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Scavenger Hunt: The Search for Sulfur Mustard-Neutralizing Compound in 2-Chloroethyl Ethyl Sulfide (CEES) Treated Human Keratinocyte (HACAT) Epithelial Cells

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SCAVENGER HUNT: THE SEARCH FOR SULFUR MUSTARD-NEUTRALIZING COMPOUNDS IN 2-CHLOROETHYL ETHYL SULFIDE (CEES) TREATED HUMAN KERATINOCYTE (HACAT) EPITHELIAL CELLS

BY

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THESIS ACCEPTANCE PAGE

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This thesis work is dedicated to my family and friends, who have been a constant source of motivation and encouragement.

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TABLE OF CONTENT

ABBREVIATIONS

A: Acetamide

AA: Acetic Acid

BAL: British Anti-Lewisite (Dimercaprol)

CEES: 2-Chloroethyl Ethyl Sulfide

CMA: Cell-Matrix Adhesion

CWA: Chemical Warfare Agent

CYS: Cysteamine

DMEM: Dulbecco's Modified Eagle Media

DMPS: 2,3-Dimercapto-1-Propanesulfonic Acid

DMSA: Dimercaptosuccinic Acid

EA: Ethyl Amine

ET: Ethanethiol

MESNA: 2-Mercaptoethane Sulfonate Sodium

METH: Methimazole

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NAC: N-Acetyl Cysteine

TAA: Thioacetic Acid

TRI: Triethylenetetramine (Trientine)

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

SCAVENGER HUNT: THE SEARCH FOR SULFUR MUSTARD-NEUTRALIZING COMPOUNDS IN 2-CHLOROETHYL ETHYL SULFIDE (CEES) TREATED HUMAN KERATINOCYTE (HACAT) EPITHELIAL CELLS

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Sulfur mustard is a highly toxic and dangerous vesicant that has been utilized as a chemical warfare agent (CWA) since World War I. Despite its extensive history, an effective antidote to sulfur mustard exposure still does not exist. With detectable levels of unreacted sulfur mustard lasting days after initial contact, a window of opportunity exists to administer a "scavenger" to affected individual immediately and in the days following exposure in hopes of reducing harm by neutralizing unreacted sulfur mustard. For this strategy to be effectively implemented, it is essential to identify a candidate compound with excellent affinity towards sulfur mustard and very low toxicity.

A deliberate stepwise process of scavenger identification was accomplished in this study. First, a group of compounds with select functional group nucleophilicity (i.e., first-generation scavengers) towards chloroethyl ethyl sulfide (CEES) (i.e., CEES is a non-CWA surrogate for sulfur mustard) was evaluated. A series of two-carbon molecules with multiple scavenging functionalities were initially tested for effectiveness in protecting human keratinocyte (HaCat) cells via MTT cell viability and cell-matrix adhesion (CMA) assays. HaCat cells were used to mimic dermal exposure. The first-generation scavengers, a set of five two-carbon compounds with various functional groups, served as a preliminary group of scavengers to survey the correlation between

scavenging ability and functional group nucleophilicity. These trials generally validated the trend of increasing scavenging-ability mirroring increasing nucleophilicity, with the exception of thioacids, which produced additive toxicity. Next, second-generation candidate scavengers (i.e., drug molecules containing promising structural features identified from the first-generation scavengers) were identified and their performance as scavengers was evaluated. With thiols and amines proving to be the most promising functional groups in terms of both excellent nucleophilicity and minimal toxicity, the second-generation candidate scavengers were selected to explore the effectiveness of these functionalities. Out of this group of seven second-generation scavengers, the thiodiamine functional group, found in the candidate scavenger methimazole, most effectively reduced cell death and loss of cell-matrix adhesion caused by CEES. Overall, this bottom-up comprehensive search for promising scavengers has narrowed the field of potential candidate molecules to those possessing thiols, thioamines, and thiodiamines.

Chapter 1. Introduction

1.1. Significance

Although the use of sulfur mustard for chemical warfare dates back to World War I, an effective antidote has not been developed. Sulfur mustard's initial reactivity and the finding that unreacted sulfur mustard remaining in the body days after initial exposure (i.e., SM has been detected in the urine of exposed individuals for up to a week post-exposure) results in a long and slow recovery process with prolonged suffering from chemical burns and internal injury.[1] With the resurgence of chemical warfare agents (CWAs) in modern unconventional warfare, the concern behind potential attacks with compound which have no countermeasure is of great concern. Therefore, there is a need to discover and develop an effective and non-toxic treatment for sulfur mustard.

1.2. Objective

The overall objective of this work was to deliberately identify scavenger candidates as sulfur mustard therapeutics and select one or two of the most successful molecules for advancement to *in vivo* murine models. While the "scavenging approach" (i.e., treatment with a relatively non-toxic compound which reacts selectively with sulfur mustard to produce reaction products with minimal toxicity) has been attempted with limited success, a deliberate structure-activity approach has not been undertaken to identify the molecular features advantageous to scavenging.[2, 3] The first phase of this project focused on evaluating a series of five two-carbon molecules with a variety of nucleophilic functional groups to determine the most effective structural features of these scavengers. In the second phase of the project, eight scavenger molecules were selected based on the most successful scavenging functionality from the first group, while actively

considering toxicity of the candidates. The scavenging ability of these molecules was evaluated in the same manner as the initial scavengers in order to select the most promising candidate chemical structures for potential translation to *in-vivo* models.

1.3. Vesicating Chemical Warfare Agents

1.3.1. Origin and Development

Out of the known blistering CWAs, the synthesis and study of sulfur mustard is the oldest. Although the purity of the product was questionable, it is likely that the first successful sulfur mustard synthesis was conducted by Despretz in 1822.[4] After a few similar experiments throughout the 1800's, Meyer synthesized a far purer sulfur mustard product in 1886; a slightly modified version of this method was utilized by the German military to produce large quantities of sulfur mustard for chemical warfare in World War I.[5] In the 1930s, nitrogen mustards were first developed as potential CWAs due to their similarity to sulfur mustard. However, no structural variant in this class of compounds was found as tactically useful as sulfur mustard.[6] One notable discovery that did arise from these studies was the cytotoxic effect of some nitrogen mustards in treating forms of leukemia, and consequently, this led to the creation of a new class of chemotherapeutic agents.[7]

The other major CWA vesicant is Lewisite. Due to its inclusion of arsenic in the chemical structure, this compound is an arsenical. Although manufactured towards the end of World War I for use on the battlefield, there is no data on Lewisite's impact from practical use; the war ended prior to its arrival on the frontline, so the stock was subsequently destroyed.[8]

1.3.2. Application and Prevalence

CWAs were initially employed in World War I to target trench warfare. Effective CWAs possessed high specific gravity, such that the gaseous form of these compounds would sink to the lowest point and often turn the trenches into a hazardous area ripe for dealing debilitating injuries to its inhabitants.[6] Aerial mustard bombs were another popular method to inflict damage over the widest possible area.[9] While vesicating CWAs can cause death, it is rare; they are much more effective at incapacitating large numbers of individuals. These CWAs produce long-term debilitating symptoms, are persistent in the environment, and inflict harm on large numbers of individuals.[10] Therefore, the underlying motives behind these agents were to remove large numbers of soldiers from the battlefield, overwhelm hospitals and healthcare services, and harm civilians.[6] The development and use of CWAs increased through World War I, leading to the Geneva Protocol of 1925 outlawing the use of CWAs and biologicals as methods of warfare.[11] A gap in this treaty was found in its lack of prohibition of the production and storage of CWAs.

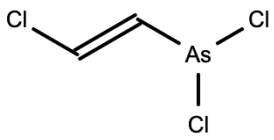
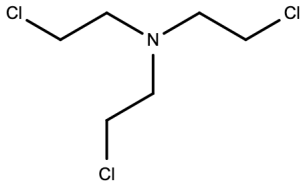
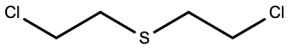
Following the Geneva Protocol, the use of CWAs was reduced worldwide, but numerous instances of CWA use occurred following the Geneva Protocol, where countries used CWAs in conflicts or simply against their own citizens.[12] Even developed countries, such as the U.S., still maintained a large stockpile of CWAs long after the Geneva Protocol was signed, likely as a deterrent for other countries in future conflicts. Finally, in 1997, the Chemical Weapons Convention added additional international protocols that restricted the production and storage of CWAs and required the destruction of existing CWAs and their production facilities.[13] Four UN-member countries have still

not fully complied to these terms: Israel, Egypt, North Korea, and South Sudan.[14] Despite these measures to eliminate CWAs from the world-wide military arsenal, radical militant groups such as ISIS recently seized stockpiles and proceeded to conduct attacks on civilians throughout the Middle East.[15]

1.3.3. Properties of the Major Vesicating Agents

The three major vesicating CWAs are lewisite, nitrogen mustard, and sulfur mustard, as shown in Table 1.1. Each of these compounds have commonalities and key differences, as discussed in further detail below.

Table 1.1. Names and chemical structures of the three main vesicating CWA.

Lewisite	**Nitrogen Mustard**	**Sulfur Mustard**
		

1.3.3.1. Lewisite

Developed near the end of World War I, Lewisite was never utilized in any battlefield operation. It is reported to smell of geraniums and was originally planned to be used in a mixture with sulfur mustard in order to lower its melting point and increase its efficacy against chemically resistant garments.[16] Categorized as an arsenous chloride, Lewisite reacts as a strong electrophile, substituting its chloride groups for various nucleophilic groups. Overall, the exact mechanism behind Lewisite's effects on living systems is yet to be fully defined, but some mechanisms are known. One well-known mechanism of action for Lewisite proceeds through permanently binding to thiol groups,

thereby inhibiting key metabolic enzymes involved with carbohydrate metabolism.[17] This, in turn, inhibits the production of acetyl-CoA and halts the Kreb's Cycle. Relying solely on glucose as an energy source, the nervous system is highly impacted by these interactions.[18]

Lewisite exposure results in pain and redness of the affected skin and occurs promptly after exposure, normally seconds to minutes after contact.[19] Lewisite does not suppress the immune system and the pain intensity and duration are relatively mild compared to other vesicating agents, but the absorption rate of Lewisite is relatively rapid, as it fully penetrates the skin in 3-5 minutes.[20] Exposure can lead to edema, and if exposure levels are high enough, a condition called Lewisite shock can occur, where proteins and plasma leak though vessels systemically, causing hypotension and hemoconcentration.[21]

Lewisite does have an antidote, called dimercaprol or British Anti-Lewisite (BAL), which is capable of decreasing the mortality rate and symptom severity if applied intramuscularly or via inhalation within 100 minutes from the initial Lewisite exposure.[22] Since BAL has a higher affinity for the arsenic atom than other nucleophilic sites within the cell, the Lewisite is displaced from the macromolecules it is initially bound to and allows normal cellular function to resume.[23] Since BAL was proven as an effective antidote, Lewisite has lost efficacy as a viable stand-alone option in warfare scenarios and is not a primary concern for further countermeasure research.

1.3.3.2. Nitrogen Mustards

Initially, nitrogen mustards were being developed around World War II as an additional CWA. However, due to their relatively low reactivity and their cytotoxic effects on white blood cells, nitrogen mustards were soon repurposed to a less maleficent use.[24] After an accidental dispersal in Bari, Italy during World War II, the United States discovered from follow up studies on the survivors that their number of lymphocytes were significantly lowered upon exposure to nitrogen mustards.[25] Upon further testing, differential effects of toxicity between tumor and control mice tissues led researchers to develop nitrogen mustards into a new class of chemotherapeutics.[24] As of today, nitrogen mustards are not considered a serious wartime threat.

1.3.3.3. Sulfur Mustard

1.3.3.3.1. Background and Uses

As stated earlier, sulfur mustard was initially developed and purified during the mid to late 19th century. Introduced during World War I by the Germans in the Battle of Flanders (near Ypres, Belgium) in 1917, sulfur mustard was soon used by both sides to target soldiers in trench warfare environments.[6] Despite the Geneva Protocol of 1925, the use of sulfur mustard has been reported in various conflicts around the world ever since, with the most recent being by the Syrian government against its own civilians in 2016.[15] Due to its ease of synthesis, widespread accessibility of starting materials, and lack of antidote, sulfur mustard is one of the largest CWA threats to this day.

1.3.3.3.2. Physical Properties and Characteristics

Purified sulfur mustard is a clear and colorless oily liquid at room temperature. Its boiling point is 227 ºC, although it decomposes at lower temperatures, and its smell resembles mustard or garlic, which inspired the name.[6] With a molar mass of 159.08 g/mol and a specific gravity of 1.27, vaporized sulfur mustard is denser than air and sinks to the lowest accessible point.[6] Due to its oily, hydrophobic nature, sulfur mustard easily penetrates membranes and fatty tissues in living systems and persists in soils and other environmental materials for weeks. Once in the environment, sulfur mustard can continue to cause harm by vaporizing and condensing multiple times as temperature fluctuates between the day and night, respectively, creating a cycle of exposure risk.[6, 26]

1.3.3.3.3. Mechanism and Toxicity

Traditional sulfur mustard is a symmetrical bifunctional alkylating agent with a sulfur atom surrounded by two chloroethyl groups as seen in Table 1.1. Sulfur mustard forms a sulfonium ion through an intramolecular cyclization process where the chloro functional group on each ethyl group acts as a leaving group. This sulfonium ion has a positive charge and a high affinity towards various biomolecular nucleophilic sites. In human cells, these nucleophilic sites are abundant in nitrogenous bases of DNA, carbohydrates, proteins, and lipids.[27] Although the full mechanism behind sulfur mustard toxicity is still not known due to the number of pathways it can affect, the most characterized mechanism within living systems is the alkylation of DNA. Most often occurring at the N-7 position on guanine, alkylation of the DNA can cause crosslinking and strand breakage.[28] Activating the DNA-repair pathway involving poly(ADP-ribose) polymerase (PARP), the cell soon uses up its storage of NAD^+.

Without a supply of NAD^+, oxidative metabolism of carbohydrates is no longer possible, energy supplies become depleted, and cell death ensues soon after.[29] Sulfur mustard exposure also raises the levels of matrix metalloproteases which serve to cleave adhesions between the cell and extracellular matrices, contributing to the vesicating effect for which sulfur mustard is known.[30] Cell death, combined with this loss of extracellular adhesion, recruits phagocytes and activates an immune response which summates to sulfur mustard's well-known vesicating effect.[29] Finally, sulfur mustard also creates adducts with glutathione. As cellular levels of this vital antioxidant molecule become diminished, it is hypothesized that free radical oxygen species that would have otherwise been eliminated by glutathione end up causing harmful oxidizing reactions throughout the cell.[31] In particular, the oxidation of lipids within the cell membrane change its fluidity and function, and ultimately, lead to its degradation.[32]

While there are a few well-documented mechanisms by which sulfur mustard effects biological systems, many others likely exist due to the plethora of nucleophilic sites which could be affected and the subsequent biochemical pathways that sulfur mustard adducts could disrupt.

1.3.3.3.4. Metabolism

As discussed above, sulfur mustard reacts with glutathione and various macromolecules. It is also directly hydrolyzed into thiodiglycol and oxidized into other metabolites (e.g., sulfur mustard oxide).[33] The elimination of sulfur mustard from the human body takes place through multiple pathways. Adducts of various macromolecules, conjugates to amines and thiols, and oxidation and hydrolysis products have been

detected in urine samples.[34] While in small quantities, the unreacted sulfur mustard molecule is detectable in urine up to one-week post-exposure.[1]

1.3.3.3.5 Short-term Physiological Implications

One of the most well-known characteristics of sulfur mustard exposure is the delayed onset of its symptoms. Unless exposed to very high quantities, individuals may not see any symptoms for 2-12 hours after exposure, depending on the exposure route.[35] These early effects are erythema of the skin, burning, itching, and tearing of the eyes, and sneezing, coughing, mucus production, and hoarseness of the airways.[6] After about a day, the full set of symptoms are finally displayed though vesicating (blistering), pain, redness, possible cornea damage, productive cough, difficulty breathing, and possible pulmonary edema seen in the airways. If the severity of exposure is great enough, corneal rupture, skin lesions with necrosis, airway obstruction, and even death are possible.[6]

Although sulfur mustard is widely known for its extremely dangerous properties, exposure to sulfur mustard rarely results in death. Out of all individuals reported to have suffered sulfur mustard exposure during World War I, only about 2.6% of British soldiers and 2.2% of American soldiers died.[6, 36] The main factors contributing to death were bone marrow failure, secondary infection, and sepsis. Specifically, hematopoiesis suppression prevents the production of vital cellular components of blood, while the rupture of blisters renders the body vulnerable to fatal bacterial infection or sepsis.[37]

Since sulfur mustard is lipophilic, it often diffuses into membranes and fatty tissues where it remains temporarily unreacted.[38] Over a course of days to weeks, these molecules slowly diffuse back out and contribute to prolonged symptoms that are characteristic of sulfur mustard exposure.[38] This lengthy symptomatic period, along with

the risk of secondary infection, significantly lengthens the recovery time and length of stay within a medical treatment facility.

1.3.3.3.6. Long-term Physiological Implications

Chronic effects are most common in severe exposure cases but can also manifest in moderate cases as well. In the eyes, the cornea can become cloudy and opaque.[10] Scarring and increased chance of carcinogenesis occurs in affected skin along with abnormal pigmentation being a common occurrence.[10] Airway epithelium can scar as well, with permanent lung damage possible as well as chronic bronchitis and chronic obstructive pulmonary disease.[10] Due to its alkylating and crosslinking effect on DNA, sulfur mustard is also a mutagen which persists as long as the effected cell lives.[39] Of those whose exposure was mild and proceeded without systemic effects or infections, a full recovery is quite common.

1.4. Sulfur Mustard Exposure Countermeasures and Treatments

1.4.1. Symptom Management

The most commonly and severely affected organs and systems within the human body are the airways, skin, and eyes. Once exposed to sulfur mustard, immediate cleansing and irrigation of the affected area with tap water and neutral soap is vital to lessen the damage sulfur mustard causes. A common cause of death is secondary infection from open wounds, so another vital intervention is administering topical bacteriostatic agents and antibiotics if necessary. To subside pain and discomfort, a variety of analgesics and antihistamines can be used to marginally decrease these unwanted symptoms.[40] Reports have suggested that debridement of larger wounds and

blisters may also help accelerate the healing process.[41] In cases of severe respiratory injury, patients may be anesthetized and put on a ventilator if breathing becomes too painful and laborious. For severe skin and eye damage, skin grafts and transplantation (i.e., corneal transplantation) may be necessary if chronic effects arise.[40]

1.4.2. Proposed and Tested Therapies

A highly studied area of sulfur mustard therapeutics involves different forms of biotherapy. This approach entails treating the sulfur mustard exposure with different biological products such as proteins, polysaccharides, and tissue transplantation. Common proteins used for biotherapy include cytokines to induce proliferation and enhance wound healing and protease inhibitors to prevent sulfur mustard's vesicating effects. Polysaccharides tested include heparin to reduce coagulation in the lungs to retain function and lipopolysaccharides (LPS) to support macrophage survival.[42, 43] Transplantation as a therapy has also been tested, including bone marrow transplantation to alleviate bone marrow suppression, mesenchymal stem cell (MSC) transplantation to counteract pulmonary disorders, and amniotic membrane (AM) transplantation to treat corneal damage.[44-46] Unfortunately, many of these proposed therapies have significant drawbacks that severely hinder their effectiveness in treating sulfur mustard exposure, such as unwanted toxic effects, counterproductive biological impacts, and ineffectiveness in severe cases.

1.4.3. Scavenger Therapy

Although sulfur mustards are highly reactive with nucleophilic sites and unstable in aqueous environments, evidence has shown that their hydrophobic nature leads to an accumulation of unreacted sulfur mustard in membranes and fatty tissues.[38] As time goes

on, these molecules slowly diffuse out of fatty tissues and lead to prolonged symptoms and delayed recovery. In response to both initial injury and accumulation of unreacted sulfur mustard, a scavenging mechanism of treatment could be employed by administering a biologically safe molecule with high affinity for reaction with sulfur mustard (e.g., highly nucleophilic molecules). Theoretically, this molecule could help reduce the immediate damage caused by sulfur mustard if administered immediately and would provide an alternate route of sulfur mustard neutralization as it diffuses out of fatty tissues. Maintaining a sufficient level in either locally affected tissues or systemically would scavenge sulfur mustard to produce a sulfur mustard-scavenger adduct, which may be excreted as waste or metabolized.

1.5. Scavengers

1.5.1. Previously Tested Scavengers

A few studies exist that explore the possibility of this scavenging model as a post-sulfur mustard exposure intervention method. Of those few, the most promising molecules explored include N-acetyl cysteine (NAC), glutathione, and 2,6-dithiopurine. NAC and glutathione were tested due to their biological relevance, known antioxidant properties, and potential scavenging ability. While NAC and glutathione provided some relief from sulfur mustard toxicity, multiple studies concluded their mechanism of action proceeds through pathways unrelated to scavenging.[2, 3] 2,6-dithiopurine was selected as a sulfur-containing thiopurine analog to the nitrogenous bases in DNA that are known to create adducts with sulfur mustard. While this molecule proved somewhat successful in alleviating mutagenesis and other effects involved with sulfur mustard exposure, it was abandoned as a potential therapeutic around 2012.[47] Therefore, the universe of potential

scavenging molecules remains largely unexplored. To date, a systematic structure-activity based study to identify candidate scavenger molecules has not been undertaken.

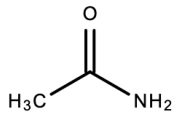
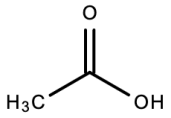
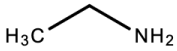
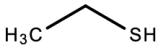
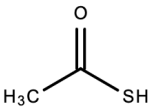
1.5.2. Potential Scavengers

While previously tested scavengers were selected based on their bioactivity or structural similarity to active sites of sulfur mustard, a structure-activity based approach was used in this study to determine the most effective structural features for scavenging sulfur mustard. To start, the initial scavenger set (i.e., first-generation scavengers) sought to evaluate various functional groups and distinguish their scavenging effectiveness as a function of functional group nucleophilicity. The five functional groups initially surveyed, in order of increasing nucleophilicity, were amides, carboxylic acids, amines, thiols, and thioacids. Based on the scavenging effectiveness of these functional groups, along with their demonstrated toxicity, a new and more refined set of scavengers (i.e., second-generation scavengers) were selected to evaluate the most effective molecular features for scavenging.

1.5.2.1. First-Generation Scavenger Set

All consisting of a two-carbon backbone, the first-generation scavenger set is shown below in Table 1.2. Functional groups were represented as follows: acetamide represented amides, acetic acid represented carboxylic acids, ethyl amine represented amines, ethanethiol represented thiols, and thioacetic acid represented thioacids.

Table 1.2. Names, chemical structures, and abbreviations of the first-generation scavenger set.

Name	Abbreviation	Structure
Acetamide	A	
Acetic Acid	AA	
Ethyl Amine	EA	
Ethanethiol	ET	
Thioacetic Acid	TAA	

1.5.2.1.1. Ethyl Amine

Ethyl amine is a very commonly used chemical building block in the synthesis of a wide range of materials including rubber products, pesticides, some anesthetic agents.[48] Consisting of a two-carbon chain connected to a nitrogen, this scavenger represents the scavenging ability of the amine functional group. Scavenging should occur between sulfur mustard and the amine by a reaction between the lone pair on the nitrogen with a carbon in the sulfur mustard sulfonium ion, mimicking the reaction between sulfur mustard and nitrogen atoms in proteins and DNA.

1.5.2.1.2. Acetic Acid

Best known for as the main organic component of vinegar, acetic acid is the scavenger representing the carboxylic acid functional group. Due to its simple structure and various possible carboxylic acid derivative products, acetic acid is a broad-range protic solvent, widely used chemical building block in polymer and chemical compound synthesis, and foodstuff typically incorporated by vinegar and pickling.[49] The carboxylic acid functional group would likely scavenge through a lone pair on the carbonyl oxygen with a carbon in the sulfonium ion. A concern with this scavenger is its effect on the pH of the cell media.

1.5.2.1.3. Ethanethiol

Ethanethiol, well-known for its putrid and easily detectable smell, is a thiol-containing two-carbon compound commonly used as an additive to dangerous natural gases in order to make leaks more detectable.[50] The structure of ethanethiol is simply a two-carbon molecule with a thiol group. The sulfur atom possesses a far greater scavenging potential due to its increased size as compared to an oxygen atom. Ethanethiol would scavenge sulfur mustard molecules by using a lone pair on the thiol group to create a bond with a carbon in the sulfonium ion. This reaction would mimic sulfur mustard's reaction with glutathione and other thiol-containing biomolecules.

1.5.2.1.4. Thioacetic Acid

Thioacetic acid is the most nucleophilic scavenger in this list, as it is a two-carbon compound containing a thioacid as the functional group. Possessing a very pungent odor, thioacetic acid exists as a yellowish liquid and is utilized in various pharmaceutical synthesis reactions.[51] The scavenging mechanism would involve the electrophilic attack

of the sulfonium on the sulfur atom of the thioacid group. Similar to acetic acid, this scavenger poses a concern due to its possible effect on the cell media pH.

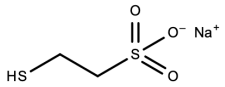
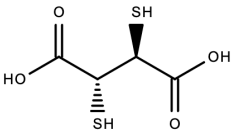
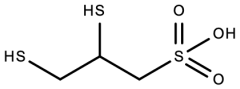
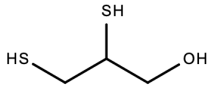
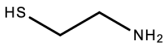
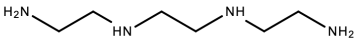
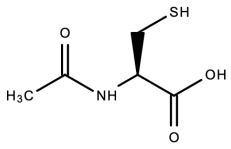
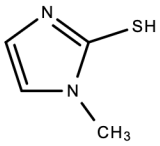
1.5.2.1.5. Acetamide

Acetamide is a two-carbon compound containing an amide functional group. It is used to create different plastics and lacquers and is also utilized as a solvent.[52] With the lowest nucleophilicity, it is suspected that this compound will possess a relatively low amount of scavenging power as compared to the rest in this group. The proposed mechanism of scavenging would occur by a nucleophilic attack of the carbonyl oxygen of the acetamide molecule on the sulfonium ion.

1.5.2.2. Second-Generation Scavenger Set

Each containing thiols, amines, or a combination of the two, the names, abbreviations, and chemical structures of the second-generation scavenger set is shown below in Table 1.3.

Table 1.3. Names, chemical structures, and abbreviations of the second-generation scavenger set.

Name	Abbreviation	Structure
2-Mercaptoethane Sulfonate Sodium	MESNA	
Dimercaptosuccinic Acid	DMSA	
2,3-Dimercapto-1-propanesulfonic Acid	DMPS	
Dimercaprol	BAL	
Cysteamine	CYS	
Trientine	TRI	
N-Acetyl Cysteine	NAC	
Methimazole	METH	

1.5.2.2.1. 2-Mercaptoethane Sulfonate Sodium

2-Mercaptoethane sulfonate sodium, commonly known as MESNA, is a medication utilized by patients undergoing chemotherapy to reduce harmful side effects.[53] MESNA contains a thiol functional group, possessing potential scavenging activity, and a sulfonate group, which only serves to increase aqueous solubility of the

molecule. Like ethanethiol, the sulfonium ion would attack a lone pair on the thiol group and form a sulfide group.

1.5.2.2.2. Dimercaptosuccinic Acid

Also known as succimer, dimercaptosuccinic acid (DMSA) is a simple four-carbon compound that possesses two thiol groups as well as two carboxylic acid groups. DMSA is a chelating agent used to treat heavy metal poisoning, including lead, mercury, and arsenic.[54] The thiol bifunctionality gives DMSA an advantage for scavenging, as the concentration required to scavenge a given amount of sulfur mustard would likely be lower than a monofunctional compound. The thiol groups in this molecule would likely be the major scavenging sites for sulfur mustard.

1.5.2.2.3. 2,3-Dimercapto-1-Propanesulfonic Acid

Similar to DMSA, 2,3-dimercapto-1-propanesulfonic acid (DMPS) is also a heavy metal chelating agent.[55] This molecule is similar in structure to MESNA (another scavenger tested), but the parent chain consists of three carbons and it contains two thiol groups instead of one. In a similar fashion to DMSA, having multiple thiol groups allows a single molecule to theoretically scavenge more than one sulfur mustard molecule.

1.5.2.2.4. Cysteamine

Cysteamine is another simple two-carbon molecule, but it contains both a thiol and an amine, two potentially useful functional groups for scavenging. A biochemical product within humans, cysteamine is not only safe in living systems but is also capable of being produced in them. Approved for medicinal use for cases of cystinosis, cysteamine provides a valuable example of how a biologically safe scavenger containing both a thiol and an amine may work.[56]

1.5.2.2.5. Dimercaprol

Best known as British anti-Lewisite (BAL), this three-carbon molecule is very similar to DMPS as it contains two thiol groups and an alcohol instead of the sulfonic acid. Developed during World War II, BAL is a chelating agent of heavy metals and proved extremely useful antidote to the arsenic-based chemical warfare agent, Lewisite.[57] Despite its relatively high toxicity, it is still currently useful for treatment of heavy metal toxicity. Exploring this as a sulfur mustard scavenger provides a twofold advantage; it serves as a valuable real-world example for comparison to the other scavengers and it could become a multipurpose antidote for both Lewisite and sulfur mustard.

1.5.2.2.6. Trientine

Another chelating agent, trientine (TRI) contains four amine groups with two carbons between each. Primarily used to treat Wilson's disease by chelating excessive copper in the body, this polyamine is also used in epoxy resins as a hardener.[58] Although amines are not the most nucleophilic functional group or best predicted scavenging group, the presence of four potentially active scavenging sites on a single molecule poses a promising scavenging candidate. One note of interest is that drug companies drove the price of this drug up to astronomical levels, so cost is a drawback to this scavenger.

1.5.2.2.7. N-Acetyl Cysteine

Acetylcysteine, or N-acetylcysteine (NAC), is a drug with very low toxicity that is a precursor to glutathione. Through increasing the amount of this antioxidant, NAC is able to treat toxic conditions such as acetaminophen overdose.[59] Its low price and widespread availability make it a very promising candidate. The functional groups it

contains are a thiol, amide, and a carboxylic acid. It also serves as a benchmark comparison since it has been tested in other similar studies.

1.5.2.2.8. Methimazole

Methimazole is a unique scavenger on this list, as it contains a thiodiamine functional group. With the central carbon surrounded by two amine groups and a thiol, this functional group has unique properties due to both its ability to tautomerize as well as the close proximity of multiple promising nucleophilic functional groups. Currently this drug is used to treat hyperthyroidism.[60]

1.5.3. Cellular Assays

In order to determine the efficacy of these compounds as sulfur mustard scavengers, two cellular assays were conducted: 1) the MTT assay to assess reduction of cellular toxicity, and 2) the cell matrix adhesion (CMA) assay to evaluate protection of extracellular adhesions. Using the HaCat cell line, derived from healthy human keratinocytes, the effectiveness of scavenger molecules was observed for epithelial cells, the type of cell which sulfur mustard primarily targets.[61]

1.5.3.1. MTT Assay

As one of the most well-known and widely used assays to estimate cell viability, the MTT assay was used to select working concentrations for both the sulfur mustard and the scavengers as well as assaying viability-protecting capabilities of the scavengers on cells treated with sulfur mustard. On a fundamental level, MTT assays measure the activity of oxidoreductase enzymes within the cell through the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its reduced formazan salt. This insoluble product is purple in color and can be resuspended in a solvent, such as

DMSO, to measure absorbance at 570 nm. The absorbance is linearly corelated to the cellular metabolism over a large range of absorbances and cellular densities, giving it a wide margin of applicability. Within this measure of metabolism is an established correlation with the relative cell viability that can be compared between differing experimental conditions.

1.5.3.2. Cell-Matrix Adhesion Assay

The second cellular assay used to survey the efficacy of potential scavengers is the CMA assay. Cells are grown and subjected to the desired experimental conditions using plates coated with a chosen type of extracellular matrix (ECM) protein. The extracellular matrix protein used for this assay was collagen I due to it being the most abundant ECM protein throughout the skin and body.[62] After a 24-hour treatment period, the plates are gently rinsed three times with PBS. This rinsing step removes non-adherent cells, so this step is what links the activity level of acid phosphatases to the level of cellular adherence with extracellular proteins.[63] A constitutively expressed enzyme in regularly functioning cells, acid phosphatase activity is directly correlated to the quantity of cells present.[63] Active acid phosphatase cleaves the substrate p-nitrophenyl phosphate into p-nitrophenol, and this product is subjected to basic conditions to deprotonate an alcohol group and create its yellow-colored conjugate base.[63] The absorbance of this final product is linearly correlated with acid phosphatase activity, measured using a wavelength of 405 nm, and analyzed similarly to the data from an MTT assay. Measuring the level of cell-matrix adhesion is a highly useful way to assay a scavenger's ability to prevent sulfur mustard's vesicating action.

1.6. Research Goal

Sulfur mustard is an easily synthesized chemical warfare agent that is still used in modern day conflicts over a hundred years after its introduction, yet a viable antidote to neutralize sulfur mustard has still not been created. Therefore, there is a critical need to conduct a thorough and comprehensive search for a highly effective “scavenger” therapeutic with a high affinity to react with sulfur mustard while producing biologically safe conjugates. The goal of this study was to use two cellular assays on agent-exposed and scavenger treated human keratinocyte (HaCat) cells to determine the therapeutic properties of potential scavenger molecules.

Chapter 2: Scavenger Hunt: The Search For Sulfur Mustard-Neutralizing Compounds In 2-Chloroethyl Ethyl Sulfide (CEES) Treated Human Keratinocyte (HaCat) Epithelial Cells

2.1. Abstract

Sulfur mustard is a highly toxic and dangerous vesicating agent which has been utilized as chemical warfare agent since World War I. Despite its extensive history, an effective antidote to sulfur mustard exposure still does not exist. With detectable urinary levels of unreacted sulfur mustard found in humans days after exposure, a window of opportunity exists from initial exposure to a few days later to administer a therapeutic which "scavenges" sulfur mustard. An effective scavenger would neutralize unreacted sulfur mustard prior to it producing harmful effects and have very low inherent toxicity. For effective implementation of this strategy, it is essential to identify promising candidate scavengers through fundamental, structure-based evaluation of molecular scavenging features, while also assessing scavenger toxicity. A set of five two-carbon compounds with various nucleophilic functional groups served as a preliminary group of scavengers to survey the correlation between scavenging ability and functional group nucleophilicity. MTT cell viability and cell-matrix adhesion assays where used to assess the effectiveness of these potential scavengers. Increasing scavenger promise generally mirrored increasing nucleophilicity, with thiol (ethanethiol) and amine (ethyl amine) functionalities proving to be the most promising functional groups. A set of eight second-generation scavengers was selected based on these functionalities and known toxicity. Out of this group, the thiodiamine functional group, found in the candidate scavenger

methimazole, most effectively reduced cell death and loss of cell-matrix adhesions. The development of methimazole, or related molecules, to scavenge sulfur mustard may provide a viable treatment to reduce the immediate and long-term toxicity of sulfur mustard.

2.2. Introduction

Sulfur mustard has a long history of use in warfare. While it was discovered in the early 1800's, Meyer proposed a simple and high-yield method of synthesis of the molecule in 1886 which allowed mass production after slight modification.[5] Years later, Germany first used sulfur mustard as a chemical warfare agent (CWA) in World War I (WWI), taking advantage of its dangerous properties.[5] It proved extremely useful as its high density and moderate vapor pressure allowed it to penetrate into enemy trenches and bunkers.[6] In fact, it caused more casualties than any other chemical agent used in WWI, even though it was introduced late in the war.[6] In 1925, the Geneva Protocol internationally outlawed sulfur mustard and all other CWAs in military conflicts.[11] However, sulfur mustard, along with other CWAs, have seen continued use throughout the past century and even into recent years by militant groups and rogue governments.[15] Despite the generally accepted Chemical Weapons Convention prohibitions on CWAs, their persistent use in terrorism and unethical warfare continues and creates a critical need for effective therapies.

The three main vesicating CWAs are sulfur mustard, Lewisite, and nitrogen mustards. Due to its ease of synthesis, toxic effects, and lack of an antidote, sulfur mustard is the most used vesicating CWA. When exposed to sulfur mustard, there is a delayed onset of symptoms for about 24 hr; initial symptoms include blistering of the

skin, lung irritation with a productive cough, and burning and itching of the eyes.[35] Although sulfur mustard is highly dangerous, it rarely causes death.[6, 36] Death typically occurs via secondary infection of open wounds from rupturing blisters, so individuals exposed to sulfur mustards usually require a prolonged stay of up to a month in a medical facility, potentially overwhelming the medical system if many soldiers and/or civilians are severely exposed.[37] Sulfur mustard has also been shown to readily diffuse into membranes and fatty tissues due to its hydrophobicity. This property allows sulfur mustard to reside in these tissues for days and to slowly diffuse back out, leading to prolonged toxicity.[38]

The mechanism of sulfur mustard toxicity is alkylation of biomolecular nucleophilic sites (e.g., amines), disrupting the function of the parent biomolecule. Sulfur mustard's effectiveness at alkylating biomolecules is based on its extreme electrophilicity. This property creates an opportunity to administer a "scavenger" therapeutic, possessing high affinity for sulfur mustard. The most effective theoretical scavenger would have very strong nucleophilic character for reaction with sulfur mustard and very low toxicity. While the scavenger concept has been previously suggested, only a few molecules have been investigated for this purpose, including N-acetyl cysteine, glutathione, and dithiopurines.[2, 3] These molecules were selected based on proposed natural biochemical reaction of sulfur mustard with these compounds such that biological detoxification mechanisms could be modeled. While these molecules were shown to somewhat mitigate the toxicity of sulfur mustard, studies showed that the mechanism of these potential therapeutic agents was not scavenging, and the development of these targeted scavengers was abandoned early in the 2010s.[47] Because these initial potential

sulfur mustard scavengers were selected based on a targeted approach, an opportunity exists for a more comprehensive structure-activity approach to discover a therapeutic agent possessing high affinity for scavenging sulfur mustard. Fundamental evaluation of structural features which lead to effective scavenging may allow identification of extremely effective scavenging molecules.

The objective of this study was to identify sulfur mustard scavenging molecules for further development by conducting a ground-up search for candidate scavengers, focusing on unique molecular structural features. By utilizing chloroethyl ethyl sulfide (CEES, i.e., the monofunctional surrogate/simulant of sulfur mustard) exposed and scavenger-treated human keratinocyte (HaCat) epithelial cells in MTT cell-viability assays and cell-matrix adhesion (CMA) assays, a wide variety of chemical structures were surveyed to narrow in on the most promising scavenging structural features.

2.3. Material and Methods

2.3.1. Materials

To measure cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent purchased from Sigma-Aldrich was mixed with PBS to create a stock solution with a concentration of 5 mg/mL. The stock was stored in a light-protected container and at 4°C. The CEES stock solution was made in anhydrous ethanol at a concentration of 1 M and was stored at 4°C. Each scavenger (acetic acid (AA), acetamide (A), ethyl amine (EA), ethanethiol (ET), thioacetic acid (TAA), 2-mercaptoethane sulfonate sodium (MESNA), methimazole (METH), dimercaptosuccinic acid (DMSA), 2,3-dimercapto-1-propanesulfonic acid (DMPA), N-acetyl cysteine (NAC), cysteamine (CYS), dimercaprol (BAL = British Anti-Lewisite), and trientine (TRI)) was purchased

from Sigma-Aldrich. Stock solutions for the scavengers were created in PBS. Besides DMSA (100 mM), all scavenger stock solutions were created at concentrations of 1 M. Higher concentrations of DMSA could not be created because of lower solubility.

2.3.2. Cell Culture

The human keratinocyte epithelial HaCat cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 10,000 IU/mL penicillin, 10,000 µg/mL streptomycin and 0.5 µg/ml amphotericin B at 37°C equilibrated with 5% (v/v) CO_2 in humidified air. The HaCat cells used in this study were frozen in liquid nitrogen when not in use and were not passaged more than 15 times.

2.3.3. MTT Assay

Changes in cell viability due to CEES and scavengers were observed by measuring cellular dehydrogenase activity through the spectrophotometrically-based MTT assay. HaCat cells were seeded in 96-well plates at a density of 1.5-2.5 x 10^4 cells/100 µL cellular media per well. The plates were incubated overnight and treated with CEES, scavengers, or both at the indicated working concentrations with eight wells per condition. CEES treatments were added within 15 s of mixing the treatment dilution in cell media in order to keep dosage effects consistent between trials, and scavenger treatments were added 15 s following CEES treatments to mimic a post-exposure response. The stated CEES concentration reflected the solution volume prior to the addition of scavenger, and the stated scavenger concentration reflected the solution volume following scavenger addition. Analysis was performed following incubation for 4, 8, 24, and 48 hr. To prevent unwanted reactions between the sulfur-containing

scavengers and the MTT reagent, 200 µL of PBS was added to each well and then removed to remove scavenger molecules. The wells were subsequently filled with 100 µL/well of cell media and 20 µL/well of MTT reagent. Plates were incubated for 1.75-2 hr and the formazan crystals were dissolved in 200 µL dimethyl sulfoxide (DMSO). Absorbance was measured using a BioTek Cytation 3 Cell Imaging Multimode reader (Winooski, VT, USA) at a wavelength of 570 nm. Relative cell viability was measured as a percentage compared to vehicle (ethanol)-treated control cells.

2.3.4. Cell-Matrix Adhesion Assay

Relative loss of extracellular matrix (ECM) adhesions was observed by measuring acid phosphatase activity through the CMA assay. HaCat cells were seeded in collagen type I coated 96-well plates pre-rinsed with PBS at a density of 2.5 x 10^4 cells/100 µL cellular media per well. The plates were incubated overnight prior to treatment with CEES and/or scavengers at the indicated working concentrations with eight wells per condition. Since this assay investigates loss of adhesion rather than cell death, a lower concentration of CEES was required to prevent large amounts of cell death interfering with the intended results. After 24 hours of incubation, the plates were gently rinsed three times with PBS to remove non-adherent cells. The substrate solution, 12 mM p-nitrophenyl phosphate (pNPP) and 50 mM sodium acetate trihydrate in 0.2% Triton X-100 adjusted to a pH of 5-6, was added at a volume of 100 µL to each well. After one hour of incubation, 50 µL of 1N NaOH was added to each well to deprotonate the substrate's product and give the wells a yellow color. Absorbance was measured using a BioTek Cytation 3 Cell Imaging Multimode reader (Winooski, VT, USA) at a wavelength of 405 nm. Relative cell viability was measured as a percentage compared to

vehicle (ethanol)-treated control cells. To accommodate for interassay variability, a normalized value was developed and dubbed the scavenging effectiveness value (SEV). This was obtained by taking the relative quantity of adhered cells in the CEES/scavenger condition divided by the relative quantity of adhered cells in the CEES control. Therefore, SEVs above one indicate improved retention of cell-matrix adhesion while SEVs below one indicates reduced cell matrix adhesions relative to CEES exposed controls.

2.4. Results and Discussion

2.4.1. CEES MTT Assay

2.4.1.1. CEES Stability and Treatment Timing for MTT Assay

The functional range of CEES concentrations for evaluation of scavenger effectiveness in the MTT assay was evaluated. The goal was to determine working CEES concentrations for each assay, such that a range of cell viability for testing scavenger effectiveness over the course of 4-48 hr was produced. Initial attempts at evaluating CEES concentration showed that CEES was reactive with components of the media. Because treating the cells required an initial dilution of CEES in aqueous cell media, CEES's instability in the aqueous media required further investigation. Therefore, an MTT assay was conducted to determine how long CEES would remain active in DMEM.

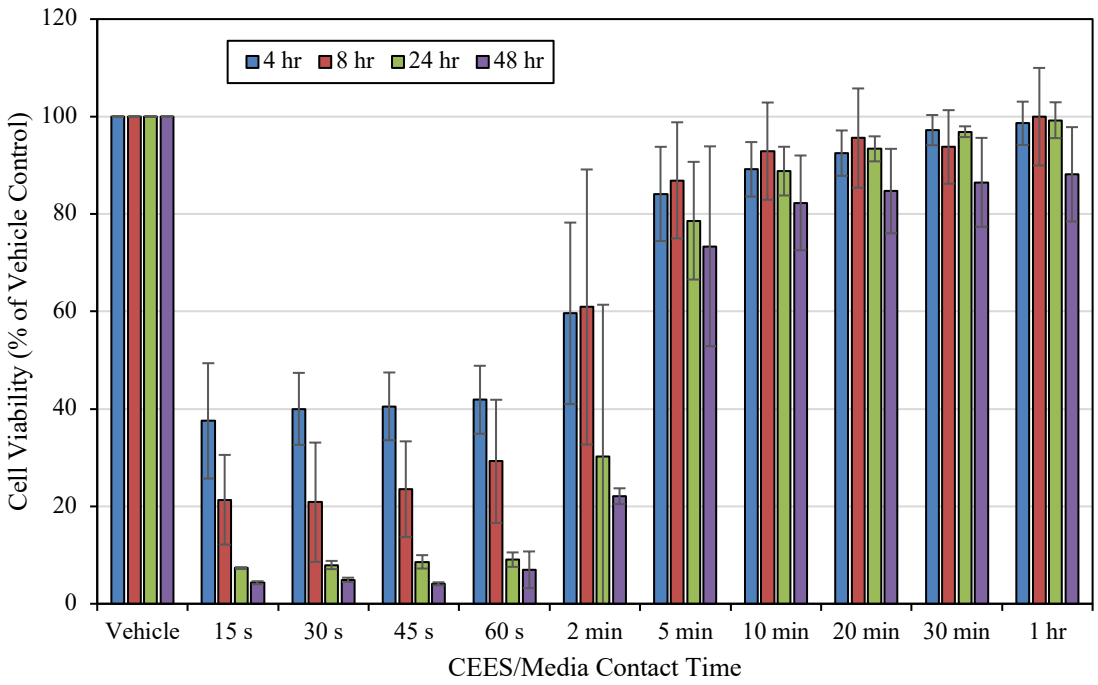


Figure 2.1. Treatment mixture standby times varying from 15 s to 1 hr. The four differently colored bars represent the duration of cell growth from addition of CEES to analysis via the MTT assay (4 hr, 8 hr, 24 hr, 48 hr), and the x-axis time stands for the length of time that passed between when the CEES was initially diluted in DMEM until the mixture was added to the cells. The cell viability levels were calculated relative to the vehicle control which was ethanol.

Figure 2.1 shows how varying the contact time of CEES in DMEM prior to addition of this mixture to the cells affected the toxicity of CEES, as measured by cell viability. The percent cell viability was clearly affected by the length of time between mixing the CEES with DMEM and treatment of the cells, generally in a bimodal fashion. With contact times less than 2 min, the cell viability was greatly reduced, but with contact times above 2 min, the cell viability was similar to the vehicle control. The higher contact times of CEES in DMEM reduced the toxicity of CEES towards the cells likely

because the CEES reacted with components of the media. This observation strengthens the potential viability of the scavenger concept. It is obvious from Figure 2.1 that for CEES to produce the desired toxicity, the contact time between CEES and DMEM needed to be short, with a maximum of a 1 min. For practical effectiveness and maximum consistency, 15 s was selected as the time between dilution of CEES within DMEM to the addition of that mixture to the cells.

2.4.1.2. CEES Working Concentration for MTT Assay

After establishing the contact time between media and CEES, a working concentration to ensure a desired range of CEES toxicity was established. Figure 2.2 displays the effect of multiple concentrations of CEES (750 μM to 5 mM) on cell viability at 4-48 hr. For MTT assays, it was desired for CEES to produce a small loss of cell viability at 4 hr (70-90% remaining cell viability) but significant loss of cell viability at 48 hr (20-40% remaining cell viability). Therefore, a CEES concentration of 2 mM was chosen based on these desired metrics. For the cell-matrix adhesion assay, a higher cell viability was desired because the CMA assay measures the loss of cellular adhesion to extracellular proteins, which can occur without cell death. To best measure the effect of the scavengers on these adhesions, the concentrations producing around a 60-80% cell viability at the 24-hour timepoint (0.75 or 1 mM) provided a reasonable CEES toxicity.

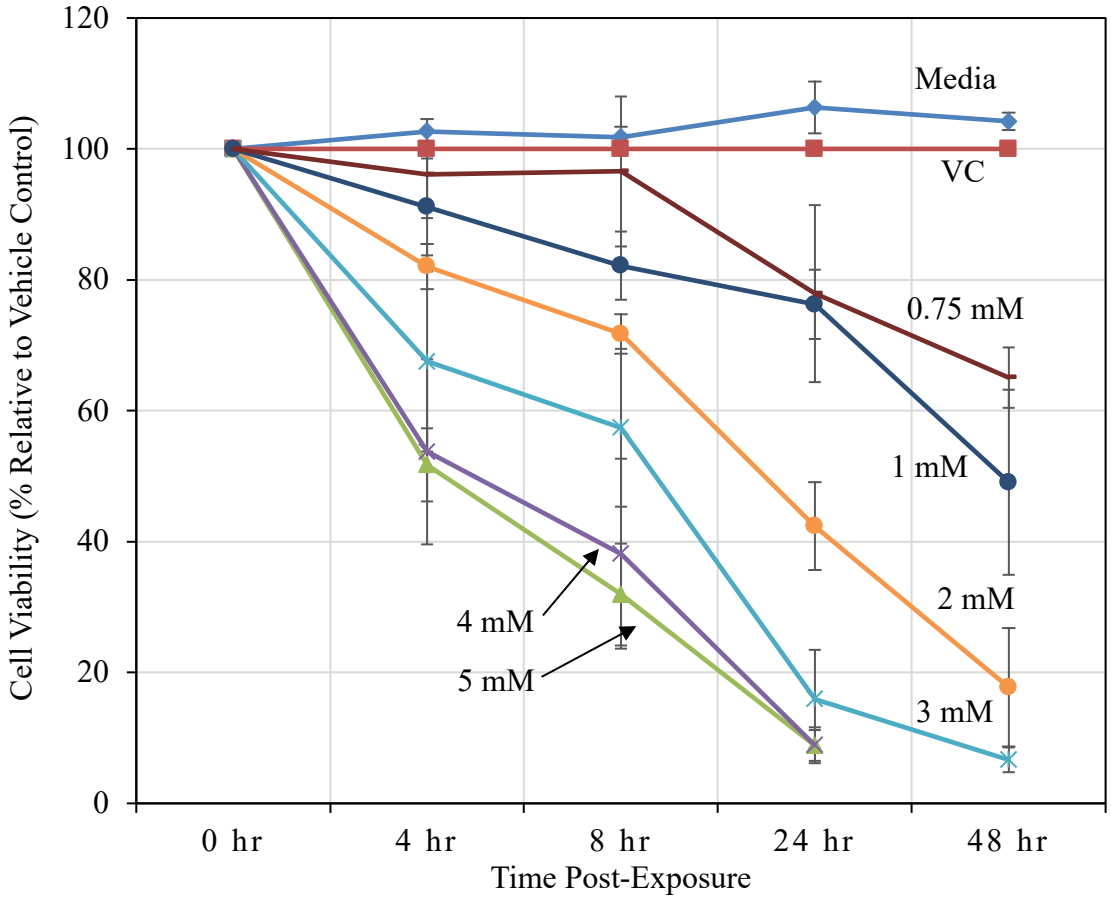


Figure 2.2. MTT assay surveying different concentrations of CEES at varying timepoints in order to establish set concentrations for desired cell viability. The VC (Vehicle Control) is 1:200 ethanol:DMEM.

2.4.2. First-Generation MTT Cell Viability Assay

Working concentrations for evaluation of the first-generation scavengers (i.e., two-carbon molecules with a variety of nucleophilic functionalities) were determined using the MTT assay. The goal of this evaluation was to identify the structure-activity relationship for their ability to neutralize CEES, and by extension, sulfur mustard. Any level of scavenger cytotoxicity is unwanted, but some is undoubtedly expected based on their nucleophilic character. The working concentration selected was the highest

concentration which resulted in at least 75% remaining cell viability after 48 hr. Figure 2.3 shows the highest concentration for each scavenger which met the desired criteria (75% cell viability line marked in red). The concentrations for the five scavengers were chosen as follows: acetamide (A) at 5 mM, acetic acid (AA) at 5 mM, ethyl amine (EA) at 2.5 mM, ethanethiol (ET) at 5 mM, and thioacetic acid (TAA) at 750 µM.

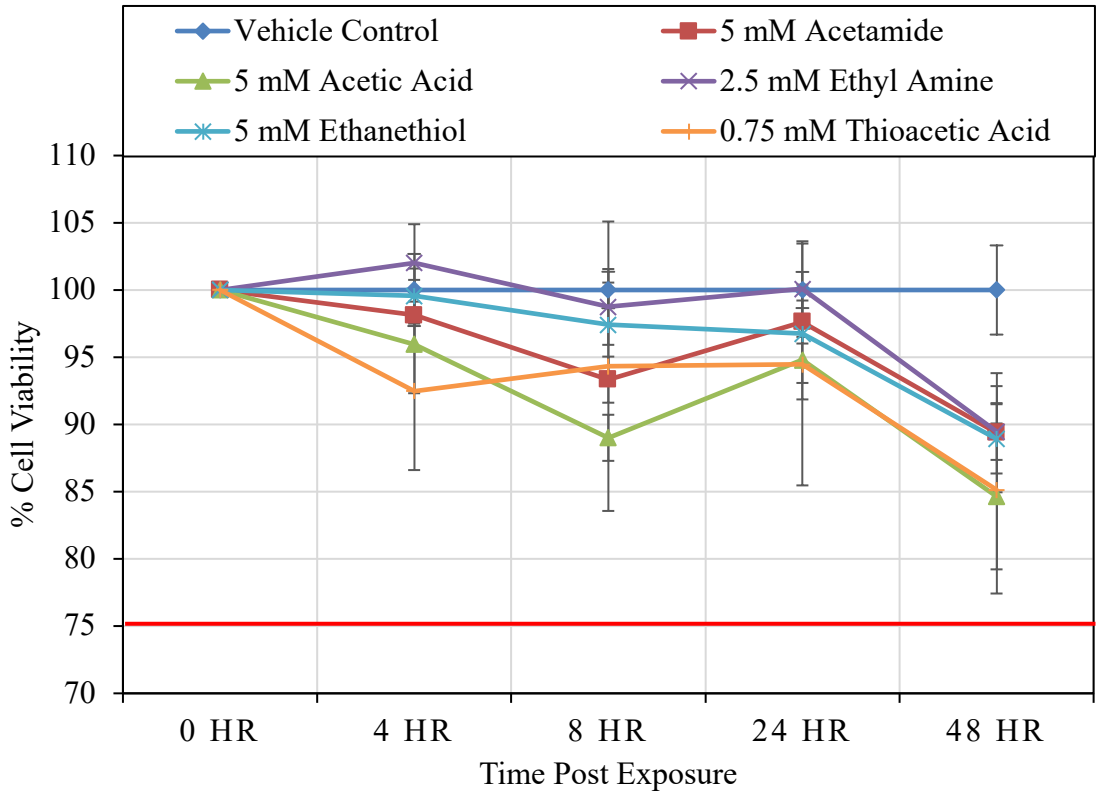
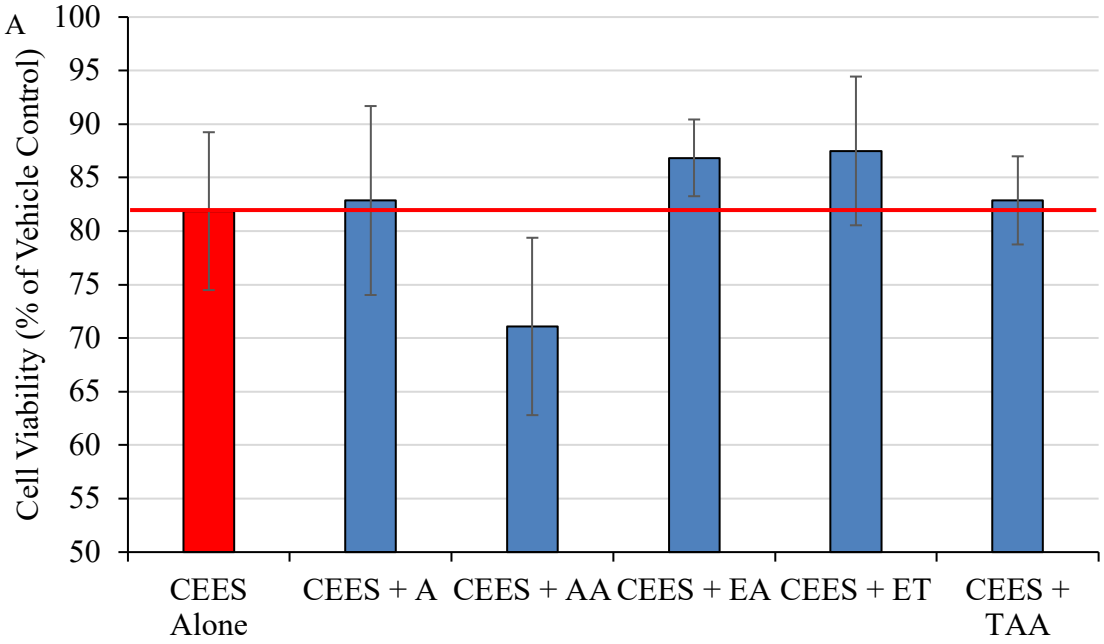
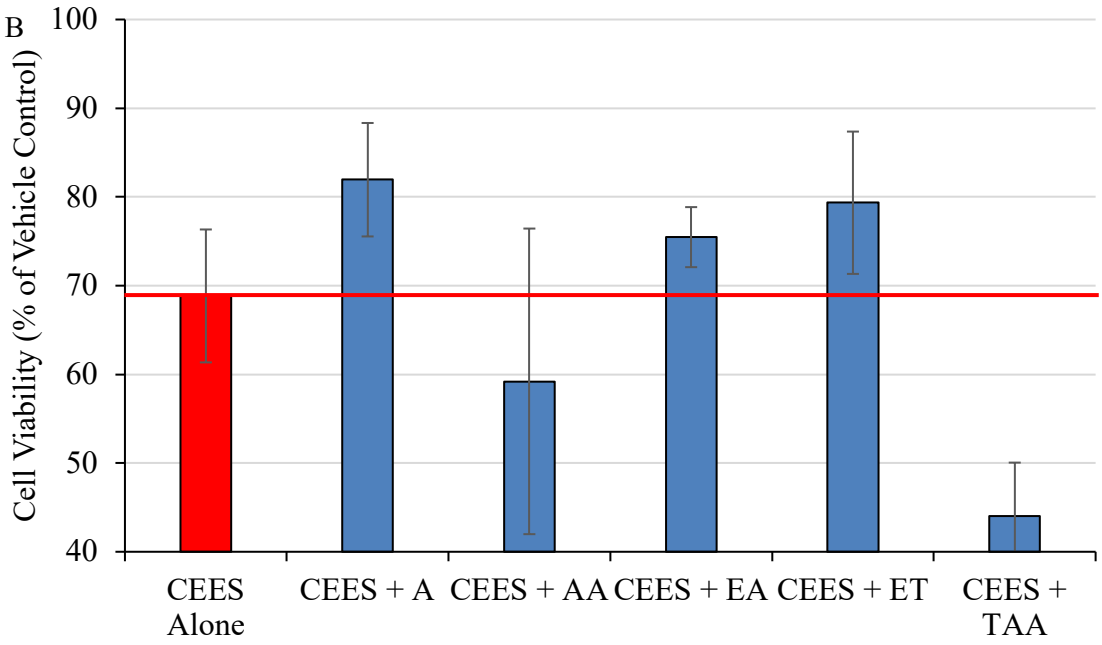


Figure 2.3. MTT assay determining the working concentrations for the first-generation scavengers. The chosen working concentrations are shown relative to the selected level (75%) of generally acceptable cell viability post-exposure to scavengers. The vehicle control is 1:200 PBS:DMEM

2.4.3. Evaluation of First-Generation Scavenging Ability via MTT Assay

The scavenging effectiveness of the first-generation scavengers was evaluated via MTT assays to determine the effectiveness of the scavengers at maintaining cell viability. This is important in two main ways. First, the desirable effect of scavenger treatment is an overall decrease in toxicity of sulfur mustard. Second, although the scavenger may not be overly toxic on its own, it is important to test whether the toxicity of the scavenger is additive, or even synergistic, with CEES toxicity.




B
Cell Viability (% of Vehicle Control)
100
90
80
70
60
50
40
CEES Alone
CEES + A
CEES + AA
CEES + EA
CEES + ET
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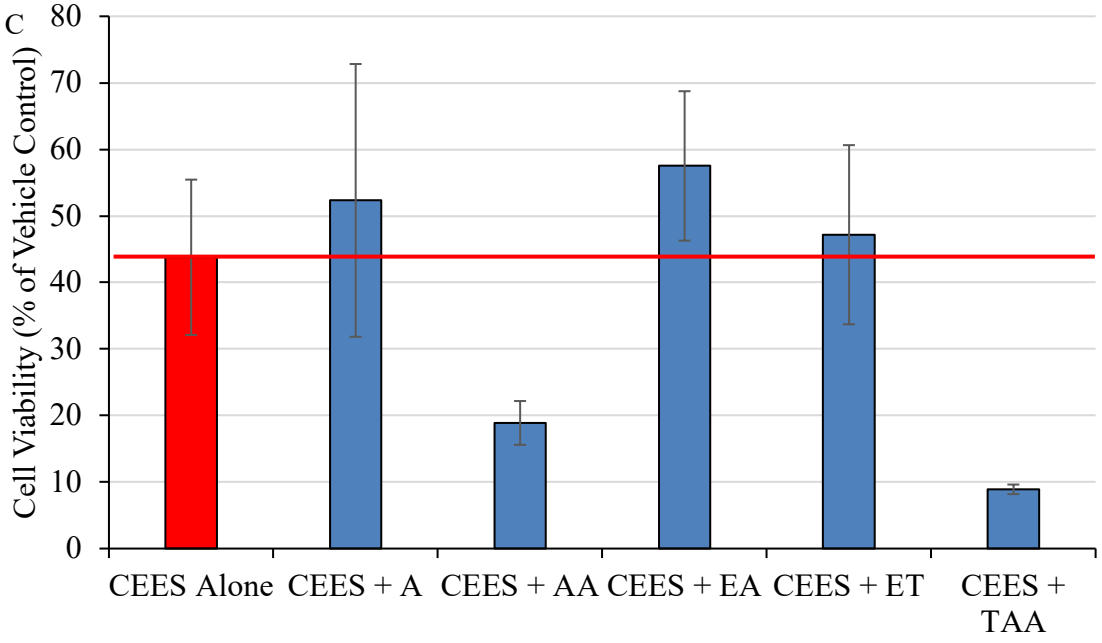

C
Cell Viability (% of Vehicle Control)
80
70
60
50
40
30
20
10
0
CEES Alone
CEES + A
CEES + AA
CEES + EA
CEES + ET
CEES + TAA

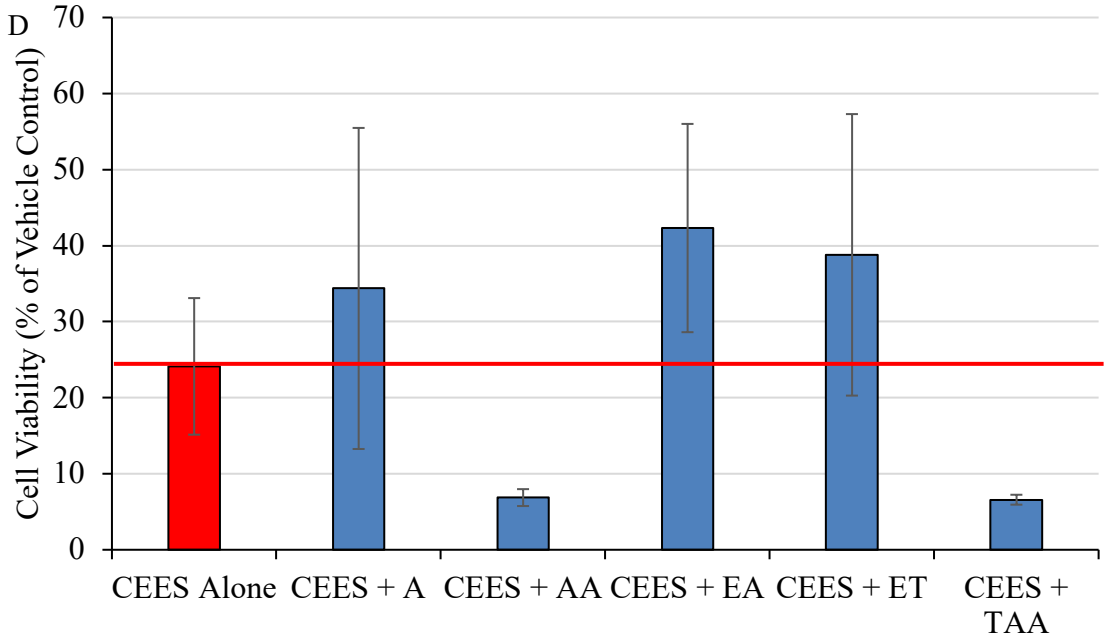


Figure 2.4. MTT assays of CEES-exposed cells treated with the indicated scavenger from the first-generation scavenger set. Figures A-D display the results obtained after 4, 8, 24, and 48 hr post treatment, respectively. CEES was added at 2 mM, and the scavengers were added at the following concentrations: acetamide 5 mM, acetic acid 5 mM, ethyl amine 2.5 mM, ethanethiol 5 mM, and thioacetic acid 0.75 mM.

As seen in Figure 2.4, three scavengers exhibited a consistent increase in cell viability: acetamide, ethyl amine, and ethanethiol representing amide, amine, and thiol functionality, respectively. While the most effective scavengers for the MTT assay were generally expected, they did not perfectly mirror nucleophilicity. Thiols are the second-most nucleophilic functionality tested and effectively decreased CEES's toxicity. This was expected as the thiol should be effective at reacting with CEES and many thiol containing compounds have relatively low toxicity, including some with biological function (e.g., glutathione). Ethyl amine, representing the amine functional group, was also one of the most successful scavengers in producing a consistent increase in cell viability. It is less nucleophilic than the thiol functionality but has significant

nucleophilicity. Additionally, it has relatively low cellular toxicity, similar to many amines essential to biological function (e.g., amino acids). Acetamide, which possessed the least amount of nucleophilic character of the functional groups tested, still showed a consistent increase in cell viability to CEES-treated cells. This effect could be attributed to its relatively low toxicity as compared to the other scavengers in this group and some moderate nucleophilic character.

Two of the scavenger molecules reduced cell viability compared to CEES alone: thioacetic and acetic acid. Thioacetic acid, was the most nucleophilic molecule tested, but actually performed the worst at maintaining cell viability. This poor performance was attributed to the molecule's toxicity, owning to its high nucleophilicity and its acidic nature, potentially altering the pH in the cellular environment. Acetic acid performed similarly to thioacetic acid, as it decreased the cell viability significantly, but somewhat less so compared to thioacetic acid for some time points. The poor performance of acetic acid could also be attributed to the change in the pH of the cellular environment combined with its poor nucleophilicity.

2.4.4. Evaluation of First-Generation Scavenging Ability via CMA Assay

To determine the effect the first set of scavengers have on HaCat cells' ability to retain extracellular matrix adhesions, the CMA assay was used. Collagen I is one of the most abundant ECM proteins in the skin and body. Sulfur mustard is known to break down ECM proteins, ultimately leading to vesication.[30] Consequently, Collagen I-coated plates were utilized to specifically investigate the role these scavengers may play in preserving this key interaction and theoretically reducing vesication.

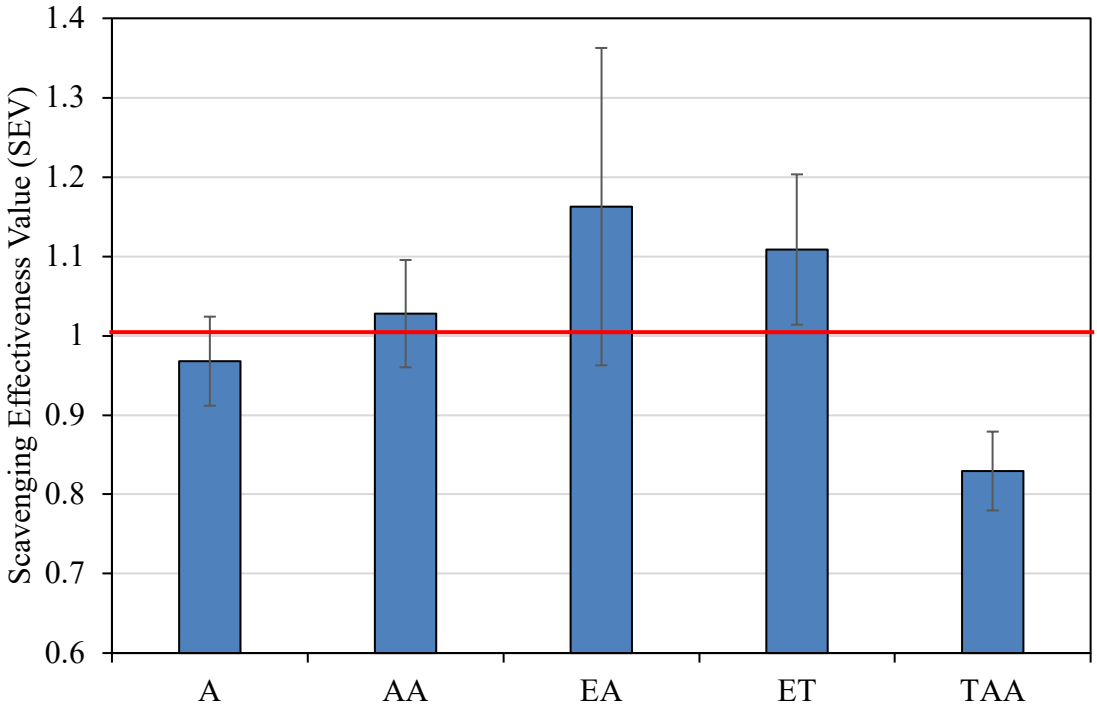


Figure 2.5. Scavenging Effectiveness Values (SEVs) obtained for five first-generation scavengers through CMA assays. Cells were treated with 1 mM CEES followed by 2 mM of each scavenger, except thioacetic acid (0.75 mM).

In Figure 2.5, SEVs for the first-generation scavengers are visualized against the red line indicating the CEES control value. With but one notable exception, the trend of increasing SEVs mirrors increasing nucleophilicity of the functional groups in the scavenger molecules. Once again, the most nucleophilic scavenger (TAA) exhibited counterproductive effects by exacerbating CEES's effects on the cells. ET displayed a promising SEV, and taken along with the MTT assay results, showed consistency in scavenging CEES. Finally, acetamide's performance in the CMA assay was more consistent with its relative nucleophilicity than in the MTT assay. Although it showed increased viability in the MTT assays, the CMA assay results show it had very little impact on helping cells retain their extracellular adhesions.

2.4.5. Second-Generation MTT Cell Viability Assay

Results from the first scavenger set indicated that thiols and amines were the most promising functional groups to further investigate. Based on this, eight second generation scavengers were selected based on their molecular structure, toxicity, uses, biological effects, and diversity. This second group included 2-mercaptoethane sulfonate sodium (MESNA), dimercaptosuccinic acid (DMSA), methimazole (METH), 2,3-dimercapto-1-propanesulfonic acid (DMPS), cysteamine (CYS), N-acetyl cysteine (NAC), trientine (TRI), and British anti-Lewisite (BAL). Each of these scavengers contained at least one thiol or amine, with most containing multiple of these functional groups. Each of these compounds are approved medicines for use in the human body.

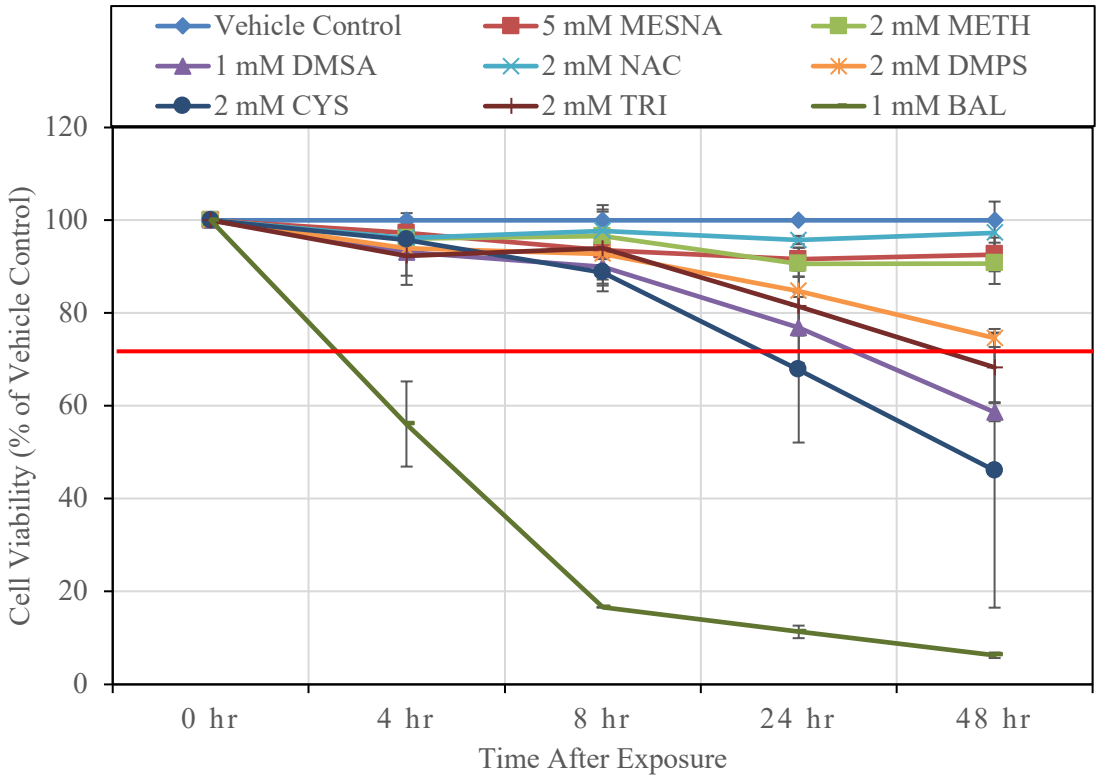


Figure 2.6. MTT assay determining the working concentrations for the second-generation scavengers. The chosen working concentrations are shown relative to the selected level (75%) of generally acceptable cell viability post-exposure to scavengers. The vehicle control was 1:200 PBS:DMEM.

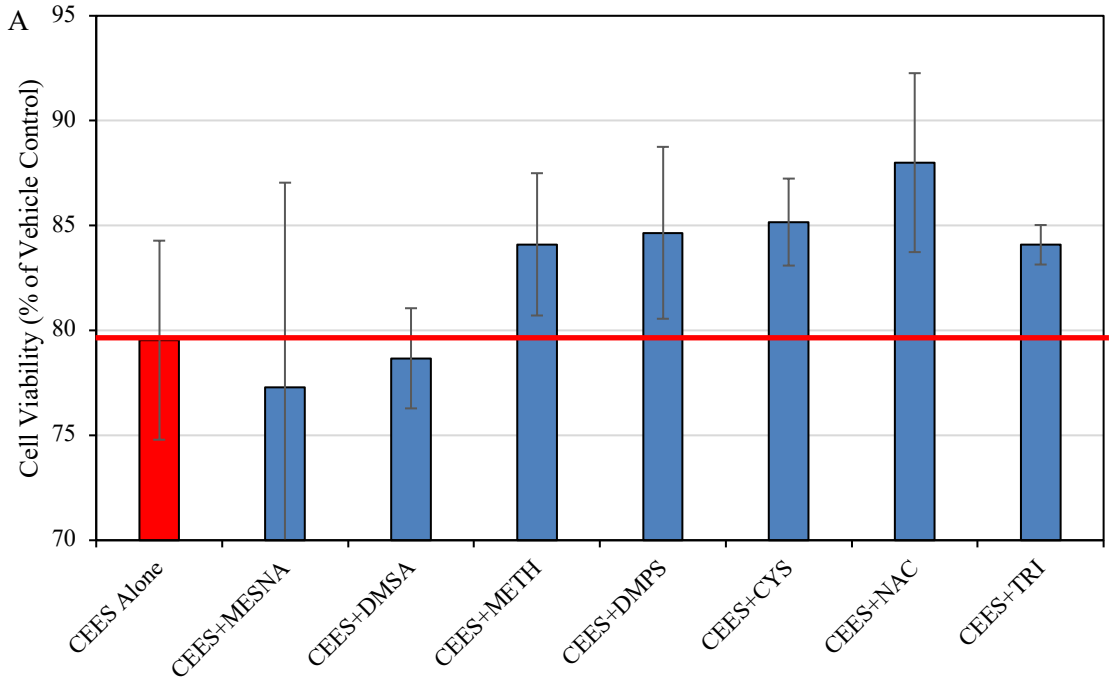
Similar to the first-generation scavengers, the working concentrations for the second-generation scavengers were found using MTT assays. Figure 2.6 shows the concentrations used for each of these scavengers. The most notable result was the high toxicity of British anti-Lewisite. Used as the primary countermeasure to Lewisite exposure, BAL is approved for use in humans. With a toxicity far greater than any of the other scavengers, BAL was considered too toxic to be used as a scavenger, since high scavenger concentrations are important to ensure scavenging effectiveness. Because the CEES concentration for the CMA assay was 1 mM, at least equimolar concentrations of

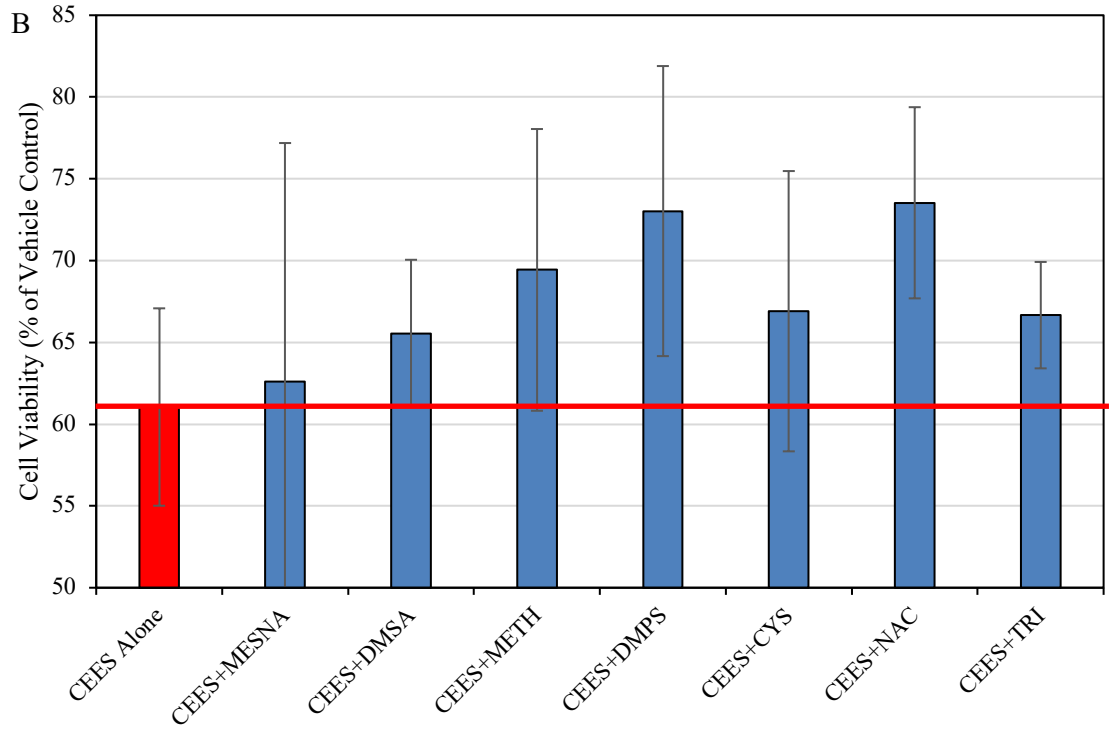
the scavenging molecules was desired, therefore BAL was excluded from future experiments.

The concentrations of the second-generation scavengers was set at 2 mM besides MESNA and DMSA, which were 5 mM and 1 mM, respectively. MESNA initially showed promise for use at 5 mM, but after lackluster results in the MTT assays, it was reduced to 2 mM for the CMA assay. DMSA's toxicity was higher than desired (i.e., at least 75% remaining cell viability) but was still evaluated as a scavenger in subsequent MTT and CMA trials, because it had multiple thiol functionalities. Finally, it was difficult to set a working concentration for CYS since it showed extreme variability in levels of toxicity, ranging from acceptable to unacceptable, as shown by the standard deviation in Figure 2.6. To keep concentrations consistent between the scavengers, besides DMSA and MESNA, CYS's working concentration was set at 2 mM.

2.4.6. Evaluation of Second-Generation Scavenging Ability via MTT Assay

Similar to the first-generation scavengers, the second-generation scavengers were added to cells treated with 2 mM CEES. As seen in Figures 2.7, five of the scavengers consistently increased cell viability over the all four timepoints of the MTT assay. Of those, DMPS and METH were the two most successful scavengers in reducing the toxicity of CEES.


