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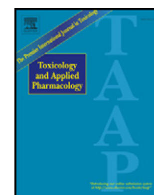

Protection against 2-chloroethyl ethyl sulfide (CEES) — induced cytotoxicity in human keratinocytes by an inducer of the glutathione detoxification pathway

Erika L. Abel [a], Jennifer D. Bubel [a], Melissa S. Simper [a], Leslie Powell [a], S. Alex McClellan [a], Michael Andreeff [b], Michael C. MacLeod [a], John DiGiovanni [a,*]

[a] Department of Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center, Smithville, TX 78957, USA
[b] Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

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ABSTRACT

Sulfur mustard (SM or mustard gas) was first used as a chemical warfare agent almost 100 years ago. