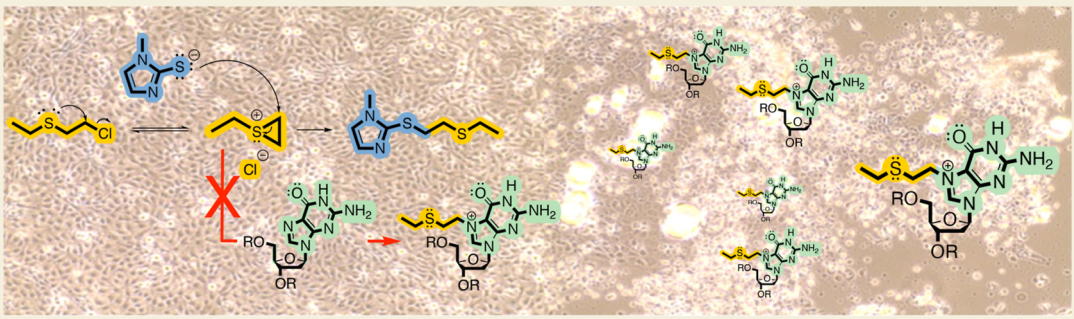
ACS BIO & MED CHEM Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

pubs.acs.org/biomedchemau
Article

Methimazole, an Effective Neutralizing Agent of the Sulfur Mustard Derivative 2-Chloroethyl Ethyl Sulfide

Albert Armoo,[§] Tanner Diemer,[§] Abigail Donkor,[§] Jerrod Fedorchik,[§] Severine Van slambrouck, Rachel Willand-Charnley, and Brian A. Logue*

Cite This: *ACS Bio Med Chem Au* 2023, 3, 448−460
Read Online
ACCESS | Metrics & More | Article Recommendations | Supporting Information



ABSTRACT: Sulfur mustard (SM), designated by the military as HD, is a highly toxic and dangerous vesicant that has been utilized as a chemical warfare agent since World War I. Despite SM's extensive history, an effective antidote does not exist. The effects of SM are predominantly based on its ability to alkylate important biomolecules. Also, with the potential for a fraction of SM to remain unreacted up to days after initial contact, a window of opportunity exists for direct neutralization of unreacted SM over the days following exposure. In this study, we evaluated the structure−activity relationship of multiple nucleophilic molecules to neutralize the toxic effects of 2-chloroethyl ethyl sulfide (CEES), a monofunctional analogue of SM, on human keratinocyte (HaCaT) cells. Cell viability, relative loss of extracellular matrix adhesions, and apoptosis caused by CEES were measured via MTT, cell−matrix adhesion (CMA), and apoptosis protein marker assays, respectively. A set of five two-carbon compounds with various functional groups served as a preliminary group of first-generation neutralizing agents to survey the correlation between mitigation of CEES's toxic effects and functional group nucleophilicity. Apart from thioacids, which produced additive toxicity, we generally observed the trend of increasing protection from cytotoxicity with increasing nucleophilicity. We extended this treatment strategy to second-generation agents which contained advantageous structural features identified from the first-generation molecules. Our results show that methimazole (MIZ), a currently FDA-approved drug used to treat hyperthyroidism, effectively reduced cytotoxicity, increased CMA, and decreased apoptosis resulting from CEES toxicity. MIZ selectively reacts with CEES to produce 2-(2-(ethylthio)ethylthio)-1-methyl-1*H*-imidazole (EEMI) in media and cell lysate treatments resulting in the reduction of toxicity. Based on these results, future development of MIZ as an SM therapeutic may provide a viable approach to reduce both the immediate and long-term toxicity of SM and may also help mitigate slower developing SM toxicity due to residual intact SM.

KEYWORDS: *sulfur mustard, 2-chloroethyl ethyl sulfide, methimazole, chemical warfare agent, blister agent*

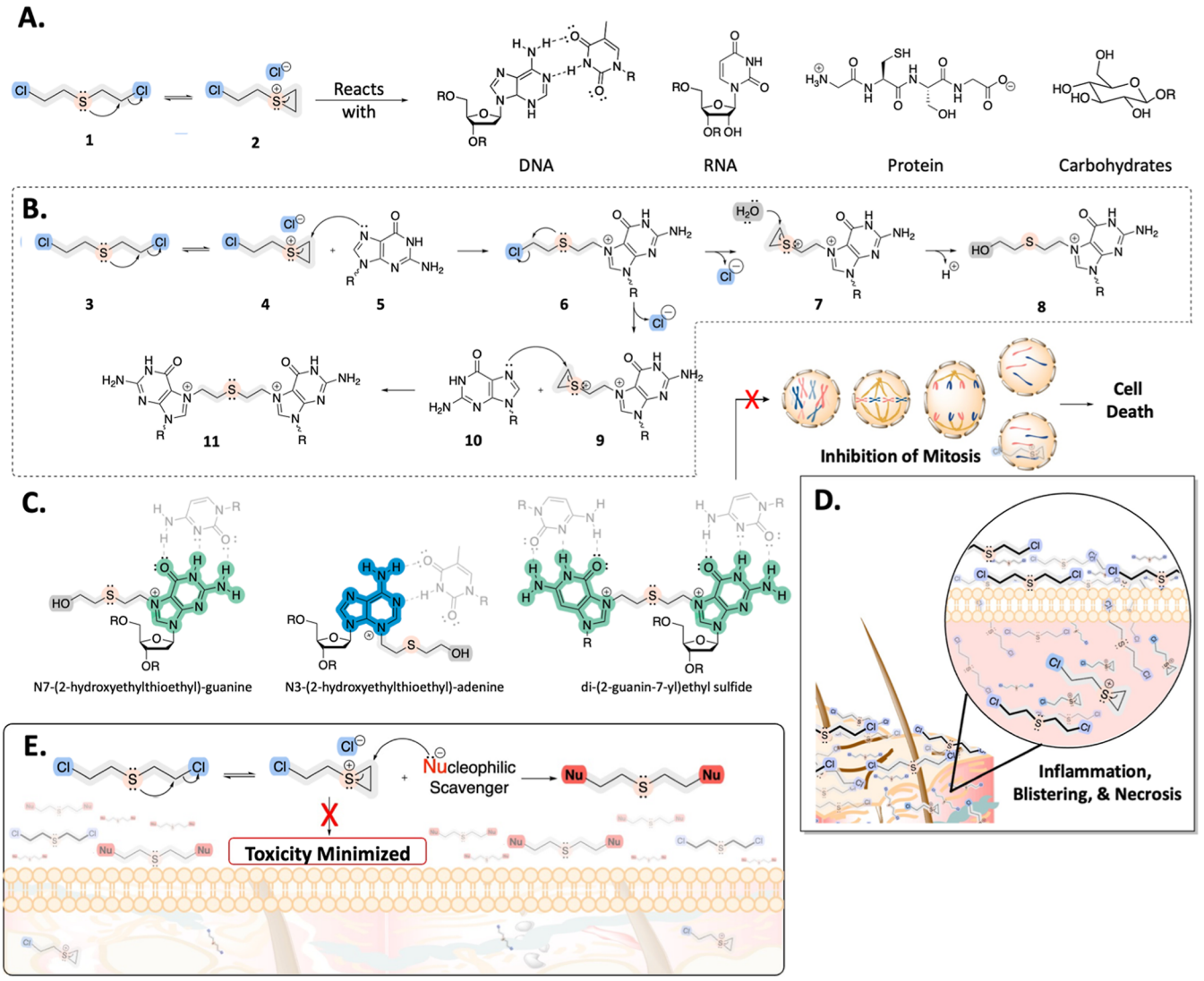
INTRODUCTION

Sulfur mustard (SM), bis(2-chloroethyl)sulfide, is a powerful bifunctional alkylating agent used as a chemical warfare agent (CWA) dating back to World War I. The lack of an effective treatment and the easy and inexpensive manufacture and storage of SM are major reasons why SM remains a threat for both civilian and military targets today. SM undergoes a spontaneous intramolecular cyclization to form the highly reactive electrophilic ethylene episulfonium intermediate by eliminating a chloride ion via an intramolecular nucleophilic substitution (Scheme 1A).[1−3] The episulfonium ion is a highly alluring electrophile toward various nucleophilic atoms found in DNA, RNA, carbohydrates, and proteins, leading to alkylation of these biomolecules (Scheme 1A). For example, SM preferentially reacts with DNA at the nucleophilic N7 of deoxyguanosine,[3] yielding a monoalkylated product which undergoes a subsequent round of spontaneous intramolecular cyclization to form a second ethylene episulfonium intermediate, which can be hydrolyzed to result in the

Received: December 29, 2022
Revised: July 17, 2023
Accepted: July 18, 2023
Published: August 9, 2023

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

Scheme 1. Mechanisms of Sulfur Mustard (SM) Toxicity and Mitigation via Nucleophilic Scavengers; (A) SM Reacting with Macromolecules; (B) Mechanism of Action between SM and the Nucleophilic N7 of Deoxyguanosine Leading to DNA Cross-Linkage; (C) Alkylated SM Results in Cell Death; (D) Effects of SM on Skin; and (E) SM Nucleophilic Scavenger Treatment