A
95
90
85
80
75
70
Cell Viability (% of Vehicle Control)
CEES Alone
CEES+MESNA
CEES+DMSA
CEES+METH
CEES+DMPS
CEES+CYS
CEES+NAC
CEES+TRI


B
85
80
75
70
65
60
55
50
Cell Viability (% of Vehicle Control)
CEES Alone
CEES+MESNA
CEES+DMSA
CEES+METH
CEES+DMPS
CEES+CYS
CEES+NAC
CEES+TRI

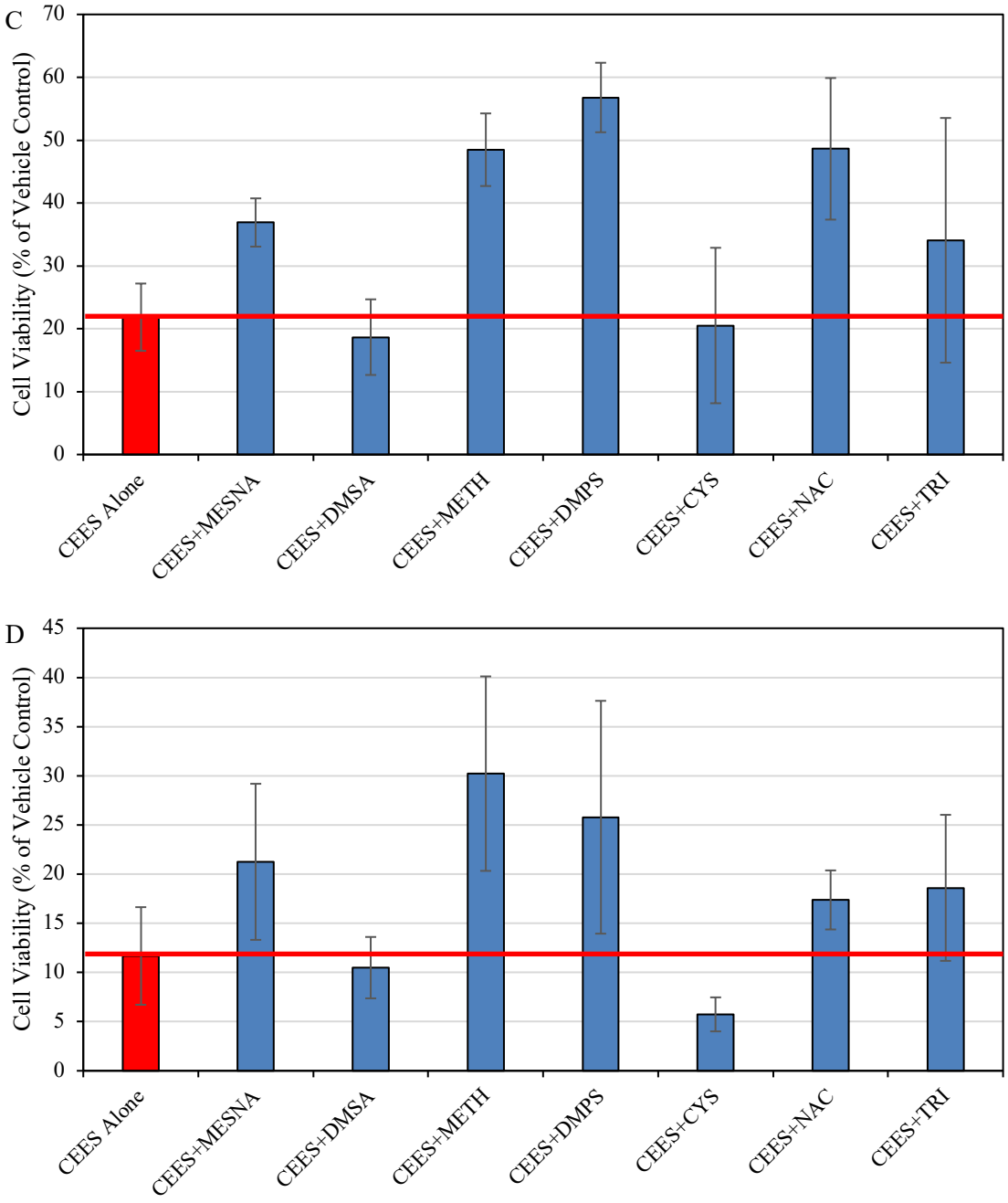


Figure 2.7. MTT assays of CEES-exposed cells treated with the indicated scavenger from the second-generation scavenger set. Figures A-D display the results obtained after 4, 8, 24, and 48 hr post treatment, respectively. CEES was added at 2 mM, and the scavengers were added at the following concentrations: MESNA 5 mM, DMSA 1 mM, METH 2 mM, DMPS 2 mM, CYS 2 mM, NAC 2 mM, and TRI 2 mM.

Of the remaining scavengers, NAC performed well during the time points prior to 48 hr, but the relative cell viability decreased relatively heavily at 48 hr. Both MESNA and TRI showed moderate increases in cell viability, while CYS and DMSA both performed poorly. Out of these results, the performance of DMSA and METH were the most surprising. Initially, DMSA was hypothesized to perform the best out of the second scavenger set due to its formidable nucleophilic character and low toxicity. Also, METH was included predominately to diversify the set of scavengers with a unique functional group (i.e., thiodiamine) which was a combination of a thiol and amine in a single functionality but performed even better than scavengers containing more rigorously-defined amine or thiol functional groups.

2.4.7. Evaluation of Second-Generation Scavenging Ability via CMA Assay

The second-generation scavengers were tested using the CMA assay in the same fashion as the first group. Figure 2.8 shows the effectiveness of these scavengers in maintaining cellular adhesion. METH performed the best once again, as its SEV was nearly 1.4. MESNA, DMPS and NAC were not nearly as effective at preserving cell-matrix adhesions as they were at protecting cell viability. TRI showed large variation in its effectiveness but was not successful in consistently preserving cell-matrix adhesions. On the other hand, CYS and DMSA showed more promising results in this assay and appear to both moderately conserve epithelial extracellular junctions with Collagen I. Overall, METH's thiodiamine functional group proved to be the most effective structure to both increase cell viability and preserve extracellular junctions to collagen I in CEES-treated

epithelial cells. It should be noted that METH performed much better than NAC (SEV = 0.97), a molecule previously suggested for use as a scavenging molecule.

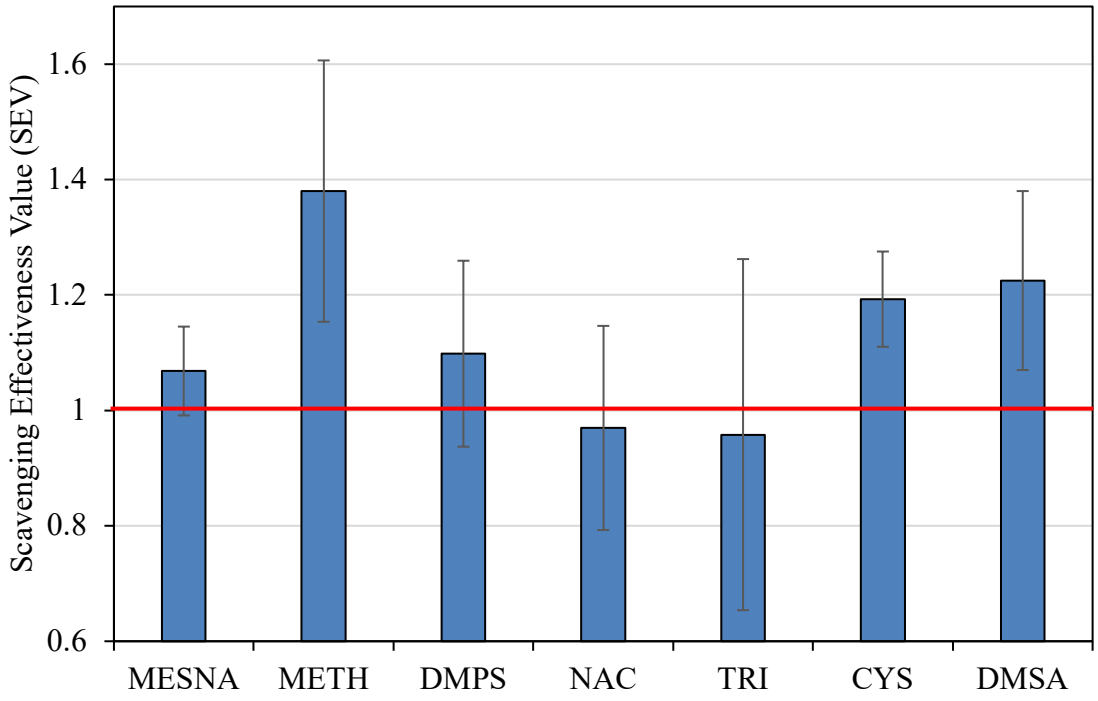


Figure 2.8. Scavenging Effectiveness Values (SEVs) obtained 24-hours post-exposure for all eight second-generation scavengers through cell-matrix adhesion assays. Cells were treated with 1 mM CEES followed by the working concentration of each scavenger.

Furthermore, METH recovered nearly half the cell adhesions lost in sulfur mustard exposure. Figure 2.9 shows average cell-matrix adhesions relative to the vehicle control from the CMA assays. The CEES condition lowered the relative CMAs to 57% while the METH treatment improved CMA retention to 78%. Nearly half of the CEES-induced CMA damage was prevented with post-exposure METH treatment. Wrapping up this segment of the scavenger hunt, these preliminary results show promise for the development of methimazole, or related molecules, to scavenge sulfur mustard and

potentially provide a viable treatment to reducing the immediate and long-term toxicity of sulfur mustard.

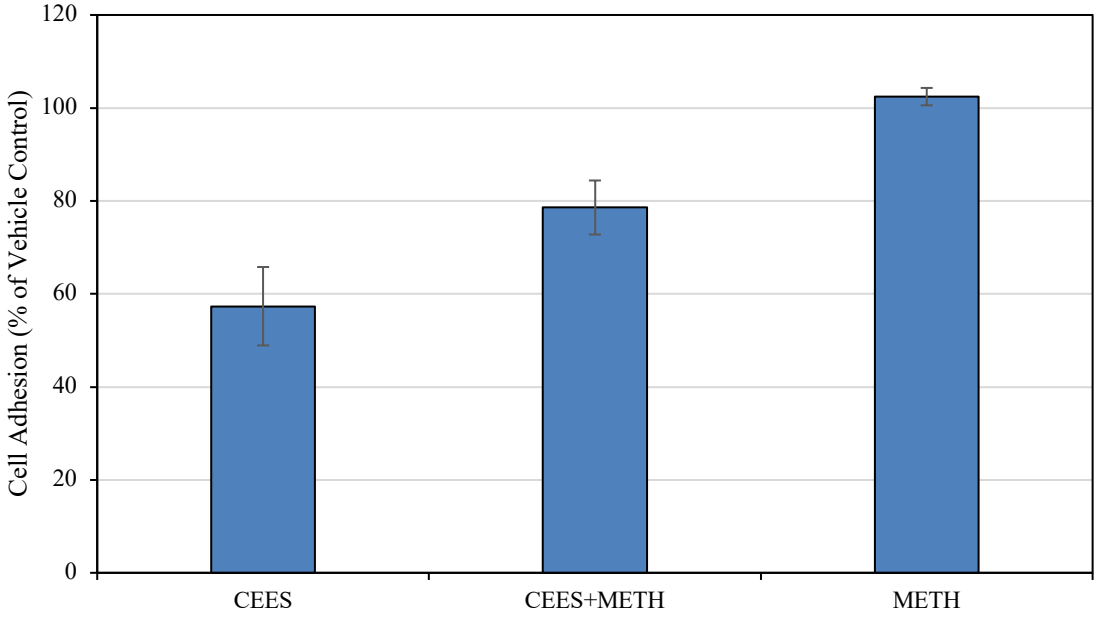


Figure 2.2. Relative cell-matrix adhesion obtained 24-hours post-exposure for methimazole. Cells were treated with 1 mM CEES and the working concentration of METH. The vehicle control was 1:1000 ethanol:DMEM.

2.5. Conclusion and Future work

While the scavenging effectiveness does seem to generally parallel functional group nucleophilicity, the toxicity of certain groups, such as thioacids, restricts those scavengers' efficacy in living systems. Based on the first-generation scavenger's toxicity and performance in CMA and MTT assays, candidate scavengers containing thiols, amines, and a mixture of the two were chosen for evaluation. Other factors in scavenger selection included cost, availability, known biological effects, and structural simplicity. Out of the second-generation scavenger group (N = 8), the individual and CEES-

combined toxicity and SEVs narrowed the leading candidate scavengers down to methimazole.

Moving forward, two main areas must be explored. First, continuing to extend the scavenger search by finding and assaying promising compounds containing thioamines and thiodiamines. Secondly, other dangerously toxic chemicals such as methyl isocyanate (MIC) react and damage living systems in a very similar mechanism to sulfur mustard. Exploring whether our scavengers are capable of neutralizing other highly toxic electrophilic species would shed light on how broadly these scavengers could be effectively utilized.

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