stenger and coworkers [71] used aquaporin-transfected HEK293 cells to examine the role of the cation channel TRPA1 in H-induced intracellular calcium (Ca_i^{2+}) flux. They found that H not only activated this channel with concomitant Ca_i^{2+} increases, but that these responses were reduced by NAC pre-treatment (15 min; 0.1–1.0 mM) in a concentration-dependent manner. No correlations were made with cellular viability in these studies, although it was concluded that chemical scavenging of H by NAC was not the major route through which these effects occurred [71], a conclusion later supported by mass spectrometric studies where NAC and H were co-incubated in either phosphate buffered saline or human serum. In this work, although reaction products of NAC and H were detected and identified, “analyses clearly documented minor reactivity not significantly contributing to reduction of SM concentrations” [72].

2.2. Sulphur mustard animal studies; non-pulmonary (Table 2)

Relatively few studies exist on the use of NAC in animal models not related to H or H simulant pulmonary toxicity. In the mouse ear vesicant model, NAC topical pre-treatments (1 mg in ethanol: 120 min; 10 mg in cream: 15 min) did not prevent the edema and morphological changes induced by 0.16 mg H application to the ear [73]. Intraperitoneal (IP) NAC pre-treatment (10 min) of guinea pigs treated topically with H was slightly effective in reducing skin lesions while post-treatment (5–10 min) was ineffective [74] (cited in Papirmeister and coworkers [3]). *N*-Acetylcysteine was included in a study examining the protective effects of amifostine and several analogues (DRDE-07, -10, −30, −35) against H and three different nitrogen mustards [75]. Mice were exposed dermally to one LD_{50} of agent and the antidote was administered orally; one dose (NAC; 250 mg/kg) 30 min prior to agent exposure and the remaining doses daily for the next 3 or 7 days. At 4 and 8 days body weights, percent spleen weights, haematological endpoints and biochemistry were assessed. Compared

Table 1
Effect of *N*-acetylcysteine against mustard vesicant treatment in tissue culture models.

Reference/Authors	Culture Model	Treatment and Exposure Time	NAC Treatment	Agent Effect on Endpoint(s)	NAC Effect on Agent-induced Endpoints
[79] **Atkins *et al.*, 2000**	Bovine pulmonary artery endothelial cells	250, 500 μM H 5-6 hr	50 mM NAC - overnight pre-treatment with washout	↓ Viability ↑ NFκB activation	↑ ↓
[70] **Balszuweit *et al.*, 2016**	HaCat human skin keratinocyte cell line	30, 100, 300 μM H 24 hr	1-10 M NAC - 1 hr pre-treatment with washout - 1 hr pre-treatment with no washout - 1 hr post-treatment	↑ Apoptosis/necrosis ↑ Inflammatory cytokines	↓ ↓ Pre-treatment effective in reversing H effects; washout reduced effects Post-treatment not effective
[65] **Cowan *et al.*, 1992**	Human peripheral blood lymphocytes	100-500 μM H 16 hr	10 M NAC - 24 hr pre-treatment	↑ Protease activity	↓ (33%)
[78] **Dabrowska *et al.*, 1996**	Bovine pulmonary artery endothelial cells	10-1000 μM H 6 hr	50 mM NAC - 20 hr pre-treatment followed by washout	↑ Apoptosis ↑ Necrosis ↑ DNA cleavage ↓ ATP levels	↓ No effect ↓ ↑ Low H concentrations = apoptosis High H concentrations = necrosis
[66] **Gross *et al.*, 1993**	Human peripheral blood lymphocytes	10, 30, 300 μM H 48 hr	10 mM NAC - 48 hr pre-treatment followed by washout	↓ Viability	↑ (only at 10 μM H)
[92] **Han *et al.*, 2004**	Jurkat T human T lymphocyte cell line	600 μM CEES 5-18 hr	5 mM NAC - 1 hr pre-treatment	↓ 18 hr viability ↑ 5 hr ROS ↓ ? hr GSH levels ↓ 5 hr mitochondrial membrane potential ↑ 12 hr caspase-3	↑ ↓ ↑ ↑ ↓
[100] **Hoesel *et al.*, 2008**	Alveolar macrophages from rat lung lavage	500 μM CEES 4 hr	50 μl NAC containing liposomes (concentration not identified) - co-administration	↑ Inflammatory cytokines	↓
[81] **Jost *et al.*, 2017**	A-549 human lung alveolar epithelial cell line	50 μM H 1, 24, 72 hr	2 mM NAC - 30 min pre-treatment	↓ 24 and 72 hr viability ↑ 1 and 24 hr DNA damage	No effect ↓ Addition of MESNA did not potentiate
[99] **Lee and Kagan, 2014**	Primary human bronchial epithelial cells	200 μM HN-2 15 min – 24 hr	1-10 M NAC - 30 min pre-treatment	↑ 24 hr IL-6 ↑ 30 min EGFR phosphorylation ↑ 30 min ROS ↑ 24 hr protein carbonylation ↑ 15 min NADPH oxidase	↓ ↓ ↓ ↓ ↓
[67] **Mahmoudabad *et al.*, 2008**	HF2FF human skin fibroblast cell line	180 μM H 24 hr	100 μM NAC - 1 hr pre-treatment followed by washout prior to H exposure 24 hr later	↓ Viability ↓ GSH levels ↑ Reactive oxygen species	↑ ↑ ↓
[93] **Paramov *et al.*, 2008**	LPS stimulated RAW 264.7 mouse macrophage cell line	500 μM CEES 24 hr	5, 10 mM NAC - 5 hr pre-treatment - co-administration - 5 hr post-treatment	↓ NO generation ↓ GSH levels ↓ Thiol levels ↑ Carbonyl protein levels ↓ Viability	No effect ↑ ↑ ↓ ↑ Maximal with NAC pre-treatment or co-administration Reduced with post-treatment NAC was co-administered for all other endpoints

(*continued on next page*)

Table 1 (*continued*)

Reference/Authors	Culture Model	Treatment and Exposure Time	NAC Treatment	Agent Effect on Endpoint(s)	NAC Effect on Agent-induced Endpoints
[98] **Rappeneau *et al.*, 2000a**	16HBE14o- human bronchial epithelial cell line	500 μM HN-2 3, 24 hr	1, 10 mM NAC - 24 hr pre-treatment with wash-out prior to HN-2 - co-administration - 1-3 hr post-treatment	↓ 4 hr metabolic assays ↓ 24 hr viability ↓ 3 hr sulfhydryl levels ↑ 3 hr peroxides	↑ (NAC co-administration and post-treatment) ↑ (NAC co-administration effective only) ↑ ↓ Pre-treatments ineffective
[80] **Rappeneau *et al.*, 2000b**	16HBE14o- human bronchial epithelial cell line	100 μM H 24 hr	10 mM NAC - co-administration	↓ Viability	↑ (PR = 1.6) NAC still effective up to 15 min post-H exposure
[80] **Rappeneau *et al.*, 2000b**	16HBE14o- human bronchial epithelial cell line	100 μM HN-2 24 hr	10 mM NAC - co-administration	↓ Viability	↑ (PR = 4.9)
[68] **Saberi and Mahmodabady, 2009**	HF2FF human skin fibroblast cell line	180 μM H 24 hr	1.1 mM NAC - co-administered with H for 1 hr, followed by washout	↓ Viability ↓ GSH levels ↓ Catalase activity	↑ ↑ ↑ NAC combined with acetaminophen and OTC slightly increased protection
[96] **Sabnam and Pal, 2019**	JB6 mouse skin keratinocyte cell line HaCaT human skin keratinocyte cell line	500 μM CEES 24 hr	5 mM NAC - 24 hr pre-treatment	↑ ROS ↓ L-cysteine, GSH ↓ Glutathione peroxidase, catalase ↓ 6-phosphofructo-2-kinase ↑ Protein carbonyls ↑ Lipid peroxidation ↓ Viability	↓ ↑ ↑ ↑ ↓ ↓ ↑
[97] **Sabnam *et al.*, 2020**	JB6 mouse skin keratinocyte cell line HaCaT human skin keratinocyte cell line	500 μM CEES 24 hr	5 mM NAC - 24 hr pre-treatment	↑ Mitochondrial superoxide dismutases ↓ Mitochondrial membrane potential ↑ Inflammatory cytokines ↓ Cell-cell communication	↓ ↑ ↓ ↑
[94] **Sagar *et al.*, 2014**	LPS stimulated RAW264.7 mouse macrophage cell line	500 μM CEES 24 hr	3-10 mM NAC - co-administration	↓ Viability/Apoptosis ↑ Reactive O_2/N_2 ↓ Catalase ↓ Superoxide dismutase ↓ GSH ↑ Akt, Erk1/2/tuberin phosphorylation ↓ DNA repair	↑ ↓ ↑ ↑ ↑ ↓ ↑
[69] **Steinritz *et al.*, 2010**	Mouse embryoid bodies	100 μM H 7 days	20 mM NAC - 1 hr exposure followed by washout - immediately post-H - 3 days post-H - 6 days post-H	↓ Endothelial tube formation ↑ Proliferation ↑ Caspase-3	↑ (only with day 6 NAC treatment) No effect ↓ (only with day 0 NAC treatment)
[90] **Steinritz *et al.*, 2014**	Mouse endothelial cells	12.5 μg/ml chlorambucil 24 hr	20 mM NAC - 1 hr co-administration	↓ endothelial cell migration	↑
[71] **Stenger *et al.*, 2017**	HEK293-A1-E human epithelial kidney cell line	12.5-5000 μM H 15-60 min	0.1, 0.5, 1.0 mM NAC - 15 min pre-treatment followed by wash-out	↑ TRPA1-dependent calcium flux ↑ DNA alkylation	↓ No effect

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Table 1 (*continued*)

Reference/Authors	Culture Model	Treatment and Exposure Time	NAC Treatment	Agent Effect on Endpoint(s)	NAC Effect on Agent-induced Endpoints
[95]	JB6 mouse skin keratinocyte cell line	350-500 μM CEES	- 25, 50 mM NAC	↓ Viability	↑
Tewari-Singh *et al.*, 2011	HaCaT human skin keratinocyte cell line	24-48 hr	- 1 hr pre-treatment - 1 hr post-treatment		Effective with both pre- and post-treatment in both cell lines
[88]	C3H mouse lymphocytes	2.0 μM HN-2	0.1-10 mM NAC	↑ 12 hr apoptosis	↓ NAC post-treatment only effective out to 30 min
Weltin *et al.*, 1996		12-48 hr	- co-administration - 30, 60 min post-treatment	↓ 48 hr mitogenic stimulation	↑
[82]	Rat lung slices	100, 1000 μM H	2.5, 5.0 mM NAC	↓ Viability	No effect (attributed to limited NAC tissue uptake)
Wilde and Upshall, 1994		7 days	- 30 min pre-treatment		

Abbreviations: CEES: 2-chloroethyl ethyl sulphide; EGFR: epidermal growth factor receptor; H: sulphur mustard; HN-2: bis(2-chloroethyl) methylamine; NAC: *N*-acetylcysteine; MESNA: 2-mercaptoethane sulfonate; OTC: 2-oxo-thiazolidine; ROS: reactive oxygen species.

to the amifostine analogues, NAC protection was generally poor, or non-existent. The authors discussed that lipophilicity is of critical importance for orally effective drugs and relate the prophylactic effectiveness of their test analogues to their higher fat solubility, enabling them to more easily cross cell membranes and increase penetration into the cells. It would be of interest to examine the efficacy of the more highly lipophilic NACA in this model system. *N*-Acetylcysteine (1 g/kg) was administered to mice 10 min prior to H treatment and increased survival with a protective ratio of 3.9. However, no additional details were located for this study [76] (cited in Papirmeister and coworkers [3]). The effect of NAC in reducing H tissue distribution and induced ocular toxicity was investigated in rabbits [77]. While under anaesthesia, a 0.4 μl (0.51 mg, 29 μCi of ^{14}C–H) droplet of neat H was placed onto the center of the cornea of both eyes. The eyes were treated with 50 μL of a 10% NAC aqueous solution 10 min prior to H exposure and 10 min after. Sulphur mustard was found to distribute to all eye components, with the highest concentrations in the cornea at all time points (1, 6 and 24 h after H exposure). Treatment with NAC reduced the radioactivity in all ocular tissues except the nictitating membrane. Although NAC treatment reduced eyelid edema, it had no effect on prostaglandin, aqueous protein or GSH content. The authors concluded that "NAC reduced ^{14}C binding and caused a small reduction in some aspects of HD-induced ocular toxicity" [77].

2.3. Sulphur mustard tissue culture studies; pulmonary (Table 1)

Several research groups have utilized *in vivo* and *in vitro* injury models to examine the effect of NAC on H-induced lung toxicity. In a model using early passage bovine pulmonary artery endothelial cells, H was found to produce significant levels of both apoptotic and necrotic cell death. Pretreatment with high (50 mM) NAC concentrations followed by washout was found to largely eliminate H-induced apoptotic, but not necrotic cell death [78], a finding that was later attributed to NAC-induced increases in GSH synthesis and concomitant reduction of nuclear transcription factor NF-κB activation [79]. Rappeneau and co-workers [80] used human bronchial epithelial 6HBE14o-cells to screen a number of drug combinations to protect against H toxicity. *N*-Acetylcysteine alone (10 mM) or in combination with doxycycline, N,N′-dimethylthiourea or hexamethylenetetramine provided protection against co-administered H. Further, NAC and NAC + doxycycline were still significantly protective when treatment was delayed up to 15 and 90 min, respectively after H treatment. Human lung alveolar epithelial A549 cells were treated (30 min) with a number of potential protective drugs including 2.0 mM NAC, in the presence or absence of the radioprotective compound mercaptoethanesulfonate (MESNA) prior to 50 μM H exposure. Both treatments (NAC, NAC + MESNA) were found to reduce DNA damage induced by H, but did not prevent cytotoxicity [81]. A 30 min pre-treatment of lung slices with 2.5–5.0 mM NAC failed to elicit protection against H in a 24 h lung slice model, although the authors attributed this to the limited capacity of the tissue to take up NAC [82].

2.4. Sulphur mustard animal studies; pulmonary (Table 2)

In a rat model of lung injury animals were administered H vapour (0.35 mg H in 0.1 ml ethanol for 50 min by intratracheal (IT) installation) that produced consistent, non-lethal pathology at 24 h [83]. At the beginning of this exposure, NAC (816 mg/kg) was administered IP and then bronchoalveolar lavage fluid was recovered at 24 h for analysis. The NAC treatment attenuated H-induced changes in lactate dehydrogenase, albumin, total protein, glutathione peroxidase, γ-glutamyl transferase and neutrophil counts, leading this group to conclude that NAC may be useful as a potential treatment for H-induced lung injury [83]. These findings were augmented in additional studies using the same rat model, where nebulized NAC (dose not identified) was administered every 2 h for 12 h after H exposure. Oxygen saturation levels were improved and pulmonary flow obstruction (50% expiratory

Table 2
Effect of *N*-acetylcysteine against mustard vesicant treatment in animal models.

Reference/ Authors	Animal Model	Agent Treatment	NAC Treatment	Agent Effects on Endpoints	NAC Effects on Agent-induced Endpoints
[77] **Amir *et al.*, 2003**	Rabbit, 2.0-2.5 kg New Zealand White, female	^{14}C-labeled H onto the central cornea of both eyes - 0.4 µl (0.51 mg) - 1, 6, 24 hr	50 µL of 10% aqueous NAC/eye - 10 min before and 10 min after H exposure	↑ ^{14}C-labeled H in eye tissues ↑ Prostaglandin content ↑ Aqueous protein ↑ Glutathione ↑ Histopathology	↓ In all tissues at 1 hr, except for the nictitating membrane, which was increased No effect No effect No effect Aggravated at 1 hr, small improvements at 6 and 24 hr
[83] **Anderson *et al.*, 2000**	Rat, 250-300g Crl:CD SD BBR Male	H vapour (IT) - 0.35 mg H in 0.1 ml EtOH for 50 min - 24 hr assessment	- 816 mg/kg NAC (IP) - administered at the beginning of the H exposure	Bronchiolar lavage: ↑ Lactate dehydrogenase ↑ Albumin, total protein ↑ Glutathione peroxidase ↑ γ-glutamyl transferase ↑ Neutrophil counts	↓ ↓ ↓ ↓ ↓
[76] **Anari *et al.*, 1988**	Mouse Strain/weight unknown	H (unknown route) - unknown dose/time	- 1 g/kg NAC - unknown route - 10 min pre-treatment	↑ Mortality	↓ Protective ratio = 3.9
[73] **Casillas *et al.*, 2000**	Mouse, 25-35g CD1, female	H (topical) - 0.16 µl of 195 mM H (0.16 mg) applied to the right ear - 24, 48, 72 hr assessments	- 1.0 mg NAC in EtOH; 120 min topical pre-treatment - 10 mg NAC in cream; 15 min topical pre-treatment	↑ Ear edema ↑ Histopathology	No effect No effect
[107] **Chatterjee *et al.*, 2004**	Guinea pig, 400g Hartley, male	CEES (IT) - 0.5-6 mg/kg in 100 µl EtOH - 27 day assessment	- 0.5g/day NAC in drinking water (PBS) for 30 days starting 3 days prior to CEES infusion	↑ Hair loss	↓
[103] **Das *et al.*, 2003**	Guinea pig, 400g Hartley, male	CEES (IT) - 0.5-6 mg/kg in 100 µl EtOH - 1 hr to 21 day assessments	- 0.5 mg NAC in PBS by gavage 10 min before CEES - 0.5g/day NAC in drinking water (PBS) for 3 or 30 days prior to CEES	↑ 1 hr lung ^{125}I leakage ↑ 1 hr TNF-α ↑ 1 hr NFκB activation ↑ 4 hr sphingomyelinases ↑ 4 hr ceramides ↓ 4 hr ROS enzymes ↑ 4 hr caspase activation ↑ 1 hr to 21 day mucin secretion ↑ 1 hr to 21 day histopathology	↓ ↓ ↓ ↓ ↓ ↑ ↓ No effect ↓ NAC treatment by gavage was not protective for any endpoint
[100] **Hoesel *et al.*, 2008**	Rat, 275-325g Long-Evans, male	CEES (IT) - ~6 mg/kg (2 µl/rat in 58 µl EtOH + DPBS) - 340 µl/rat instilled into left lung mainstem bronchus - 4 hr, 3 week assessments	- 3 mg/kg NAC in 100 µl DPBS (^{125}I leakage only) - 3 mg/kg NAC or NAC + α/γ-tocopherol in 100 µl liposomes (100 nm dia) - IT instillation immediately after CEES	↑ 4 hr lung ^{125}I leakage ↑ 4 hr inflammatory cytokines (lavage) ↑ 3 week histopathology and hydroxyproline	↓ (only liposomal NAC effective) ↓ ↓ liposomal (NAC + α/γ-tocopherol) protective, but not liposomal NAC-only
[77] **Jugg *et al.*, 2013**	Swine, 49-59 kg White (strain not identified), female	H (inhalation) - 100 mg/kg in ~10 min - 12 hr assessment	- 1,200 mg NAC total - nebulized and delivered via inhalation over 5 min - doses delivered at 0.5, 2, 4, 6, 8, 10 hr post-H exposure	↓ Survival ↑ Neutrophils/protein levels (lavage) ↓ PaO_2 and oxygen saturation levels ↑ $PaCO_2$ ↑ Heart rate ↑ Shunt fraction ↓ Arterial blood pH ↓ Bicarbonate levels ↑ Histopathology	↑ (8/8 vs 5/6) ↓ ↑ ↓ ↓ ↓ No significant effect ↑ No significant effect
[84] **Keyser *et al.*, 2014**	Rat, 250-300g Crl:CD SD BBR Male	H vapour (IT) - 0.35 mg H in 0.1 ml ethanol for 50 minutes - 24 hr assessment	NAC, doses not identified - nebulized NAC given by inhalation immediately following H exposure, and every 2 hr for 12 hr	↑ Pulmonary flow obstruction ↓ Tissue oxygenation	↓ ↑
[86] **Langenberg *et al.*, 1998**	Hairless guinea pig, 400-500g Crl:IAF(HA)BR, male	H (nose only inhalation) - 5 min nose-only exposures to H vapour - 96 hr LCT_{50}	5 mMol/kg NAC (IP) - 5 min pre-treatment	↑ Mortality	No statistical effect LCT_{50} = 800 mg/min/m^3 (H) versus 1285 mg/min/m^3 (H + NAC)

(*continued on next page*)

Table 2 (*continued*)

Reference/ Authors	Animal Model	Agent Treatment	NAC Treatment	Agent Effects on Endpoints	NAC Effects on Agent-induced Endpoints
[102] **McClintock *et al.*, 2006**	Rat, 275-325g Long-Evans, male	CEES (IT) - ~6 mg/kg (2 μl/rat in 58 μl EtOH + DPBS) - 340 μl/rat instilled into left lung mainstem bronchus - 4 hr assessment	- 1.5 mg/kg NAC in 100 μl liposomes (100 nm dia) - IT NAC instillation immediately after CEES - 1.5 mg/kg NAC time course; injected 10 min prior to or 10-180 min after CEES treatment	↑ Lung ^{125}I leakage	↓ (60%) immediately post-CEES treatment ↓ NAC effective when administered 10 min before or up to 60 min after CEES treatment ↓ (55%) GSH + NAC effective up to 90 min post-CEES
[101] **McClintock *et al.*, 2002**	Rat, 275-325g Long-Evans, male	CEES (IT) - ~6 mg/kg (2 μl/rat in 58 μl EtOH + DPBS) - 340 μl/rat instilled into left lung mainstem bronchus - 4 hr assessment	- 5-40 mg/kg NAC (IV) dose response; injected 10 prior to CEES treatment - 20 mg/kg NAC (IV) time course; injected 10-180 min after CEES treatment	↑ Lung ^{125}I leakage	↓ Injection of NAC 10 min prior to CEES treatment; optimal at 20 mg/kg NAC (70%) ↓ Protective effects obtained with 20 mg/kg up to 90 min after CEES treatment (54%)
[104] **Mukherjee *et al.*, 2009**	Guinea pig, 400g Hartley, male	CEES (IT) - 2 mg/kg in 100 μl EtOH - 2 hr, 30 day assessments	- liposomal (75 mM NAC + α,γ,-tocopherols) or α,γ,δ-tocopherols (IT) - 200 μl (1000 nm dia) - 5 or 60 min after CEES treatment	↑ 2 hr lung ^{125}I leakage ↑ 30 day lipoperoxidation ↑ Histopathology, lipoperoxidation	↓ 5 and 60 min NAC post-treatment ↓ 5 min NAC post-treatment ↓ 5 min NAC post-treatment Optimal protection in liposomes containing NAC and α,γ,δ-tocopherols
[105] **Mukhopadhyay *et al.*, 2009**	Guinea pig, 400g Hartley, male	CEES (IT) - 0.5 mg/kg in 100 μl EtOH - 1 hr, 30 day assessments	- liposomal 75 mM NAC + α,γ,δ-tocopherols (IT) - 200 μl (size not defined) - 5 min after CEES treatment	↑ Histopathology ↑ TNF-α ↑ MAPK activation (ERK-1, p38, JNK1/2) ↑ AP-1 signaling ↑ D2 cyclinD1, PCNA	↓ ↓ No effect ↓ ↓ Protection at 1 hr and 30 days post-CEES treatment
[106] **Mukhopadhyay *et al.*, 2010**	Guinea pig, 400g Hartley, male	CEES (IT) - 0.5 mg/kg in 100 μl EtOH - 1 hr, 30 day assessments	- liposomal 75 mM NAC + α,γ,δ-tocopherols (IT) - 200 μl (size not defined) - 5 min after CEES treatment	↑ Histopathology ↑ IL-1β, IL-6 ↑ SAF1/MAZ transcription factors	↓ ↓ ↓ Protection at 1 hr and 30 days post-CEES treatment
[74] **Parsaie *et al.*, 1988**	Guinea pig Strain/weight unknown	H (topical) - unknown dose/time	- NAC (IP) - unknown dose/time - prophylactic or therapeutic	↑ Dermal lesions	↓ slight decrease with prophylaxis No effect therapeutically
[75] **Sharma *et al.*, 2010**	Mouse, 25-30g Swiss, female	H (topical) - 1x LD50 in PEG-300	- 250 mg/kg NAC (oral) - 30 min before agent and daily for the 3 or 7 subsequent days	↓ Body weight ↓ Spleen weight ↓ White blood cell count ↑ Lipoperoxidation ↑ DNA fragmentation ↓ Glutathione	No significant effects on any endpoints
[75] **Sharma *et al.*, 2010**	Mouse, 25-30g Swiss, female	HN-1, HN-2, HN-3 (topical) - 1x LD_{50} in DMSO	- 250 mg/kg NAC (oral) - 30 min before agent and daily for the 3 or 7 subsequent days	↓ Body weight ↓ Spleen weight ↓ White blood cell count ↑ Lipoperoxidation ↑ DNA fragmentation ↓ Glutathione	No effect No effect No effect No effect ↓ HN-1 at 4 days, HN-2 at 8 days ↑ HN-1: reduced GSH at 4 days ↑ HN-2: reduced GSH at 4 days and oxidized GSH at 8 days

Abbreviations: CEES: 2-chloroethyl ethyl sulphide; DPBS: Dulbecco's phosphate buffered saline; EtOH: ethanol; H; sulphur mustard; HN-1: (bis(2-chloroethyl) ethylamine); HN-2: bis(2-chloroethyl) methylamine; HN-3(tris(chloroethyl) amine); IP: intraperitoneal; IT: intratracheal; IV: intravenous; LCT_{50}: median lethal concentration x time dose; NAC: *N*-acetylcysteine; NO: nitric oxide; PaO_2: arterial blood oxygenation; $PaCO_2$: arterial blood CO_2; PCNA: proliferating cell nuclear antigen.

Table 3
Effect of *N*-acetylcysteine in case reports or clinical trials of H-exposed patients.

Reference	Trial Parameters or Case Reports	Inclusion Criteria	NAC Dose Regimen	Outcomes
[119] **Abtahi *et al.*, 2016**	Retrospective 5 yr follow-up study NAC-treated bronchiolitis obliterans group compared to asthma and COPD groups - 38 asthma patients - 16 COPD patients - 19 bronchiolitis obliterans patients	H exposure between 1984 and 1987 Patients with documented obstructive pulmonary disease (asthma, COPD, bronchiolitis obliterans)	Asthma and COPD groups: as per existing guidelines with no NAC Bronchiolitis obliterans group: NAC 1200–1800 mg/day orally, inhaled Seretide 125–250/25 (2 puffs BID), azithromycin (250 mg, 3x/week)	1) Yearly mean FEV1 decline was significantly higher in COPD and bronchiolitis obliterans groups compared to asthma groups 2) No significant difference in yearly mean FEV1 decline between COPD and bronchiolitis obliterans groups 3) The authors conclude that considering the poorer natural history of bronchiolitis obliterans in comparison to COPD, the insignificant differences between the two groups suggest the efficacy of NAC treatment in bronchiolitis obliterans patients
[116] **Ghanei *et al.*, 2004**	Open-label study No placebo controls 17 patients	H exposure between 1985 and 1988 Patients with chronic bronchitis and bronchiolitis obliterans and not responsive to conventional treatment	NAC 600 mg/day and clarithromycin 500 mg/day orally for 6 months	1) Cough and sputum production in 10/17 patients at baseline; 10/10 improved 2) FEV1 and FVC were improved 3) No significant change in FEV1/FVC 4) The authors speculate that the results may also be due to the therapeutic effects of clarithromycin
[117] **Ghanei *et al.*, 2008**	Placebo controlled Double blind, randomized clinical trial 72 NAC patients 72 control patients	H exposure in 1988 Patients with chronic pulmonary disease due to H exposure (bronchiolitis obliterans syndrome class 0) ie. normal pulmonary function	NAC 1200 mg/day in 2 doses orally for 4 months Patients not allowed other drugs 1 month before and during the trial other than salmeterol (50 μg, 2x daily) and fluticasone (250 μg, 2x daily)	1) Cough, dyspnea and sputum production improved 2) FEV1 and FVC were improved 3) FEV1/FVC was improved 4) The authors noted that a 4 month NAC treatment improved not only symptoms, but also pulmonary function even 18 years after H exposure
[118] **Shohrati *et al.*, 2008**	Placebo controlled Double blind, randomized clinical trial 72 NAC patients 72 control patients	H exposure in 1988 Patients with chronic pulmonary disease due to H exposure (bronchiolitis obliterans syndrome class 1 and 2) ie. impaired pulmonary function	NAC 1200 mg/day in 3 doses orally for 4 months Patients not allowed other drugs during the trial other than salmeterol (50 μg, 2x daily) and fluticasone (250 μg, 2x daily)	1) Cough, dyspnea and sputum production improved 2) FEV1 and FVC were improved 3) FEV1/FVC was improved 4) The authors noted that a 4 month NAC treatment improved not only symptoms, but also pulmonary function even 18 years after H exposure
[18] **Willems, 1989**	Case reports No control groups	H-exposed casualties evacuated to Europe from 1984 to 1986 NAC treatment ~5–17 days after H exposure	9 patients: aerosolized NAC treatment 26 patients: 150 mg NAC intravenously every 3 h	No conclusions could be made as to the efficacy of NAC treatment due to the absence of control groups, the small group sizes and the large variability in patient histories. However, no note was made of adverse effects due to treatment

Abbreviations: BO: COPD: chronic obstructive pulmonary disease; FVC: forced vital capacity; FEV1: forced expiratory volume (1 s); H: sulphur mustard; NAC: *N*-acetylcysteine.

flow [EF50]) was reduced in rats 24 h after exposure [84]. These results were further supported in a domestic swine model where NAC was also found to protect against inhaled H [85]. Female white domestic swine (49–59 kg) were anaesthetized and baseline measurements taken. Once stabilized, the animals were exposed to H vapour via the endotracheal tube at a target inhaled dose of 100 μg/kg delivered over 10 min. Drug treatment was administered through the endotracheal tube as an aerosol (1 ml of 200 mg/ml NAC solution of Mucomyst™) at 30 min, 2, 4, 6, 8 and 10 h post-H treatment. Animals were followed for 12 h and sacrificed. Five of six H treated animals survived, compared to eight of eight NAC treated animals, although average histopathological lung injury scores were not significantly different between groups. Animals treated with NAC exhibited improved arterial blood oxygen saturation, pH and bicarbonate levels, as well as fewer neutrophils and lower protein levels in lavage fluid compared to the values obtained in H controls. The authors attributed the beneficial effects to NAC's antioxidant and mucolytic properties. Unfortunately, this group has not published additional studies investigating longer term outcomes of NAC treatment of H using this powerful animal model. In contrast to the above studies, NAC was not protective against H in hairless guinea pigs. The animals were administered NAC (IP, 5 mMol/kg) 5 min prior to 5 min nose-only H vapour exposures and although pre-treatment increased the 96 h median lethal concentration x time (LCT_{50}) values compared to H-only exposures [LCT_{50} = 800 mg/min/m^3 (H) versus 1285 mg/min/m^3 (H + NAC)], they were not statistically significantly different [86]. This study did not make note of any observations at earlier time points.

2.5. Summary of NAC and sulphur mustard

Early studies used tissue culture models and NAC pre-treatment strategies with or without washout, to demonstrate its protective efficacy against H, and to show that in addition to direct scavenging activity, NAC also served as a substrate for increased GSH synthesis. Ensuing *in vitro* work demonstrated the protective efficacy of NAC in reducing H-induced cytotoxicity, oxidative stress and inflammatory responses in a wide range of culture models. Animal studies using IT infused or inhaled H confirmed the protective value of NAC administered immediately after or 30 min after H exposure. However, although this work showed significant improvements in a variety of lung function and biochemical endpoints, the studies were of only 12–24 h duration. In the one longer term study carried out, although a single IP administration of NAC 5 min prior to H inhalation exposure reduced the 96 h LCT_{50} in guinea pigs, the values were not statistically different. Unfortunately, the authors did not indicate whether significant but transient protection was present at earlier time points. Although the short-term protective efficacy of NAC against H has been well demonstrated, there is a requirement for additional work examining longer term dosing regimens of NAC by different routes. While 30 day studies have been carried out with the H simulant 2-chloroethyl ethyl sulphide (CEES; see below), it has been shown that there may be some differences in the mechanisms of action between these two chemicals [87] and the long-term persistence of NAC protection against H should also be investigated. Given the well demonstrated effectiveness of NAC in protecting against the toxicity of H in a wide range of tissue culture systems, as well as against inhaled H in animal models, it may also be useful to re-examine its potential as a protectant against cutaneously applied H, especially using the more strongly lipophilic NACA.

3. NAC and sulphur mustard simulants

3.1. Simulant tissue culture studies; non-pulmonary (Table 1)

The protective properties of NAC have also been examined with putative H simulants such as the nitrogen mustards and CEES (Fig. 1). Mechlorethamine (HN-2; bis(2-chloroethyl) methylamine, Fig. 1) induced apoptotic cell death was reduced in primary C3H murine spleen cultures by the addition of 2.5 mM NAC at the time of treatment, slightly reduced with a 30 min post-treatment, and unaffected with a 60 min post-treatment [88]. In a survey of sulphur containing compounds Valeriote and Grates [89] showed that the colony forming ability of bone marrow cells in a spleen colony assay was enhanced (protection index = 26) when mice were treated with a maximally tolerated dose of NAC (8 mg/mouse, IP) 15 min prior to HN-2 administration (0.3 mg IP), compared to bone marrow cells from animals with no pre-treatment. Early endothelial cells were isolated from mouse embryoid bodies and used to assess the effects of NAC on chlorambucil-induced deficits in wound healing parameters [90]. Co-administration of NAC with chlorambucil was only modestly effective in protecting against its effects, a result that the authors hypothesized may have been due to only transient increases in reactive oxygen and nitrogen species. Pre-incubation of 17 mM NAC for 5 min at room temperature with purified chymotrypsin was shown to completely prevent its inactivation by 4.17 mM chlorambucil, an effect postulated as possibly being a result of the covalent interactions of the thiol and carboxyl groups of NAC with the agent [91]. In studies utilizing both human Jurkat cells and normal human lymphocytes, 5.0 mM NAC pre-treatment prevented the effects of CEES on the accumulation of ROS, intracellular GSH levels and apoptotic cell death markers [92]. In LPS-stimulated murine macrophages (RAW 264.7), 10 mM NAC pre-treatment or co-administration with 500 μM CEES prevented toxicity but had no effect on CEES-induced nitric oxide inhibition inhibition. Delaying the NAC treatment for 5 h yielded much reduced protective effects. Co-administration of 3–10 mM NAC also reversed several CEES-induced oxidative stress parameters [93]. Similar results were found in the same model system where co-administration of 500 μM CEES and 3–10 mM NAC reduced the adverse effects of the simulant on viability, reactive oxygen and nitrogen species, GSH, catalase, superoxide dismutase, Akt phosphorylation, ERK1/2 and tuberin, as well as on markers of DNA repair [94]. Tewari-Singh and coworkers [95] showed that a 25 mM NAC treatment 1 h before or after CEES treatment provided significant protection against cytotoxicity in both JB6 mouse skin and HaCaT human skin cells. The effects of NAC on CEES-induced inflammation, apoptosis and mitochondrial dysfunction were also investigated in these same two cell lines. Pre-incubation of 5 mM NAC attenuated 500 μM CEES-induced oxidative stress, inflammation and apoptosis [96], as well as mitochondrial superoxide dismutases and membrane potential, and cell-cell communication [97].

3.2. Simulant animal studies; non-pulmonary (Table 2)

Mice were utilized in work comparing the efficacy of several amifostine derivatives and NAC against H, as well as the closely related CW nitrogen mustard agents HN-1 (bis(2-chloroethyl) ethylamine), HN-2 and HN-3 (tris(chloroethyl) amine). Oral administration of NAC (see Section 2.2) was largely ineffective against the topical application of these agents compared to the amifostine derivatives [75].

3.3. Simulant tissue culture studies; pulmonary (Table 1)

In studies using the human bronchial epithelial cell line 16HBE14o-, 10 mM NAC was found to reduce HN-2 induced cell death, but only if present during the exposure [98]. Co-administration of NAC with HN-2 in this culture model resulted in a protective ratio of 4.9, significantly better than that obtained for H (1.6) [80]. The authors concluded that in both cases, protection was mainly due to the direct chemical scavenging of NAC with the agents in the culture medium. Primary human bronchial epithelial cells were used to evaluate the role of oxidative stress and epidermal growth factor receptor (EGFR) activation in CEES-induced cytokine secretion. The elevation of interleukin-6, ROS, protein carbonylation, EGFR phosphorylation and NADPH oxidase activation induced by 200 μM CEES was largely eliminated by a 30 min

pre-incubation with NAC [99]. Primary rat alveolar macrophages from bronchoalveolar lavage were incubated with 500 μM CEES with or without antioxidant liposomes containing NAC (final NAC concentration unclear) for 4 h [100]. Liposomal NAC was shown to significantly reduce the levels of the CEES-induced inflammatory response (CINC-1, IL-1β, IL-6, TNF-α).

3.4. Simulant animal studies; pulmonary (Table 2)

The oxidative stress known to be produced by H and CEES in the lungs and respiratory tract has also resulted in a series of studies examining the effect of NAC against the pulmonary toxicity caused by IT introduction of CEES (6 mg/kg) into the lungs of guinea pigs or rats. McClintock and co-workers [101] showed that intravenous pre-treatment (−10 min) of 20 mg/kg NAC in rats elicited protection against CEES-induced lung toxicity at 4 h (70%), and that treatment was still effective when given up to 90 min after CEES exposure (54% protection). Studies utilizing liposome encapsulated NAC were carried out in an effort to obtain higher and more persistent drug tissue concentrations [102]. In 4 h experiments, rats were exposed IT to ~6.0 mg/kg CEES and IT installation of liposomal NAC was found to be effective in reducing lung damage whether administered 10 min before (60%) or 60 min after (55%) CEES treatment [102]. Similar results were also reported in the same rat model system by Hoesel et al. [100], who showed that liposomal NAC (3 mg) administered IT immediately after CEES treatment was effective in eliminating the short-term effects (4 h; increased lung permeability and elevated pro-inflammatory mediators in bronchoalveolar lavage fluids) induced by ~6.0 mg/kg CEES. However, these studies also showed that similar NAC dosing regimens were ineffective in decreasing CEES-induced fibrosis three weeks after exposure, unless α/γ-tocopherol was also included in the liposomal preparations. This study also included a direct comparison of the protective effects of liposomal NAC encapsulation compared to treatment with NAC in solution (3 mg; Dulbecco's phosphate buffered saline) against CEES when administered immediately after agent treatment. This work indicated that liposomal NAC reduced the CEES-induced leakage of ^{125}I-albumin from lung parenchyma into the bloodstream at 4 h by 59%, compared to less than 10% for NAC in solution [100].