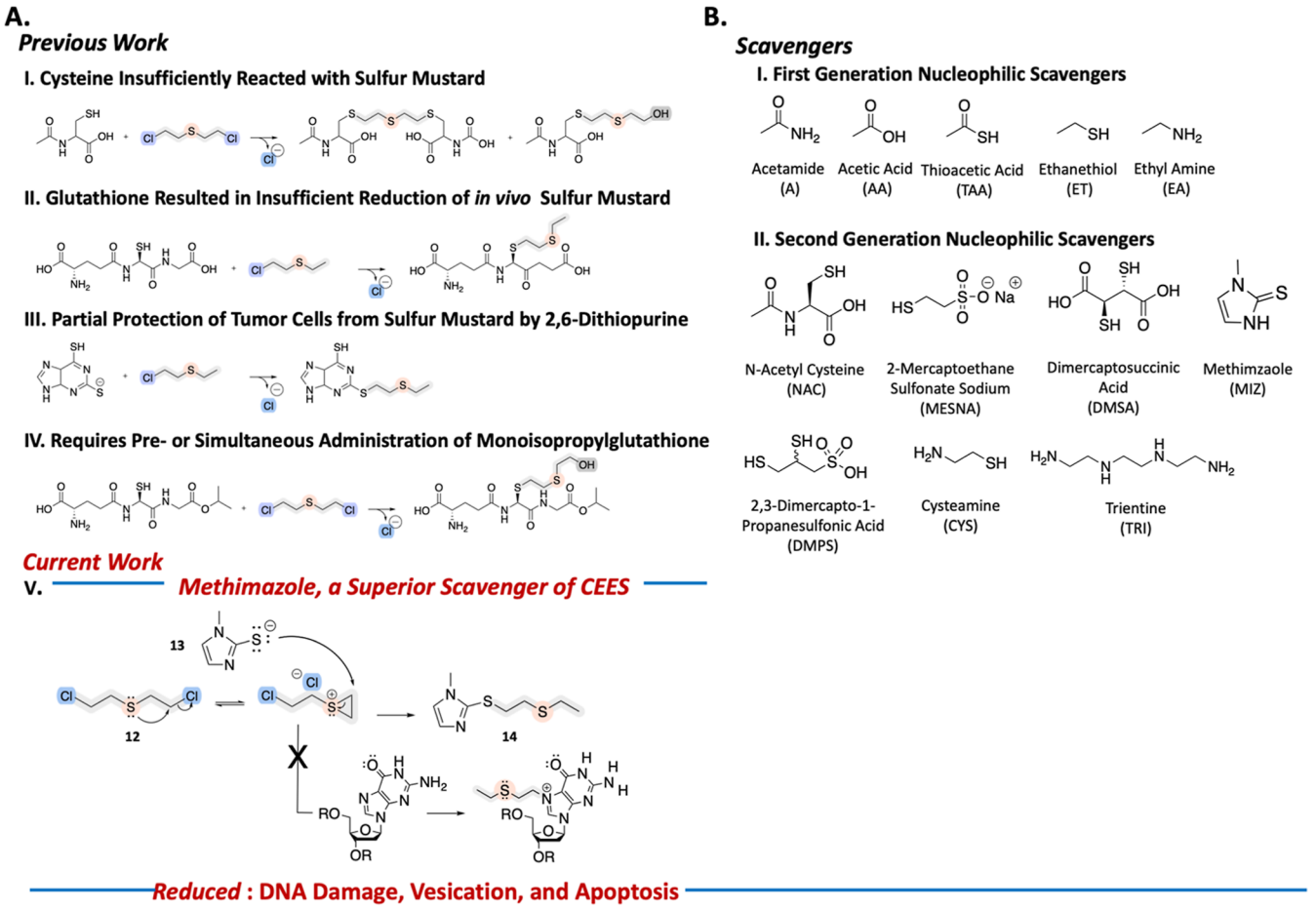
monoalkylated N7-(2-hydroxyethyl-thioethyl) adduct (Scheme 1B).[4,5] The ethylene episulfonium intermediate is also subject to nucleophilic attack by an adjacent deoxyguanosine to form the cross-linked di-(2-guanin-7-yl)ethyl sulfide. SM can additionally alkylate the N3 of deoxyadenosine following a similar mechanistic pathway which does not result in a cross-linked product.[5] SM-alkylated products induce cytotoxicity.[6,7] For example, the bi-alkylated intra- and interstrand cross-links result in the inhibition of mitosis, leading to cell death (Scheme 1C). Another mechanism of SM toxicity is depletion of intracellular NAD+ levels which leads to inhibition of glycolysis. The enzymes of the pyruvate oxidase system are also sensitive to direct interaction with SM. In addition to the damage occurring in intracellular membranes, SM can lead to the depletion of cellular glutathione (GSH) and other antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, resulting in the accumulation of reactive oxygen species (ROS), followed by lipid peroxidation, protein oxidation, and DNA damage.[8,9]

SM exposure damage occurs in both intracellular and extracellular matrixes. The vesicating effects of SM on mammalian skin (Scheme 1D) have been shown to cause inflammation and extensive blistering.[2,4] Clinical symptoms of SM exposure, which are based on early alkylation of key biomolecules, appear hours after exposure.[10] There is also evidence that a significant fraction of the SM dose remains biologically active within the victim's body for several days after exposure.[11−13] Therefore, a window of opportunity exists to administer a molecule with a high affinity for SM to an affected individual immediately and in the days following exposure to reduce harmful alkylation by neutralizing unreacted SM. This strategy can be considered "scavenging" or direct "neutralization" of SM. If a scavenging molecule can be delivered to fatty tissues, or sufficient intra- and intercellular concentrations can be maintained, SM could preferentially react with this compound to form a non-toxic byproduct.

Due to the safety concerns and regulatory requirements of procuring and handling SM, many studies of mustard toxicity utilize nitrogen mustards[14] or CEES[15] as simulants for SM. CEES was chosen to simulate SM in this study based on its structural and mechanistic similarity to SM.[11,16,17]

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

Scheme 2. Previous vs Current Work; (A) Pitfalls of Previous Efforts to Treat Sulfur Mustard and Sulfur Mustard Derivatives, e.g., CEES, Poisoning; (I) NAC and GSH; (II) Were Effective but Did Not Reduce the SM Concentration by Chemical Scavenging; (III) 2,6-Dithiopurine Was Successful in Alleviating Mutagenesis in Tumor Cells but Only Provided Partial Protection; (VI) MIPE Application Was Required before or Immediately after SM Exposure to be Effective in Protecting A549 Cells via Extracellular Inactivation of SM by MIPE; (V) Current Work Results Using Methimazole to Scavenge CEES Resulting in Reduced DNA Damage, Vesication, Apoptosis, and Necrosis of the Cells; and (B) Suite of Nucleophilic Scavengers Tested for Their Reactivity with CEES and Ability to Reduce DNA Damage, Vesication, and Apoptosis



The scavenger concept has been previously suggested (Scheme 1E), but only few molecules have been investigated for this purpose, including *N*-acetyl cysteine (NAC),[18] glutathione (GSH),[6,19] 2,6-dithiopurine (DTP),[20] and mono-isopropylglutathione (MIPE).[21] These molecules were selected to mimic the reactions of SM with naturally occurring biomolecules. While these scavengers were shown to mitigate the toxicity of SM to some degree, studies showed that the mechanism of some of these potential therapeutic agents was not based on direct reaction with SM, while others were not effective intracellularly. For example, Siegert et al.[22] documented that NAC was effective but did not reduce the SM concentration by chemical scavenging, suggesting that protection was due to physiological processes (Scheme 2A(I)). In 2018, Siegert et al.[23] also evaluated GSH scavenging and concluded that there was not a significant reduction in the SM concentration, suggesting that the mechanism of GSH protection was also based on physiological effects instead of direct reaction with SM (Scheme 2A(II)). Liu et al.[5] and Powell et al.[20] studied 2,6-dithiopurine as a sulfur-containing thiopurine analogue for its effectiveness as an SM scavenger. This molecule proved successful in alleviating mutagenesis in tumor cells but only provided partial protection (Scheme 2A(III)). Lindsay et al.[21] studied MIPE and its ability to protect A549 cells (i.e., lung carcinoma epithelial cells) from SM toxicity. Although A549 cells were 2 times more viable when protected with MIPE than SM-exposed cells, the authors observed the protection to be likely due to extracellular inactivation of SM by MIPE. Furthermore, the authors concluded that MIPE would need to be applied prior to, or immediately after, SM exposure to be effective (Scheme 2A(IV)).

For a scavenging strategy to be effectively implemented, it is essential to identify a candidate compound with excellent affinity toward SM and low toxicity. Based on previous studies of scavenger treatment, an opportunity exists for a more comprehensive structure−activity approach to discover a therapeutic agent possessing high affinity for scavenging SM. Fundamental evaluation of structural features which lead to effective direct reactivity may allow identification of extremely effective scavenging molecules.

Methimazole (MIZ), which is an antithyroid drug, contains a thiodiamine functional group. The two amine groups and the thiol that surrounds the central carbon gives MIZ its unique property to tautomerize and act as a nucleophile. MIZ has numerous advantageous physiological properties such as anti-

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

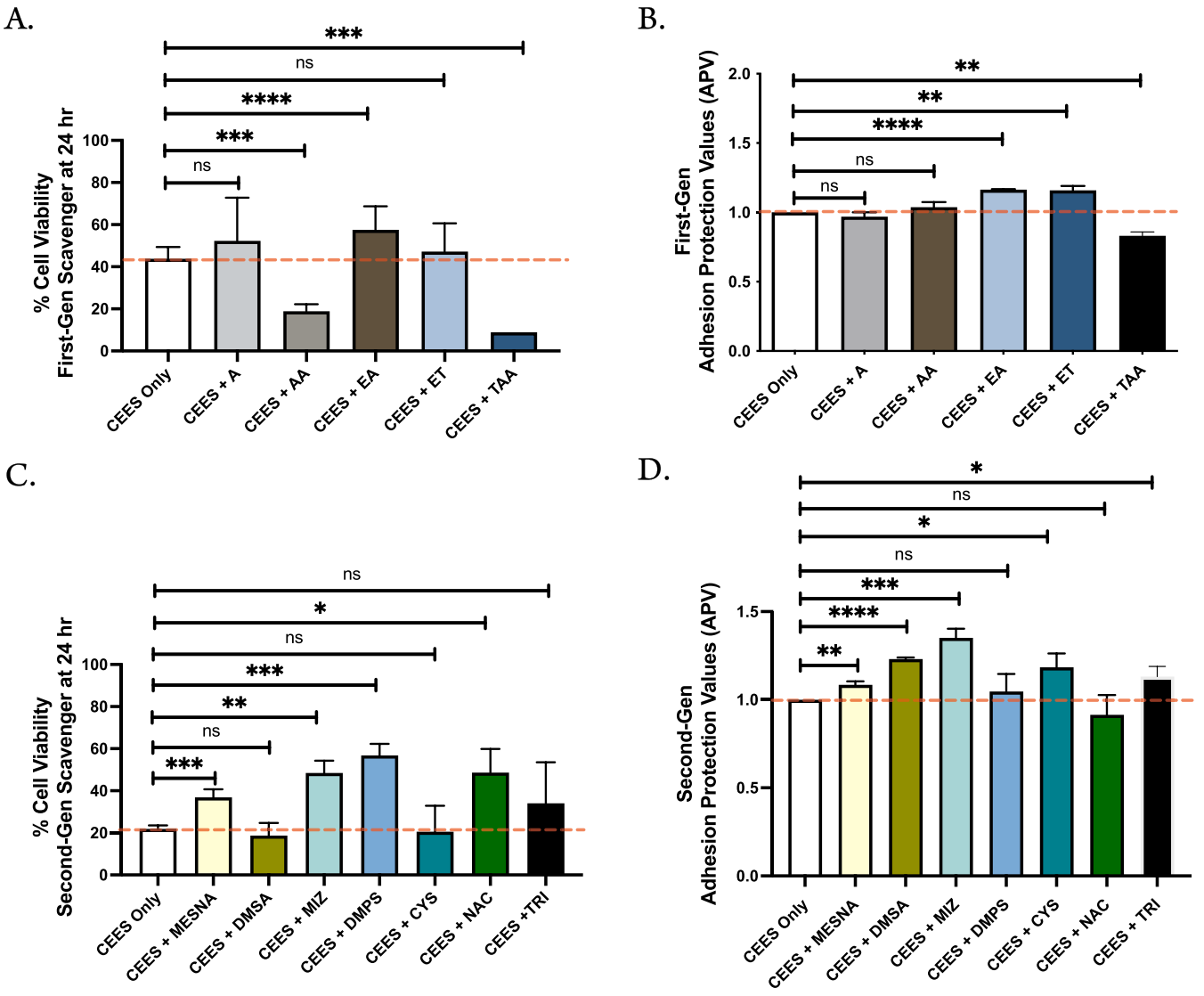


Figure 1. MTT and CMA assays of CEES-exposed HaCaT cells treated with the indicated scavenger from the first- and second-generation scavenger set. Human epidermal HaCaT cells were seeded in 96-well plates and incubated overnight. The HaCaT cells were exposed to 2 mM CEES in the MTT assay and 1 mM CEES in the CMA assay for 24 h and various concentrations of first-generation scavengers (A) and second-generation scavengers (C) were used to treat the cells in the MTT assays. Adhesion Protection Values (APVs) for first-generation scavengers (B) and second-generation scavengers (D) were calculated via CMA assays. All data are means of ±s.d of triplicates from an experiment that was repeated a total of three times. (*) $P < 0.05$ as compared to the CEES-exposed group. ns = not significant. See Scheme 2 for acronym designations.

inflammatory, antimicrobial, and antioxidant properties. MIZ can neutralize different forms of free radicals such as ROS and thyroid peroxidases (TPO).[24] Moreover, MIZ can also regulate certain main inflammatory pathways involving the interferon-γ (IFN-γ)-activated signaling pathway in thyroid cells.[25]

The objective of the current study was to identify and evaluate scavenging molecules as potential therapeutics to decrease the toxicity of CEES. A deliberate, stepwise, structure−activity evaluation to identify the best SM scavenging molecules was accomplished in this study. Specifically, a group of compounds with varying functional group nucleophilicity toward CEES was evaluated (Scheme 2B). These compounds were tested for effectiveness in protecting human keratinocyte (HaCaT) cells from SM toxicity via MTT, cell viability, and cell−matrix adhesion (CMA) assays. Additionally, this study sought to investigate the potential role of MIZ in decreasing molecular damage caused by CEES via probing apoptosis (Scheme 2A(V)).

RESULTS

Scientific Rigor

All reagents/antibodies/cell lines were selected based on published figures and purchased from companies that provide validation of purity. All studies include the appropriate isotype and secondary antibody-only controls. Experiments were performed in technical and then biological replicates. Experiments were performed in the hands of another appropriately trained researcher in a partial blind study for further validation (Supporting Information: Materials, Methods, NMR Spectra, and HPLC Chromatogram of MIZ). We used *t*-tests or ANOVA followed by Bonferroni or Dunnett post-tests for multiple comparisons using GraphPad Prism 8. Data is always presented as mean ± standard deviation with *P*-values < 0.05 indicating significance.

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

CEES Toxicity and MIZ Dose

To best observe the effects of treatments on the cell viability of CEES-exposed cells as measured via the MTT assay, a cell viability of approximately 30% for untreated cells was targeted. After observing the cell viability of CEES-exposed HaCaT cells at 24 h post-exposure, we determined that 1 mM was the most appropriate concentration of CEES to obtain the target cell viability (Supporting Information: Evaluation of CEES Toxicity and MIZ Dose). The effective dose of MIZ was determined by treating CEES-exposed HaCaT cells with 0.5−4 mM MIZ. The HaCaT cells treated with 2 and 4 mM MIZ showed excellent recovery of cell viability as compared to CEES-exposed but untreated cells (Supporting Information: Evaluation of CEES Toxicity and MIZ Dose). While 2 and 4 mM MIZ worked equally well to reduce the toxicity of CEES, the working dose of MIZ was set to 2 mM. The lower dose was chosen to minimize any potential toxic effects of higher concentrations of MIZ.

Cytotoxicity Protection by First-Generation Scavengers

A series of two-carbon molecules (Scheme 2B group I) with multiple scavenging functionalities were initially tested for effectiveness in protecting HaCaT cells via MTT cell viability and CMA assays. The first-generation scavengers, a set of five two-carbon compounds with various functional groups, served as a survey group to evaluate the structure−activity correlation between scavenging ability and functional group nucleophilicity. Specifically, acetamide, acetic acid, ethyl amine, ethanethiol, and thioacetic acid were evaluated as first-generation scavengers for mitigating the cellular toxicity of CEES.

Although increases in cell viability for the two-carbon molecules appear relatively small, including a non-significant increase in cell viability for acetamide, acetamide, ethyl amine, and ethanethiol representing amide, amine, and thiol functionality, respectively, produced a generally consistent increase in cell viability after 24 h (Figure 1A) and 48 h (data not shown). While the most effective scavengers in the MTT assay were generally expected, they did not perfectly mirror increased nucleophilicity. This is likely because of multiple factors, including the cellular toxicity of the compound itself. Ethanethiol, representing thiol functionality, was the second most nucleophilic compound tested but only slightly increased cell viability. Ethyl amine, representing the amine functional group, was the most successful molecule for producing a consistent increase in cell viability. While it is less nucleophilic than the thiol functionality, it has significant nucleophilicity and relatively low cellular toxicity, like many amines essential to biological function (e.g., amino acids). Acetamide, which possessed the least amount of nucleophilic characteristic of the functional groups tested, still showed a consistent increase in cell viability for CEES-treated cells. This effect could be attributed to its moderate nucleophilic characteristic paired with relatively low toxicity. It is likely that relatively high toxicity of these two carbon molecules played a significant role in mitigating their ability to increase cell viability compared to CEES-exposed (untreated) cells. Thioacetic acid, one of the most nucleophilic molecules tested, produced increased cell death when compared to CEES alone (Figure 1A). This is likely due to its additive toxicity owing to significant pH changes or high relative nucleophilic characteristic resulting in off-target binding. Acetic acid additionally reduced cell viability when compared to CEES alone, likely due to lowering the cellular pH in combination with CEES toxicity.

Protection of Cell Adhesion by First-Generation Scavengers

We investigated the effect of CEES on extracellular matrix (ECM) protein adhesions using collagen I, the most abundant protein found in the skin. To accommodate for interassay variability, a normalized value was developed and dubbed the adhesion protection value (APV). 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. APVs above 1 indicate improved retention of cell−matrix adhesion, while APVs below 1 indicate reduced cell matrix adhesions relative to CEES-exposed controls. APVs for the first-generation scavengers are visualized against the red line, which indicates the CEES control value (Figure 1B). Aside from thioacetic acid, increasing APVs generally correlated with increasing nucleophilicity of the functional groups of the first-generation scavenger molecules (Figure 1B). Ethanethiol and ethylamine displayed promising APVs and, taken along with the MTT assay, showed consistency in mitigating the toxic effects of CEES. Acetamide's performance in the CMA assay was more consistent with its relative nucleophilicity than in the MTT assay. Although acetamide showed increased viability in the MTT assay (Figure 1A), the CMA assay showed that it had very little impact on helping cells retain their extracellular adhesions (Figure 1B). While thioacetic acid was the most nucleophilic scavenger, it exhibited counterproductive effects by exacerbating CEES's effects on cellular adhesion, which aligned with the MTT assay and likely relates to its relatively high toxicity. As with the MTT assay, the protective effects of the two-carbon scavengers appear relatively small. This is likely because of the inherent toxicity of these molecules. Although the effect may not appear sizable, the effect is significant and clearly shows the effectiveness of the amine and thiol moieties to protect matrix adhesion.

Taken together, the cell viability and protection of cellular adhesion generally validated the trend of increasing scavenging ability mirroring increasing nucleophilicity, with the main deviation occurring with thioacetic acid, which produced additive toxicity to CEES alone. Based on these assays, compounds with thiol and amine functional groups with low toxicity were sought for evaluation of their protection against SM toxicity.

Protection against CEES Toxicity by Second-Generation Scavengers

Second-generation scavengers were identified based on best performing structural features from the first-generation scavengers along with their safety profile, affordability, and availability. With thiol- and amine-containing first-generation scavengers proving to be the most promising in terms of both protection and minimal toxicity, eight second-generation scavengers were selected to explore the effectiveness of these functionalities: *N*-acetyl cysteine (NAC), 2-mercaptoethane sulfonate sodium (MESNA), dimercaptosuccinic acid (DMSA), methimazole (MIZ), 2,3-dimercapto-1-propanesulfonic acid (DMPS), cysteamine (CYS), trientine (TRI), and dimercaprol (DMP) (Scheme 2B, group II). In part, these molecules were selected because they contained at least one thiol or amine, with most containing multiple combinations of these functional groups.

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

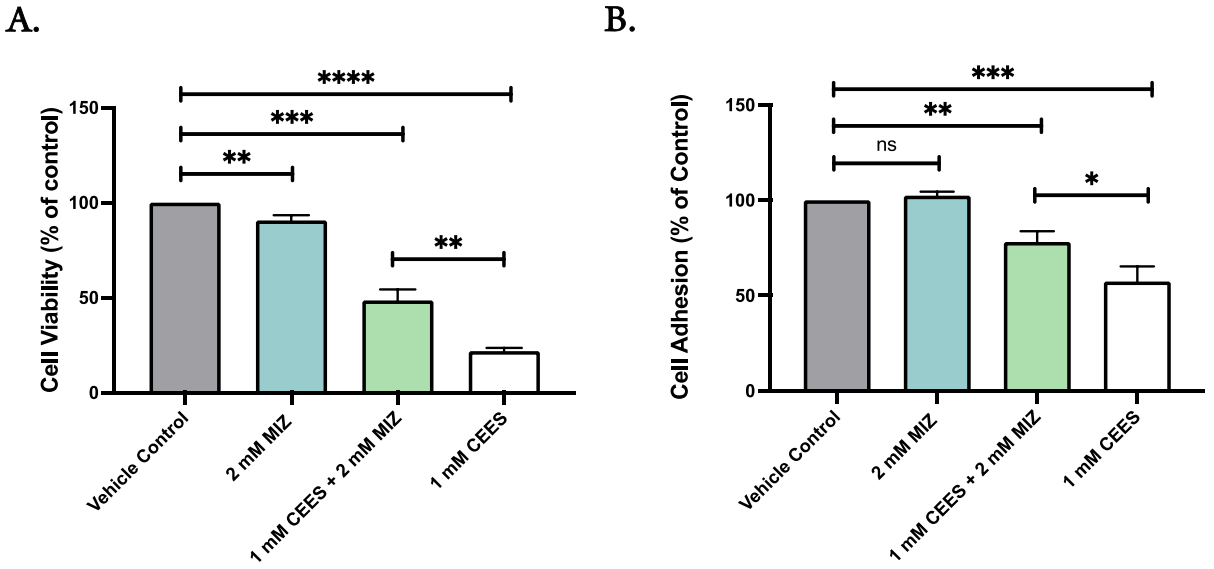
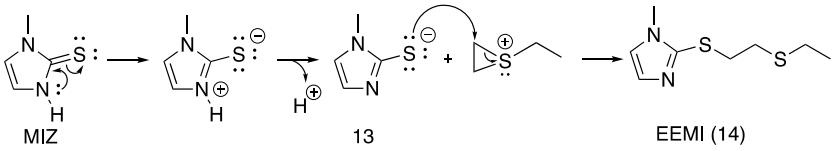


Figure 2. MIZ treatment alleviates CEES-induced cytotoxicity in HaCaT cells. Human epidermal HaCaT cells were seeded in 96-well plates and incubated overnight. They were then exposed to ethanol alone as vehicle control, 2 mM MIZ alone, 2 mM CEES alone, and 2 mM MIZ added after 1 mM CEES exposure (A). Relative cell−matrix adhesion obtained 24 h post exposure of MIZ. Human epidermal HaCaT cells were seeded in 96-well plates and incubated overnight. They were then exposed to vehicle control, 2 mM MIZ alone, 1 mM CEES alone, and 2 mM MIZ added after 1 mM CEES exposure (B). All data are means of ±s.d of triplicates from an experiment that was repeated a total of three times with MTT assay performed after 24 hr. (*) $P < 0.05$ as compared to the CEES-exposed group. ns = not significant.

Scheme 3. Proposed Mechanism of Action for the Formation of EEMI under Aqueous Reaction Conditions[a]



[a]Methimazole (MIZ) aromatizes to form a negatively charged sulfur (13) residue that reacts with the electrophilic sulfonium ion intermediate via an intramolecular nucleophilic substitution mechanism forming EEMI (14).

As with the two-carbon scavengers, the ability of second-generation scavengers to reduce CEES toxicity was evaluated via the MTT and CMA assays. MESNA, MIZ, DMPS, NAC, and TRI all consistently increased cell viability via the MTT assay after 24 h of CEES incubation (Figure 1C). Of those compounds, DMPS and MIZ were the most successful in reducing the toxicity of CEES. Of the remaining scavengers, NAC performed well during the time points prior to 48 h, but the relative cell viability decreased relatively quickly at 48 h (data not shown). Both MESNA and TRI showed moderate increases in cell viability, with TRI showing high variability, which led to the increase in cell viability not being statistically significant. CYS and DMSA both did not significantly increase cell viability.

For the CMA assay, MIZ provided the most effective protection of cellular adhesion of the second-generation scavengers, with an APV of approximately 1.4 (Figure 1D). MESNA provided statistically significant protection of matrix adhesion, but only a small absolute increase was observed. DMPS and NAC were not nearly as effective at preserving cell−matrix adhesions as they were at protecting cell viability. TRI and CYS were only moderately successful in preserving CMA and showed large variations in their effectiveness. While DMSA was ineffective at increasing cell survival (Figure 1C), it showed more promise in conserving epithelial extracellular junctions with collagen I.

MIZ Increases Cell Viability/Adhesion of CEES-Exposed Cells

Based on the promise MIZ showed compared to the other second-generation treatments, it was explored in more detail as a treatment for CEES toxicity. The MTT assay was again performed following CEES exposure (1 mM) and treatment with an optimized concentration of 2 mM MIZ. Compared to vehicle controls, CEES exposure caused a decrease in cell viability to approximately 22% at 24 h (Figure 2A). When CEES-exposed cells were treated with MIZ, a reversal of CEES-induced cytotoxicity was observed (Figure 2A). MIZ treatment was able to reduce the cytotoxicity of CEES, increasing cellular viability to approximately 48% of control, a 118% increase when compared to CEES only cells.

The CMA assay was also performed using the optimized MIZ dose of 2 mM following CEES exposure (1 mM). At 24 h, we observed a decrease in cell adhesion to approximately 57% of the vehicle control when exposing the cells to CEES (Figure 2B). When treated with MIZ, cell adhesion increased to approximately 78% of vehicle control, a 37% increase.

In both assays, MIZ treatment alone did not cause a significant change in HaCaT cell behavior as compared to the vehicle control (Figure 2), indicating that this concentration of MIZ is not measurably toxic to the HaCaT cells via these assays.

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

MIZ and CEES React to Form EEMI

When CEES reacts directly with MIZ, it forms 2-(2-(ethylthio)ethylthio)-1-methyl-1*H*-imidazole (EEMI) (Scheme 3). The reaction of CEES and MIZ in PBS buffer at room temperature produced EEMI as determined via mass spectrometric analysis of the resulting product. Figure 3 shows

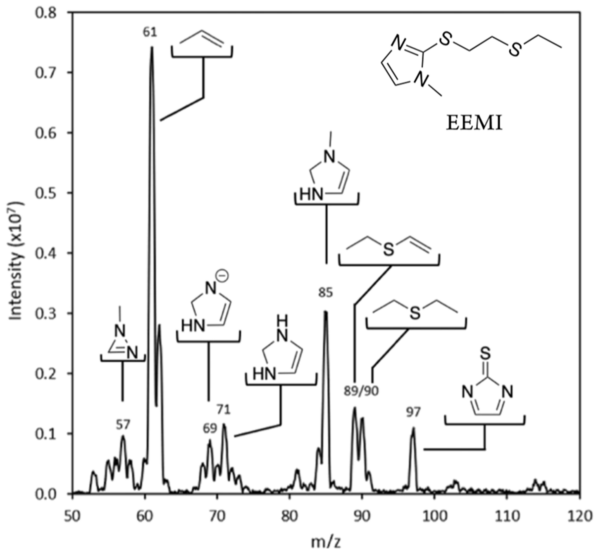


Figure 3. ESI (+) product ion mass spectra with the identification of the EEMI abundant ions. Molecular ion of EEMI [M + H]+ corresponds to 204.30 *m*/*z*. Insets: structure of EEMI with abundant fragments indicated.

the product ion mass spectra of the EEMI-abundant ions along with the suggested structures of the abundant fragments. Although it was clear that a product with the mass of EEMI was formed, the reaction site for CEES (i.e., N vs S) was inconclusive. Purification of EEMI with subsequent NMR analysis revealed that the CEES reacts with MIZ at the sulfur atom (Supporting Information). This result was expected since the sulfur atom is the most nucleophilic site of MIZ. Scheme 3 shows the suggested mechanism for CEES reaction with MIZ. MIZ aromatizes to form a negatively charged sulfur (13) residue that reacts with the electrophilic sulfonium ion intermediate via an intramolecular nucleophilic substitution mechanism to form EEMI (14). EEMI was fully characterized via nuclear magnetic resonance (NMR) and by ESI (+)-MS.

MIZ Selectively Reacts with CEES

The formation of EEMI was further probed under in vitro settings. CEES-affected HaCaT cell lines were treated with MIZ, the cells were lysed, prepared, and analyzed via ESI (+)-MS for the expected EEMI product. EEMI was detected from 1× PBS, media controls, and the cell lysates (Figure 4). Comparing EEMI formed in the 1× PBS to EEMI formed in the media and cell lysates, there was no significant difference. This indicates that MIZ reacts with CEES preferentially to naturally occurring electrophiles found in the biological milieu, such as fetal bovine serum (FBS), cellular components, or the aqueous solvent itself.

MIZ Reduced the Apoptotic Effects of CEES

To further confirm the ability of MIZ to protect cells from CEES toxicity, we measured the ability of MIZ to reduce CEES-induced apoptosis in HaCaT cells. The cells were seeded in a 96-well plate and incubated overnight. This

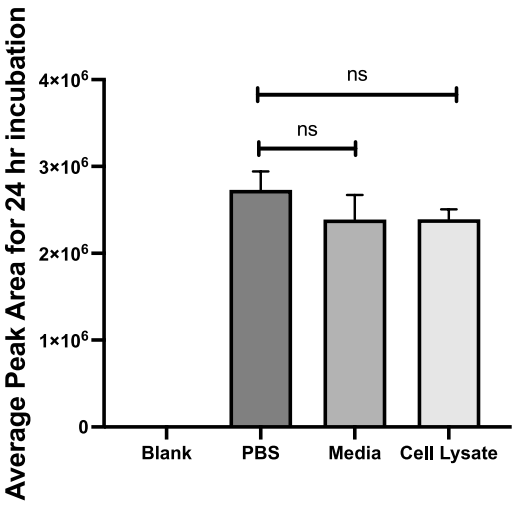


Figure 4. Comparison of the production of EEMI from the reaction of CEES and MIZ in PBS buffer, media, and cell lysates. EEMI was formed in PBS buffer, media, and cell lysates with only a slight decrease in EEMI produced in the presence of the biological milieu found in media and cell lysate. ns = not significant.

allowed the cells to adhere to the bottom of the plate. The cells were then treated with vehicle control, MIZ alone, CEES with MIZ treatment, and CEES alone. Figure 5A shows the images of the cell morphology of HaCaT cells treated with vehicle control (Figure 5A1), 2 mM MIZ (Figure 5A2), 2 mM MIZ after 1 mM CEES exposure (Figure 5A3), and 1 mM CEES exposure without MIZ treatment (Figure 5A4). After 24 h, the cells were imaged with an EVOS XL Core transmitted light inverted imaging system. The cell morphology of the cells treated with MIZ only (Figure 5A2) and the CEES-exposed MIZ-treated cells (Figure 5A3) was similar to that of the vehicle control (Figure 5A1). For the cells treated with CEES only (Figure 5A4), the cells appear to have lost their ability to adhere to the plate, which is an indication of cell injury.

We further determined the effect of MIZ on HaCaT cells exposed to CEES by measuring apoptosis. HaCaT cells were treated with 2 mM MIZ after 1 mM CEES exposure. After incubation (24 h), apoptosis was measured by Annexin V staining. Annexin V dye detects apoptotic cells by binding to phosphatidylserine from the inner face of the plasma membrane to the cell surface. Figure 5B shows the effect of 2 mM MIZ on cell viability for cells treated with CEES. Cells exposed to CEES alone resulted in a reduction in percent mean live cells (70.4%) compared to the vehicle control (93.8%). MIZ treatment after CEES exposure showed a significant increase in % mean live cells (86.1%) compared to CEES alone, producing an almost identical live cell percentage as MIZ treatment alone (87.6%).

We further examined the CEES-induced apoptotic effect on HaCaT cells by assessing the presence of caspase 3 and polyADP ribose polymerase-1 (PARP-1) enzymatic activity. Activating caspases is one of the common signaling cascades involved in apoptosis. Apoptotic cell death via caspases occurs when proteins required for cellular functioning and survival cleave PARP that contains DNA-binding domain.[26] Once activated, caspases cleave several key proteins, such as PARP-1, which is a hallmark of apoptosis.[27] Therefore, an increase in caspase 3 and PARP-1 indicates increased apoptosis. To evaluate caspase 3 and PARP-1 activity, HaCaT cells were treated with 2 mM MIZ after 1 mM CEES exposure. After

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

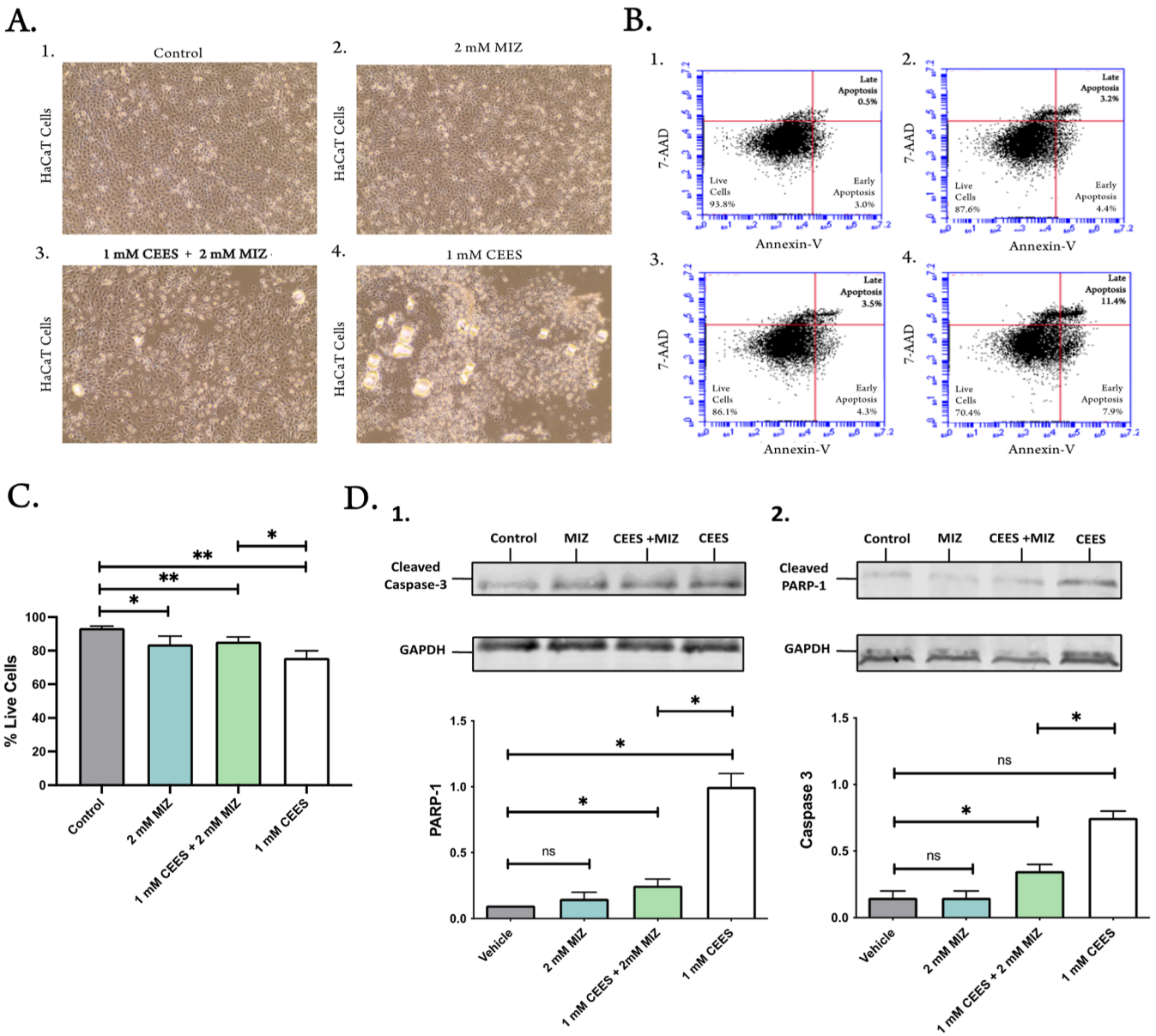


Figure 5. Effectiveness of MIZ in the reducing apoptosis and DNA damage. (A) Effect of MIZ on apoptosis in live cells exposed to 1 mM CEES. Human epidermal HaCaT cells were seeded in 96-well plates and incubated overnight. They were then exposed to ethanol as vehicle control (1), 2 mM MIZ alone (2), 1 mM CEES alone (3), and 2 mM MIZ after 1 mM CEES exposure (4) for 24 h. (B) MIZ treatment reduces the ability of CEES to cause apoptosis. Human epidermal HaCaT cells were exposed to ethanol alone as vehicle control (1), 2 mM MIZ alone (2), 2 mM MIZ after 1 mM CEES exposure (3), and 1 mM CEES alone (4) for 24 h. (C) Effects of MIZ on HaCaT cells exposed to CEES for 24 h. Human epidermal HaCaT cells were treated with vehicle control, 2 mM MIZ alone, 1 mM CEES alone, and 2 mM MIZ after 1 mM CEES exposure for 24 h. (D) Whole-cell lysates were prepared at the end of the incubations and analyzed using western blot to detect caspase-3 (1) and PARP-1 (2) enzymatic activity. All data are means of ±s.d of triplicates from an experiment that was repeated a total of three times. (*) $P < 0.05$ as compared to the CEES-exposed group.

incubation for 24 h, we quantified and normalized the apoptotic proteins present in the HaCaT cells (Figure 5D). Cells exposed to CEES alone for 24 h showed a significant increase in caspase 3 expression (0.8) when compared to vehicle control (0.2). MIZ treatment after CEES exposure significantly reduced the levels of caspase 3 expression from 0.8 to 0.4, a 50% reduction. MIZ alone (0.2) did not have a significant effect when compared to vehicle control (Figure 5D1). A similarly strong increase in apoptotic protein expression was also observed in PARP-1 protein expression following CEES exposure. Cells that were exposed to CEES only for 24 h had a significantly higher level of PARP-1 protein expression (1.0) compared to cells treated with MIZ and CEES combination (0.3), a 70% reduction in PARP-1 expression. Vehicle control and MIZ treatment alone resulted in low PARP-1 activity levels, 0.1 and 0.2, respectively (Figure 5D2), with no significant difference between them.

DISCUSSION

SM has been utilized as a CWA since World War I, but despite its extensive history and highly toxic effects, an effective antidote does not exist.[28] CEES, which has been used extensively as a simulant for SM, induces skin toxicity through its monofunctional alkylating effects, forming adducts with

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

DNA, RNA, and proteins.[29,30] Vesicating agents such as SM and CEES also interact with cellular thiols, resulting in the accumulation of ROS which induce DNA damage.[19,30] DNA-damage-related signaling pathways are activated by SM/CEES-induced DNA damage, resulting in cellular toxicity and potential cell death.[31] As demonstrated in other studies, if the effects of SM/CEES are not prevented or repaired, there is a decline in cell viability.[30,32,33]

In this study, we investigated the direct interaction between CEES and various nucleophilic scavengers to evaluate their effectiveness. Thiols interact with the cysteine residues of intracellular proteins and helps to maintain the protein structure and functions found in the intracellular matrix. Thiols can also act as scavengers for hydroxyl radicals, hydrogen peroxides, and reactive metabolites of lipids and proteins.[16] Ultimately, MIZ's thiodiamine functional group, a combination of a thiol and amines in a single moiety, proved to be the most effective scavenger to both increase the cell viability and preserve the extracellular junctions in CEES-treated epithelial cells. MIZ is a distinctive scavenger that has a strong nucleophilic characteristic and a high affinity toward various electrophilic molecules while producing limited cellular toxicity. Detection of EEMI was confirmed in cell media following exposure of cells to CEES and treatment with MIZ at levels close to the reaction of these compounds in a simple aqueous buffer (i.e., PBS), verifying the direct reaction and selective affinity of CEES and MIZ. MIZ also has numerous advantageous physiological properties such as antioxidant and anti-inflammatory properties. Studies have also shown that one of the toxic mechanisms of CEES is to reduce the level of GSH by increasing the production of ROS. However, it is possible that MIZ reacted with CEES to form the EEMI found in the buffer, media, and cell lysates and inhibited ROS accumulation and the loss of intracellular GSH induced by CEES.

Mustard vesicants target the basal epidermal layer, forming blisters on skin tissue when exposed to these agents. The blisters are caused when proteases digest anchoring filaments of the epidermal−dermal junction.[34,35] In our studies, a relative loss of extracellular matrix adhesions was observed by measuring acid phosphatase activity through the CMA assay. Collagen I-coated plates were utilized to specifically investigate the role MIZ may play in preserving this key interaction to reduce vesication. Accordingly, our CMA studies confirmed the MIZ treatment resulted in the preservation of extracellular junctions to collagen I in CEES-treated HaCaT cells.

The final event of cytotoxicity is cell death, and one of the important events in determining the mode of cell death depends on the mechanism of intoxication. The loss of mitochondrial membrane potential is an early event leading to apoptosis.[36] Our results (Figure 5B) showed that CEES induces apoptosis in HaCaT cells. However, MIZ treatment decreased the percentage of apoptotic HaCaT cells by inhibiting cleavage of procaspase-3, leading to a decrease in cleaved caspase 3. This subsequently led to a decrease in PARP-1 cleavage, as can be seen in Figure 5D1 and D2. These results are supported by Han et al.,[16] a team that determined thiol antioxidants such as GSH and NAC can protect against CEES-induced apoptosis via activation of procaspase-3 and the cleavage of PARP-1. They observed that cells pretreated with the GSH precursor *N*-acetyl cysteine or with GSH-ethyl ester were able to inhibit the effects of CEES on the accumulation of ROS and inhibited the activity of caspase-3,[16] an important protease in apoptotic death which cleaves the key proteins involved in apoptosis. PARP-1 is a major protein affected by caspase-3 activity, and its activity results in DNA strand breaks in cells that have been exposed to DNA-damaging agents.[37,38]

Papirmeister et al. proposed that SM-induced DNA damage significantly decreases the amount of NAD+ and ATP levels as a result of enhanced synthesis and release of protease. It is proposed that these proteases are responsible for the formation of blisters by separating the basal cell layer from the basement membrane and allowing fluid in this space.[39−42] Excessive activation of PARP-1 and depletion of NAD+ promote further DNA and structural damage which disrupts ion channels and results in functional loss.[43] In our studies, we have shown that there was an increase in caspase-3 activity in the presence of CEES, which correlates with an increase in cleaved-PARP-1. However, MIZ had a positive effect on CEES-induced caspase-3 activity and PARP-1 production by reducing their levels. This is likely because the application of MIZ to CEES-exposed cells converts a portion of the free CEES to a non-toxic byproduct, thereby reducing increased caspase-3 activity.

MIZ is one of the most used antithyroid drug for treating Graves' hyperthyroidism, which is an autoimmune disease sustained by autoantibodies binding to and activating the thyrotropin (TSH) receptor located on the thyroid follicular cell. These TSH receptor antibodies (TRAb) stimulate the synthesis and secretion of thyroid hormone. The MIZ mechanism of action targets the TSH receptor antibodies (TRAb) and reduces their levels significantly in treated patients.[44−46] Treatment with MIZ is done with a single daily oral dose (5−60 mg/day).[47−51] While MIZ proved to be an excellent treatment for CEES toxicity in the in vitro models presented here, it has been shown to have some toxicity for human use. MIZ can produce dose-related adverse effects, including nausea; stomach pain; tender lymph glands in the neck, armpit, or groin; and tightness in the chest.[17,52] While our models are very different from oral dosing in humans, it will be important to consider potential adverse effects of MIZ during future development of this compound for the treatment of SM exposure.

The vapor and liquid exposure concentrations of SM producing erythema are 100−300 mg·min/m^3 and 10−20 μg/cm^2, respectively, while blister formation is produced at 1000−2000 mg·min/m^3 for vapor exposure and 40−100 μg/cm^2 for neat (liquid) exposure.[55] Additionally, droplets of liquid sulfur mustard containing as little as 0.0025 mg can cause erythema.[53,54] In comparison, despite the HaCaT cells used in the present studies being exposed to 0.65 mg (1 M) concentration of CEES, MIZ was able to neutralize the effects of CEES and reduce DNA damage, vesication, and apoptosis. The conditions used in this study represent compelling early evidence concerning the applicability of MIZ to neutralize SM in the future.

CONCLUSIONS

We demonstrated the ability of MIZ to reduce the cytotoxic effects of CEES, increase CMA, and reduce apoptosis by activating caspase-3 and cleaving PARP in the HaCaT cell line. Overall, the results demonstrate that MIZ has the potential to be an effective therapeutic to protect against the toxic effects of CEES. However, additional efficacy studies both in vitro and in vivo are required to evaluate this promising therapeutic for SM injury.

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

MATERIALS AND METHODS

Reagents, Toxicants, and Compounds

All HPLC-MS/MS reagents and solvents were at least of HPLC grade, unless otherwise stated. All compounds were purchased aside from EEMI at purities > 95%. Formic acid and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent purchased from Sigma-Aldrich and 1× PBS purchased from Gibco were used to create a stock solution with a concentration of 5 mg/mL. The stock was stored in a light-protected container at 4 °C. The CEES stock solution was made in anhydrous ethanol at a concentration of 1 M. Methimazole (MIZ) stock solution was prepared in PBS to a concentration of 2 M. CEES and MIZ stock solutions were stored at 4 °C.

Cell Culture and Their Treatment

The experiments were performed using the human keratinocyte cell line (HaCaT). The cells were acquired from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin (pen/strep) (Cytiva Hyclone). Cells grown under standard culture conditions were treated with either ethanol (vehicle control) alone, CEES (1 mM) alone, MIZ (2 mM) alone, or CEES (1 mM) with MIZ (2 mM). CEES treatments were added within 15 s of mixing the treatment dilution in cell media to keep dosage effects consistent between trials. The scavenger treatments were also added 15 s following CEES treatments to mimic a post-exposure response. All CEES preparations and treatments were employed using the required and approved personal protective equipment under a safety laminar hood.

Formation of EMMI in Media

Briefly, the HaCaT cells were grown in 96-well plates at a concentration of 1.5×10^4 cells/100 μL. The cells were treated with 100 μL of 1 mM CEES and 2 mM MIZ for 24 h in media or PBS and incubated at 37 °C in a 5% CO_2 humidified atmosphere. At the end of the incubation, the media and/or PBS solutions were collected, and the aqueous and organic layers were washed with ether (3 × 30 mL), dried over sodium sulfate, filtered, and concentrated in vacuo upon which time the crude product was subject to flash column chromatography and characterized as described below.

HPLC-MS/MS Analysis of EEMI

Briefly, the HaCaT cells were grown in 96-well plates at a concentration of 1.5×10^4 cells/100 μL. The cells were treated with 100 μL of 1 mM CEES and 2 mM MIZ for 24 h in media or PBS and incubated at 37 °C in a 5% CO_2 humidified atmosphere. At the end of the incubation, the cells were collected and lysed with RIPA lysis buffer to form the cell lysates and characterized using HPLC-MS/MS as described below.

Liquid chromatography analysis was performed on a Shimazu UFLC with an LC-20ADXR controller. The column used for chromatography was an Agilent polymeric reversed phase column, Zorbax eclipse-XDB C18 (4.6 × 150 mm, 5.0 μM, part#: PN 993967−902). The chromatographic separation was achieved using isocratic elution at a flow rate of 1 mL/min at 90% B held for 7 min. Mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. The column was equilibrated for 1 min, and a volume of 10 μL was injected for HPLC-MS/MS analysis. For MS analysis, a tandem mass spectrometer (Sciex Q-Trap 5500 MS) equipped with an electrospray ionization interface in the positive polarity mode was used to detect the CEES-MIZ product (i.e., EEMI) formation in PBS, media, and cell lysates. Optimization of mass spectrometric conditions was accomplished by direct infusion of a CEES-MIZ reaction solution into the spectrometer at a flow rate of 10 μL/min. After infusion of the CEES-MIZ adduct solution into the ESI, the molecular ion of EEMI, m/z 204.30 ($[30]^+$), was identified. Multiple reaction monitoring (MRM) parameters for EEMI were optimized. Nitrogen (50 psi) was used as the curtain and nebulization gas. The ion spray voltage was 4500 V, the source temperature was 500 °C, and both the nebulizer (GS1) and heater (GS2) gas pressures were 90 psi. The collision cell was operated with an entrance potential of 10.0 V and an exit potential of 11.0 V at a "medium" collision gas flow rate. The total mass spectrometry acquisition time was 7 min.

Cell Viability Assay (MTT Assay)

The HaCaT cells were seeded on 96-well plates at a concentration of 1.5×10^4 cells/100 μL cellular media per well and allowed to reach a confluency of 80% before treatment. The cells were then treated with different concentrations of CEES (0.5, 1, 2, and 4 mM). The modulatory effect of MIZ was examined at different concentrations (0.5, 1, 2, and 4 mM) at different conditions and incubated for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. For the MIZ pre-treated conditions, the cells were incubated with MIZ for 1 h prior to CEES was added to the cells. For the co-treated condition, both MIZ and CEES were mixed and added to the wells concurrently. For the CEES-pre-treated wells, the cells were treated with CEES for 15 s before MIZ was added to the cells. The changes in cell viability were observed by measuring the cellular dehydrogenase activity using the MTT assay. Briefly, the treatment-containing medium was removed, the cells were washed with PBS, and MTT was added to each well of the plates and incubated for 2 h at 37 °C in a 5% CO_2 humidified atmosphere. After the end of incubation, DMSO was added to the purple formazan product. The absorbance was read at 570 nm using a BioTek Cytation 3 Cell Imaging Multimode reader (Winooski, VT, USA). Relative cell viability was measured as a percentage compared to vehicle-treated control cells.

Cell−Matrix Adhesion Assay

Briefly, the relative loss of extracellular matrix (ECM) adhesion was observed by measuring the 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 2.5×10^4 cells/100 μL cellular media per well. The plates were incubated overnight prior to treatment with CEES, scavengers, or both at the indicated working concentrations with eight wells per condition. After 24 h of incubation, the plates were gently rinsed three times with PBS to remove non-adherent cells. The substrate solution, 12 mM *p*-nitrophenyl phosphate (*p*NPP) and 50 mM sodium acetate trihydrate in 0.2% Triton X100 adjusted to a pH of 5−6, was added at a volume of 100 μL to each well. After 1 hour of incubation, 50 μL of 1 N NaOH was added to each well to deprotonate the substrate's product and produce 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 adherence was measured as a percentage compared to vehicle-treated control cells. To accommodate for inter-assay variability, a normalized value was developed and dubbed the adhesion protection value (APV). This was defined as the ratio of the adhered cells in the CEES/scavenger condition and the CEES control. Therefore, APVs greater than 1 indicate improved retention of cell−matrix adhesion, while APVs less than 1 indicate reduced cell matrix adhesions relative to CEES-exposed controls.

Apoptosis Detection Using Flow Cytometry

Briefly, HaCaT cells were grown in 96-well plates at a concentration of 1.0×10^5 cells/100 μL cellular media per well. The cells were treated with either vehicle (ethanol) alone or required concentrations of CEES, scavengers, or both, and after 12 h of treatment, floating and adhered cells were collected. The cells were washed twice with cold PBS (Gibco) and re-suspended in 100 μL of 1× Annexin V Binding Buffer. The cell suspension was transferred in to a 5 mL test tube. The cells were stained by adding 5 μL of 7-AAD (BD Biosciences) and Annexin V (BD Biosciences) dyes and incubated in the dark for 15 min at room temperature. A final volume of 400 μL of Annexin V Binding Buffer was added to each tube and analyzed by a flow cytometer (BD Accuri C6 Plus). Quantification of apoptotic cells was performed in triplicate for each treatment, and the number of apoptotic cells were recorded.

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

Western Blot Analysis

The cells were harvested and then lysed with RIPA Lysis and Extraction Buffer (Thermo Fisher) according to the manufacturer's instruction. Lysis buffer was prepared by adding Pierce Protease and Phosphatase Inhibitor (Thermo Fisher) to the RIPA buffer according to the manufacturer's instruction. The total protein concentration was measured using the BCA assay (Sigma-Aldrich), and immunoblot analyses per protein sample were between 20 and 100 μg. The plate was read using a BioTek Cytation Live Cell imager (BioTek). Vehicle and treated samples were denatured in SDS sample buffer and subjected to SDS−PAGE using 4−12% (w/v) Criterion TGX Precast Gel (BioRad). Separated proteins were transferred onto a Trans-Blot Turbo RTA Midi 0.2 μm nitrocellulose transfer kit and the associated Turbo-Blot Turbo Transfer System (BioRad). Membranes were blocked at room temperature for 30 min in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) BSA. Membranes were either probed with cleaved caspase-3 or caspase-9 primary antibodies (Cell Signaling Technology) at a dilution of 1:1000 and incubated at 4 °C overnight with a gentle shaking. Both antibodies were reconstituted in Intercept (TBS) Protein Free Blocking buffer (Li-COR). The membranes were washed three times in TBST for 5 min each time and incubated in the dark with goat anti-rabbit IRDye secondary antibody (Li-COR) for the detection of cleaved caspase-3 and caspase-9. Protein loading was confirmed by stripping and re-probing the membranes with GAPDH antibody. Incubation occurred for 1 h at a dilution of 1:1000 for each in TBST. Membranes were allowed to dry and analyzed using the Odyssey CLx Imager (Li-COR) and quantified using Image Studio Lite Version 5.2 (Licor).