Similar studies to the rat work were carried out using guinea pigs, a species whose lung function and structure are more similar to that of humans than are rats. In animals exposed IT to 0.5–6 mg/kg CEES, although a single 0.5 g dose of NAC administered via gavage just prior to CEES exposure was ineffective, pre-treatment with 0.5 g NAC/animal/day in the drinking water for 3–30 days protected against 69–76% of the acute (4 h) lung injury and inhibited several associated markers of CEES-induced lung damage [103]. These studies were extended to examine the efficacy of "antioxidant" liposomes comprised of different levels of NAC, GSH, cholesterol, phospholipid and tocopherols against IT CEES-induced lung injury. The IT liposomal treatments were infused at 5 or 60 min after CEES and were effective in reducing several aspects of both short- and long-term lung toxicity in guinea pigs, including increased lung permeability at 2 h, and increased lung lipoperoxidation, hydroxyproline (a measure of fibrosis), and histopathology at 30 days. Optimal protection was obtained when liposomes were comprised of 75 mM NAC, 11 mM α-tocopherol, 11 mM γ-tocopherol and 5 mM δ-tocopherol [104], and this treatment and model system were used in subsequent studies to ascertain whether it could modulate the CEES-induced MAPK/AP-1 signalling pathway in lung injury [105]. This work showed that although antioxidant liposome treatment was ineffective in reducing the CEES-induced phosphorylation of several members of the MAPK (ERK-1, p38, JNK1/2) family, it did show significant efficacy in decreasing the CEES-induced activation of AP-1 transcription factors, and in decreasing TNF-α, cyclin D1 and proliferating cell nuclear antigen (PCNA) up to 30 days, leading the authors to conclude that the protective effect of antioxidant liposomes against CEES was mediated through control of AP-1 signalling [105]. Further efforts by this group again used this model system to examine the role of the transcription factors serum accelerator factor-1 (SAF-1) and Myc-associated zinc finger protein (MAZ) in CEES-induced inflammation. The antioxidant liposome treatment (5 min post-CEES) significantly blocked the CEES-induced activation of IL-1β and IL-6, as well as SAF-1/MAZ [106], again out to 30 days post-CEES exposure. No NAC-only or liposomal NAC-only treatment groups were included in these studies to show the comparative protective efficacy of NAC or NAC-only encapsulation. As an off-shoot of the above studies, guinea pigs given NAC in their drinking water (0.5 g/day/animal) were found to be less prone to CEES-induced hair loss than IT infused CEES-only exposed animals [107].

N-Acetylcysteine has also been found to be an effective pulmonary protectant in other cases where highly reactive molecular species are produced upon chemical exposure. These include known lung toxicants such as bleomycin [108,109], perfluoroisobutene [110], paraquat [111,112] and phosgene [113,114].

3.5. Summary of NAC and sulphur mustard simulants

The protective efficacy of NAC against the H simulants CEES and HN-2 has been demonstrated in several cell culture models where it reversed or reduced the agent-induced deficits on viability, inflammation, oxidative stress and a wide range of additional endpoints. In some systems NAC post-exposure was protective, although this work tended to use relatively low agent concentrations and the protection was reduced with time post-NAC treatment. Several studies have been conducted examining the efficacy of NAC in protecting against the pulmonary injury caused by IT infusion of CEES in both rats and guinea pigs. When NAC was included in the drinking water for 3–30 days prior to CEES exposure, lung damage was reduced out to 21 days in guinea pigs. When encapsulated into liposomes and instilled IT, NAC was only effective in reducing CEES-induced endpoints of lung damage out to 4 h, unless tocopherols were also encapsulated. In these cases, several markers of lung damage were reduced out to 30 days post-CEES exposure. This work demonstrates the potential long-term efficacy of NAC treatment against mustard-induced lung injury when used with liposomal encapsulation, in combination with other drugs, and by different dosing routes.

4. NAC and sulphur mustard casualties (Table 3)

Limited studies exist examining the clinical use of NAC in H-exposed individuals. In a summary of the clinical files of Iranian H casualties evacuated to European hospitals from 1984 to 1986, Willems [18] briefly discussed the use of NAC in patients during their treatment. In 9 patients, NAC was administered as an aerosol primarily for its mucolytic properties, although it was also hoped that it might bind residual free H in the lungs. In addition, 26 patients were also treated via the intravenous infusion of 150 mg NAC every 3 h to bind and inactivate circulating free H in the body. However, no conclusion as to the benefits of these treatments could be made due to the small group sizes and the differences in patient histories. The author also expressed doubts as to the efficacy of a treatment aimed at the detoxification of H when there was no evidence of "toxicologically relevant concentrations of sulphur mustard on the patient or circulating in the blood at the time of arrival at the European clinic several days after exposure" (~5–17 days) [18].

Iranian clinical trials have been carried out examining the effect of NAC on the long-term pulmonary effects in H-exposed veterans of the Iran-Iraq War with positive results [5,115]. In 2004 Ghanei and coworkers [116] reported on a study investigating the effect of daily oral 600 mg NAC and 500 mg clarithromycin treatments on veterans documented as having been exposed to H between 1985 and 1988. These veterans were diagnosed as having bronchiolitis obliterans that was non-responsive to high dose bronchodilator (salmeterol) therapy and inhaled corticosteroid (fluticasone propionate). At six months, in

those patients exhibiting cough and sputum (10/17), all ten exhibited reduced rates of these symptoms. Forced expiratory volume over 1 min (FEV1) was improved in 14 of 17 patients with an overall improvement of 10.6 ± 9.7% ($p < 0.001$ versus baseline), while forced vital capacity (FVC) was improved in 13 of 17 patients with an overall improvement of 12.9 ± 13.6% ($p < 0.001$ versus baseline). No placebo, clarithromycin-only, or NAC-only controls were carried out in this study and the authors volunteer that the results may have been due to the clarithromycin treatment. A much larger (144 patients) placebo controlled, double-blind study was conducted by the same group examining the effects of NAC in Iranian veterans "suffering from pulmonary disorders due to a single high dose of sulfur mustard gas during the Iran-Iraq conflict in 1988", but exhibiting normal pulmonary function (bronchiolitis obliterans syndrome class 0) [117]. Patients received 600 mg NAC or placebo twice daily for four months. No other drugs were allowed one month before or during the trial. However, salmeterol (50 μg twice daily) and fluticasone (250 μg twice daily) were taken by all patients during the study. *N*-Acetylcysteine was found to be highly effective statistically in reducing dyspnea, cough, and sputum, as well as improving pulmonary function endpoints such as FEV1 and FVC. The authors considered the findings to be remarkable due to the eighteen year gap between H exposure, and the relatively short four month NAC treatment regimen. This same group published a similar study using identical parameters, but this time with inclusion criteria requiring the trial patients to be exhibiting impaired pulmonary function (bronchiolitis obliterans syndrome class 1 and 2) due to the previous 1988 H exposure, and NAC being administered in 600 mg tablets three times daily [118]. Virtually identical conclusions were obtained. However, although these studies appeared to have been run simultaneously by the same group, no comparisons were made between these two trials, even when reviewed by the same authors in later review papers [5,115].

In a retrospective five year follow-up study aimed at assessing the longer term effectiveness of therapy including NAC, Abtahi and coworkers [119] assessed nineteen Iranian veterans suffering from H-induced bronchiolitis obliterans and treated for five years with inhaled Seretide (125–250/25 μg 2 puffs BID), azithromycin (250 mg, three times/week) and NAC (1200–1800 mg/day). When compared against matched chronic obstructive pulmonary disease patient groups (asthma and chronic obstructive pulmonary disease) "treated according to guidelines", FEV1 decline was similar over the five year period. The authors interpreted this outcome, when considering the poorer prognoses for patients suffering from bronchiolitis obliterans compared to those with COPD, as proof positive of the effectiveness of this long-term treatment regimen.