Solvents/Reagents

Tetrahydrofuran (THF) was freshly distilled in our laboratory. THF was produced by distilling sodium benzophenone. Unless otherwise stated, all reagents were used exactly as they were purchased.

Chromatography

TLC was performed using silica gel 60 plates that were 175−225 μm thick, aluminum-backed, and coated with the fluorescent indicator F254. TLC tests were evaluated with a portable UV light and/or created with one of the stains listed below: 1% $KMnO_4$ in water. High-purity silica gel (pore size: 60, particle size: 230−400 mesh) was used for flash column chromatography.

Characterization

For 1H NMR, the frequency was 600 MHz, while for 13C 1H NMR, it was 150 MHz. As was already mentioned, both 1H and 13C 1H NMR spectra were obtained using CDCl3 as the solvent. The signal multiplicity, coupling constant(s) (J), and number of protons for the spectra of all compounds were determined. High-resolution mass spectrometry was performed by the Nebraska Center for Mass Spectrometry at the University of Nebraska-Lincoln using electrospray ionization (ESI) on a Bruker SolariX FT-ICR (HRMS).

Synthesis of EEMI

A flame-dried round-bottom flask with a magnetic stir bar was charged with 10 mmol MIZ (1.1 g), and 11 mmol of CEES (1.2 g) was dissolved in 10 mL of anhydrous THF. The reaction proceeded at room temperature for 1 h and monitored via TLC. Upon completion, the solvent was removed, worked up, and purified via flash column chromatography (30% EtOAc/Hex), producing the desired product, 2-(2-(ethylthio)ethylthio)-1-methyl-1*H*-imidazole, in 60% yield (1.2 g) of a solid white powder, melting point of 282.2 °F. Characterization: R_f = 0.35 (30% EtOAc/Hex); 1H NMR (600 MHz, $CDCl_3$): δ 7.06 (d, 1H), 6.95 (d, 1H), 3.63 (s, 3H), 3.30−3.20 (dd, J = 9.87, 5.98, 2H), 2.89−2.78 (dd, J = 9.56, 5.67, 2H), 2.61−2.52 (q, 2H), 2.29−2.21 (t, 3H); 13C NMR (150 MHz, $CDCl_3$): δ 147.0, 129.2, 122.1, 34.0, 33.2, 31.4, 25.7, 14.6. HRMS (FT-ICR, ESI+): m/z: calcd for $C_8H_{14}N_2NaS_2$ [30], 225.04902; found, 225.04906. (Supporting Information: Methods, NMR Spectra, and HPLC Chromatogram of MIZ).

Statistical Analysis

Data were analyzed by Student's t-test. All charts were created by GraphPad Prism 8.0 software. A P-value of <0.05 was considered statistically significant. All experiments were performed in triplicates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomedchemau.2c00087.

Experimental details, materials, methods, NMR spectra, evaluation of CEES toxicity, and MIZ dose (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Brian A. Logue − *Department of Chemistry & Biochemistry, South Dakota State University, Brookings, South Dakota 57007, United States;* orcid.org/0000-0002-2400-9339; Phone: (605) 688-6698; Email: brian.logue@sdstate.edu

Authors

Albert Armoo − *Department of Chemistry & Biochemistry, South Dakota State University, Brookings, South Dakota 57007, United States*

Tanner Diemer − *Department of Chemistry & Biochemistry, South Dakota State University, Brookings, South Dakota 57007, United States*

Abigail Donkor − *Department of Chemistry & Biochemistry, South Dakota State University, Brookings, South Dakota 57007, United States*

Jerrod Fedorchik − *Department of Chemistry & Biochemistry, South Dakota State University, Brookings, South Dakota 57007, United States*

Severine Van slambrouck − *Youngstown State University, Youngstown, Ohio 44555, United States*

Rachel Willand-Charnley − *Department of Chemistry & Biochemistry, South Dakota State University, Brookings, South Dakota 57007, United States;* orcid.org/0000-0003-2721-6955

Complete contact information is available at: https://pubs.acs.org/10.1021/acsbiomedchemau.2c00087

Author Contributions

[§]A.A., T.D., A.D., and J.F. contributed equally to this work. CRediT: **Jerrod Fedorchik** methodology (supporting).

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank the LARGE and RAWC group members for their excellent technical support.

■ ABBREVIATIONS

CEES	2-chloroethyl ethyl sulfide
CMA	cell−matrix adhesion
CWA	chemical warfare agent
DMEM	Dulbecco's modified Eagle's media

EEMI	2-(2-(ethylthio)ethylthio)-1-methyl-1*H*-imidazole
GSH	cellular glutathione
HaCaT	human keratinocyte cell line
MIZ	methimazole
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	*N*-acetyl cysteine
ROS	reactive oxygen species
SM	sulfur mustard
A	acetamide
AA	acetic acid
DMP	dimercaprol
CYS	cysteamine
DMPS	2,3-dimercapto-1-propanesulfonic acid
DMSA	dimercaptosuccinic acid
EA	ethyl amine
ET	ethanethiol
MESNA	2-mercaptoethane sulfonate sodium
TAA	thioacetic acid
TRI	triethylenetetramine (trientine)
pNPP	*p*-nitrophenyl phosphate
SEV	scavenging effectiveness value
MIPE	monoisopropylglutathione

REFERENCES

(1) Wormser, U. Toxicology of Mustard Gas. *Trends Pharmacol. Sci.* **1991**, *12*, 164−167.

(2) Dacre, J. C.; Goldman, M. Toxicology and pharmacology of the chemical warfare agent sulfur mustard. *Pharmacol. Rev.* **1996**, *48*, 289−326.

(3) Saladi, R. N.; Smith, E.; Persaud, A. N. Mustard: a potential agent of chemical warfare and terrorism. *Clin. Exp. Dermatol.* **2006**, *31*, 1−5.

(4) Fidder, A.; Moes, G. W. H.; Scheffer, A. G.; van der Schans, G. P.; Baan, R. A.; de Jong, L. P. A.; Benschop, H. P. Synthesis, characterization, and quantitation of the major adducts formed between sulfur mustard and DNA of calf thymus and human blood. *Chem. Res. Toxicol.* **1994**, *7*, 199−204.

(5) Liu, J.; Powell, K. L.; Thames, H. D.; MacLeod, M. C. Detoxication of sulfur half-mustards by nucleophilic scavengers: robust activity of thiopurines. *Chem. Res. Toxicol.* **2010**, *23*, 488−496.

(6) Paromov, V.; et al. Sulfur mustard toxicity following dermal exposure: role of oxidative stress, and antioxidant therapy. *J. Burns Wounds* **2007**, *7*, No. e7.

(7) Matijasevic, Z.; Precopio, M.; Snyder, J.; Ludlum, D. Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host cell reactivation assay. *Carcinogenesis* **2001**, *22*, 661−664.

(8) Jowsey, P. A.; Williams, F. M.; Blain, P. G. DNA damage, signalling and repair after exposure of cells to the sulphur mustard analogue 2-chloroethyl ethyl sulphide. *Toxicology* **2009**, *257*, 105−112.

(9) Jain, A. K.; Tewari-Singh, N.; Orlicky, D. J.; White, C. W.; Agarwal, R. 2-Chloroethyl ethyl sulfide causes microvesication and inflammation-related histopathological changes in male hairless mouse skin. *Toxicology* **2011**, *282*, 129−138.

(10) Romero, A.; Ramos, E.; López-Muñoz, F.; De Los Ríos, C.; Egea, J.; Gil-Martín, E.; Pita, R.; Torrado, J. J.; Serrano, D. R.; Juberias, A. Toxicology of Blister Agents: Is Melatonin a Potential Therapeutic Option? *Diseases* **2021**, *9*, 27.

(11) Tewari-Singh, N.; Rana, S.; Gu, M.; Pal, A.; Orlicky, D. J.; White, C. W.; Agarwal, R. Inflammatory biomarkers of sulfur mustard analog 2-chloroethyl ethyl sulfide-induced skin injury in SKH-1 hairless mice. *Toxicol. Sci.* **2009**, *108*, 194−206.

(12) Gould, N. S.; White, C. W.; Day, B. J. A role for mitochondrial oxidative stress in sulfur mustard analog 2-chloroethyl ethyl sulfide-induced lung cell injury and antioxidant protection. *J. Pharmacol. Exp. Ther.* **2009**, *328*, 732−739.

(13) Laskin, J. D.; Black, A. T.; Jan, Y. H.; Sinko, P. J.; Heindel, N. D.; Sunil, V.; Heck, D. E.; Laskin, D. L. Oxidants and antioxidants in sulfur mustard-induced injury. *Ann. N. Y. Acad. Sci.* **2010**, *1203*, 92−100.

(14) Sharma, M.; Vijayaraghavan, R.; Gautam, A. DRDE-07 and its analogues as promising cytoprotectants to nitrogen mustard (HN-2)–an alkylating anticancer and chemical warfare agent. *Toxicol. Lett.* **2009**, *188*, 243−250.

(15) Bennett, R. A.; Behrens, E.; Zinn, A.; Duncheon, C.; Lamkin, T. J. Mustard gas surrogate, 2-chloroethyl ethylsulfide (2-CEES), induces centrosome amplification and aneuploidy in human and mouse cells : 2-CEES induces centrosome amplification and chromosome instability. *Cell Biol. Toxicol.* **2014**, *30*, 195−205.

(16) Han, S.; Espinoza, L. A.; Liao, H.; Boulares, A. H.; Smulson, M. E. Protection by antioxidants against toxicity and apoptosis induced by the sulphur mustard analog 2-chloroethylethyl sulphide (CEES) in Jurkat T cells and normal human lymphocytes. *Br. J. Pharmacol.* **2004**, *141*, 795−802.

(17) Chung, J. H. Antithyroid Drug Treatment in Graves' Disease. *Endocrinol. Metab.* **2021**, *36*, 491−499.

(18) Jain, A. K.; Tewari-Singh, N.; Gu, M.; Inturi, S.; White, C. W.; Agarwal, R. Sulfur mustard analog, 2-chloroethyl ethyl sulfide-induced skin injury involves DNA damage and induction of inflammatory mediators, in part via oxidative stress, in SKH-1 hairless mouse skin. *Toxicol. Lett.* **2011**, *205*, 293−301.