Although the ability of thiols to bind H has been recognized for many decades, investigations into NAC as a potential H antidote have been carried out primarily after the end of the Iran-Iraq war. Thus, while the few examples of studies using NAC as a treatment in H-exposed casualties are largely positive, they were directed towards the long-term pulmonary effects that occurred many years after exposure. Fortunately, there are relatively few opportunities to investigate the efficacy of drugs against CW agents in humans. However, there exists a significant quantity of work that suggests that NAC or its derivative NACA should have great potential as an early treatment of H-induced injury in humans, either as a scavenger or in preventing the cascades of deleterious events that H has been shown to cause, particularly in the respiratory system. The safety of NAC has been demonstrated in numerous clinical trials (see Section 5.0) and strong consideration should be given to this drug as a first line treatment for H poisoning in the future.

5. NAC in other clinical applications and safety

The relative safety of NAC in humans has been well illustrated not only by its long history of clinical use, but also by the broad range of clinical applications that it is continually being tested for, or applied to. Research on NAC was initiated in the 1960s with reports of its effectiveness as a mucolytic in patients with cystic fibrosis (CF). Its development was the result of efforts to provide reduced thiols to break the disulphide bridges in the glycoprotein matrix of mucus in CF patients. Acetylation of cysteine yielded the much more stable NAC, which was shown to have significant mucolytic activity and clinical applications not only in the treatment of CF, but also in the prophylaxis and therapy of a number of other respiratory diseases [52]. In the 1970s, the ability of NAC to provide sulfhydryl moieties drove the effort to utilize it as a treatment for acetaminophen poisoning. During these studies, its protective role was also shown to include the provision of precursors to replenish GSH levels that had become depleted during phase two metabolism of acetaminophen [46]. The resultant widespread clinical use and success of NAC treatment in acetaminophen poisoning [49–51] has subsequently driven a large body of research investigating its therapeutic potential in a broad range of pathologies and disorders that are thought to be due to oxidative stress and GSH depletion such as bronchitis, doxorubicin-induced cardiotoxicity, acute respiratory distress syndrome, radio-contrast-induced nephropathy, pesticide exposure, heavy metal toxicity, ischemia-reperfusion injury and dermatological conditions [46,47,120,121]. *N*-acetylcysteine has also recently been proposed as both a preventative and therapeutic agent against COVID-19 due to its abilities to reduce the excessive mucus production, GSH depletion, oxidative stress and inflammation in the lungs that this virus causes [122–127], and several clinical trials are being carried out to assess NAC's efficacy against this disease [128–131]. Interestingly, it has also been noted "that there are some similarities in the pathogenesis of SARS-CoV-2 and mustard gas in immune system disruption and pulmonary infection" [132], including up-regulation of lung angiotensin-converting enzyme (ACE2), the receptor for SARS-CoV-2. The authors speculate that these commonalities will lead to increased susceptibility and more severe outcomes in Iranian survivors of H exposure to COVID-19 [132].

The mechanistic complexity of NAC has become increasingly obvious and with the revelation that in addition to its antioxidant and GSH stabilizing attributes, it also impacts anti-inflammatory pathways, mitochondrial function, apoptosis and neurogenesis, there has been growing evidence that this drug can also be of utility in treating a host of psychiatric and neurological disorders. Implicit in much of this work have been the discoveries that NAC also impacts neurotransmission not only through facilitation of dopamine release, but also through regulation of NMDA and AMPA due to NAC-induced activation of cysteine/glutamate antiporter activity and subsequent elevations in glutamate levels. Trials using NAC to treat a multitude of neuropsychiatric disorders have been carried out and have yielded positive evidence for its use in the treatment of Alzheimer's and Parkinson's diseases, autism, compulsive and grooming disorders, depression, bipolar disorder, schizophrenia, cocaine and cannabis dependence [53,133–138]. In a recent report reviewing NAC safety and efficacy as a protectant against traumatic brain injury (TBI), the authors found "moderate quality of evidence of efficacy and safety of the use of NAC and NACA in preclinical studies" and very limited clinical research in TBI patients [56]. They concluded that the available information warranted further animal work and clinical trials.

Deepmala and coworkers [134] reviewed clinical trials using NAC in neurological and psychiatric diseases, and highlighted the high dosages of this drug that can be tolerated, as well as the potential side effects of this drug. The trials reviewed used daily dosages ranging from 0.6 to 6 g/day with the majority of the studies using 2.0–2.4 g/day. Durations of the trials ranged from 2 days to 60 months, with positive outcomes in all cases. Side effects were rare in these studies, and very few reported were severe enough to necessitate discontinuation of NAC treatment. Gastrointestinal symptoms were the most common side effects and included mild abdominal pain and/or discomfort, nausea, vomiting, heartburn, flatulence, cramps and diarrhoea. Neurological side effects included headache and right hand tingling. Isolated

instances of adverse effects also included rash, pruritus, fatigue, dry mouth, muscle pains, insomnia, nasal congestion, runny nose, restlessness, dizziness and irritability [134]. They concluded that given NAC's long track record of safety, tolerability, affordability, availability and positive but still preliminary results, there is a requirement for well designed, larger controlled studies for its use in a variety of different psychological and neurological disorders, a theme echoed in other reviews of NAC in these areas [53,56,133–138].

6. Summary

For over one hundred years, researchers have endeavoured to understand the mechanism of toxic action of H and to develop medical countermeasures against it. Although a great deal has been learned as to how this CW agent disrupts a broad range of cellular, biochemical and molecular pathways, the links between H exposure and toxic effects have as yet to be fully understood. Of the many potential candidate molecules investigated for potential protective efficacy against H, NAC possesses the most robust record both in terms of safety and of efficacy. It is available as an over-the-counter nutritional supplement, and is also considered to be a safe and well tolerated medication for a host of diseases. Although, originally investigated for its direct antioxidant activities and maintenance of intracellular GSH levels, NAC also appears to play a role in an increasingly wide range of cellular pathways. It has been shown to be prophylactically and therapeutically protective against a broad range of drugs/chemicals, including H and H simulants. Notably, studies have repeatedly shown the efficacy of NAC in decreasing H toxicity in both *in vitro* and *in vivo* model systems of lung injury, while Iranian clinical trials have demonstrated the safe and efficacious use of NAC in treating patients suffering the long-term and chronic pulmonary effects of H in veterans of the Iran-Iraq War - up to eighteen years after the initial exposure. The well documented properties of NAC warrant further attention to it as a candidate for additional pre-clinical work, as well as for inclusion in clinical trials to facilitate its regulatory approval as a treatment for the pulmonary effects of H.

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Declaration of competing interest

None.

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