(19) Tewari-Singh, N.; Agarwal, C.; Huang, J.; Day, B. J.; White, C. W.; Agarwal, R. Efficacy of glutathione in ameliorating sulfur mustard analog-induced toxicity in cultured skin epidermal cells and in SKH-1 mouse skin in vivo. *J. Pharmacol. Exp. Ther.* **2011**, *336*, 450−459.

(20) Powell, K. L.; Boulware, S.; Thames, H.; Vasquez, K. M.; MacLeod, M. C. 2,6-Dithiopurine blocks toxicity and mutagenesis in human skin cells exposed to sulfur mustard analogues, 2-chloroethyl ethyl sulfide and 2-chloroethyl methyl sulfide. *Chem. Res. Toxicol.* **2010**, *23*, 497−503.

(21) Lindsay, C. D.; Hambrook, J. L.; Lailey, A. F. Monoisopropylglutathione ester protects A549 cells from the cytotoxic effects of sulphur mustard. *Hum. Exp. Toxicol.* **1997**, *16*, 636−644.

(22) Siegert, M.; Kranawetvogl, A.; Thiermann, H.; John, H. N-Acetylcysteine as a chemical scavenger for sulfur mustard: New insights by mass spectrometry. *Drug Test. Anal.* **2018**, *10*, 243−253.

(23) Siegert, M.; Kranawetvogl, A.; Thiermann, H.; John, H. Glutathione as an antidote for sulfur mustard poisoning: Mass spectrometric investigations of its potency as a chemical scavenger. *Toxicol. Lett.* **2018**, *293*, 31−37.

(24) Heufelder, A. E.; Wenzel, B. E.; Bahn, R. S. Methimazole and propylthiouracil inhibit the oxygen free radical-induced expression of a 72 kilodalton heat shock protein in Graves' retroocular fibroblasts. *J. Clin. Endocrinol. Metab.* **1992**, *74*, 737−742.

(25) Kim, H.; Lee, T. H.; Hwang, Y. S.; Bang, M. A.; Kim, K. H.; Suh, J. M.; Chung, H. K.; Yu, D. Y.; Lee, K. K.; Kwon, O. Y.; et al. Methimazole as an antioxidant and immunomodulator in thyroid cells: mechanisms involving interferon-gamma signaling and H(2)O(2) scavenging. *Mol. Pharmacol.* **2001**, *60*, 972−980.

(26) Kaufmann, S. H.; et al. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.* **1993**, *53*, 3976−3985.

(27) Chaitanya, G. V.; Alexander, J. S.; Babu, P. P. PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. *Cell Commun. Signaling* **2010**, *8*, 31.

(28) Kehe, K.; et al. Sulfur mustard research–strategies for the development of improved medical therapy. *Eplasty* **2008**, *8*, No. e32.

(29) Guainazzi, A.; Campbell, A. J.; Angelov, T.; Simmerling, C.; Schärer, O. D. Synthesis and molecular modeling of a nitrogen mustard DNA interstrand crosslink. *Chemistry* **2010**, *16*, 12100−12103.

(30) Inturi, S.; Tewari-Singh, N.; Gu, M.; Shrotriya, S.; Gomez, J.; Agarwal, C.; White, C. W.; Agarwal, R. Mechanisms of sulfur mustard

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460

analog 2-chloroethyl ethyl sulfide-induced DNA damage in skin epidermal cells and fibroblasts. *Free Radical Biol. Med.* **2011**, *51*, 2272−2280.
(31) Tewari-Singh, N.; Inturi, S.; Jain, A. K.; Agarwal, C.; Orlicky, D. J.; White, C. W.; Agarwal, R.; Day, B. J. Catalytic antioxidant AEOL 10150 treatment ameliorates sulfur mustard analog 2-chloroethyl ethyl sulfide-associated cutaneous toxic effects. *Free Radical Biol. Med.* **2014**, *72*, 285−295.
(32) Mol, M. A.; de Vries-van de Ruit, A. M. Concentration- and time-related effects of sulphur mustard on human epidermal keratinocyte function. *Toxicol. In Vitro* **1992**, *6*, 245−251.
(33) Ku, W. W.; Bernstein, I. A. bis-(beta-chloroethyl)sulfide (BCES)-induced changes in epidermal cell homeostasis in vitro. *Toxicol. Appl. Pharmacol.* **1988**, *95*, 397−411.
(34) Hayden, P. J.; Petrali, J. P.; Stolper, G.; Hamilton, T. A.; Jackson, G. R.; Wertz, P. W.; Ito, S.; Smith, W. J.; Klausner, M. Microvesicating effects of sulfur mustard on an in vitro human skin model. *Toxicol. In Vitro* **2009**, *23*, 1396−1405.
(35) Shohrati, M.; Haji Hosseini, R.; Esfandiari, M. A.; Najafian, N.; Najafian, B.; Golbedagh, A. Serum matrix metalloproteinase levels in patients exposed to sulfur mustard. *Iran. Red Crescent Med. J.* **2014**, *16*, No. e15129.
(36) Salvioli, S.; Ardizzoni, A.; Franceschi, C.; Cossarizza, A. JC-1, but not $DiOC_6(3)$ or rhodamine 123, is a reliable fluorescent probe to assess $\Delta\Psi$changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. *FEBS Lett.* **1997**, *411*, 77−82.
(37) Boulares, A. H.; Yakovlev, A. G.; Ivanova, V.; Stoica, B. A.; Wang, G.; Iyer, S.; Smulson, M. Role of Poly(ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis. *J. Biol. Chem.* **1999**, *274*, 22932−22940.
(38) Rhun, Y. L.; Kirkland, J. B.; Shah, G. M. Cellular responses to DNA damage in the absence of Poly(ADP-ribose) polymerase. *Biochem. Biophys. Res. Commun.* **1998**, *245*, 1−10.
(39) Cowan, F. M.; Broomfeld, C. A.; Lenz, D. E.; Smith, W. J. Putative role of proteolysis and inflammatory response in the toxicity of nerve and blister chemical warfare agents: implications for multi-threat medical countermeasures. *J. Appl. Toxicol.* **2003**, *23*, 177−186.
(40) Gross, C. L.; et al. Sulfur mustard lowers nicotinamide adenine dinucleotide concentrations in human skin grafted to athymic nude mice. *Toxicol. Appl. Pharmacol.* **1985**, *81*, 85−90.
(41) Liu, F.; Jiang, N.; Xiao, Z. y.; Cheng, J. p.; Mei, Y. z.; Zheng, P.; Wang, L.; Zhang, X. r.; Zhou, X. b.; Zhou, W. x.; et al. Effects of poly (ADP-ribose) polymerase-1 (PARP-1) inhibition on sulfur mustard-induced cutaneous injuries in vitro and in vivo. *PeerJ* **2016**, *4*, No. e1890.
(42) Papirmeister, B.; Gross, C. L.; Meier, H. L.; Petrali, J. P.; Johnson, J. B. Molecular basis for mustard-induced vesication. *Fundam. Appl. Toxicol.* **1985**, *5*, S134−S149.
(43) Zhang, D.; Hu, X.; Li, J.; Liu, J.; Baks-te Bulte, L.; Wiersma, M.; Malik, N. u. A.; van Marion, D. M. S.; Tolouee, M.; Hoogstra-Berends, F.; et al. DNA damage-induced PARP1 activation confers cardiomyocyte dysfunction through NAD(+) depletion in experimental atrial fibrillation. *Nat. Commun.* **2019**, *10*, 1307.
(44) Davies, T. F.; Evered, D.; Smith, B.; Yeo, P.; Clark, F.; Hall, R. Value of thyroid-stimulating-antibody determinations in predicting short-term thyrotoxic relapse in Graves' disease. *Lancet* **1977**, *309*, 1181−1182.
(45) Zakarija, M.; McKenzie, J. M.; Banovac, K. Clinical significance of assay of thyroid-stimulating antibody in Graves' disease. *Ann. Intern. Med.* **1980**, *93*, 28−32.
(46) Teng, C. S.; Yeung, R. T. Changes in thyroid-stimulating antibody activity in Graves' disease treated with antithyroid drug and its relationship to relapse: a prospective study. *J. Clin. Endocrinol. Metab.* **1980**, *50*, 144−147.
(47) Bouma, D. J.; Kammer, H. Single daily dose methimazole treatment of hyperthyroidism. *West. J. Med.* **1980**, *132*, 13−15.
(48) Shiroozu, A.; Okamura, K.; Ikenoue, H.; Sato, K.; Nakashima, T.; Yoshinari, M.; Fujishima, M.; Yoshizumi, T. Treatment of hyperthyroidism with a small single daily dose of methimazole. *J. Clin. Endocrinol. Metab.* **1986**, *63*, 125−128.
(49) Roti, E.; et al. Methimazole and serum thyroid hormone concentrations in hyperthyroid patients: effects of single and multiple daily doses. *Ann. Intern. Med.* **1989**, *111*, 181−182.
(50) Mashio, Y.; Beniko, M.; Matsuda, A.; Koizumi, S.; Matsuya, K.; Mizumoto, H.; Ikota, A.; Kunita, H. Treatment of hyperthyroidism with a small single daily dose of methimazole: a prospective long-term follow-up study. *Endocr. J.* **1997**, *44*, 553−558.
(51) He, C. T.; Hsieh, A. T.; Pei, D.; Hung, Y. J.; Wu, L. Y.; Yang, T. C.; Lian, W. C.; Huang, W. S.; Kuo, S. W. Comparison of single daily dose of methimazole and propylthiouracil in the treatment of Graves' hyperthyroidism. *Clin. Endocrinol.* **2004**, *60*, 676−681.
(52) Singh, G.; Correa, R. Methimazole. In *StatPearls*; StatPearls: Treasure Island (FL), 2023.
(53) National Research Council. *Review of the U.S. Army's Health Risk Assessments for Oral Exposure to Six Chemical-Warfare Agents*; National Research Council: Washington (DC), 1999.
(54) Review of the U.S. Army's health risk assessments for oral exposure to six chemical-warfare agents. Introduction. *J. Toxicol. Environ. Health, Part A*, **2000**. *59*(), 281-526.
(55) Papirmeister, B. The sulfur mustard injury: description of lesions and resulting incapacitation. In *Medical Defense Against Mustard Gas: Toxic Mechanisms and Pharmacological Implications*; Papirmeister, B.; Feister, A. J.; Robinson, S. I.; Ford, R. D., Eds.; CRC Press: Boca Raton, Florida; 1991, pp 16−17.

https://doi.org/10.1021/acsbiomedchemau.2c00087
ACS Bio Med Chem Au 2023, 3, 448−460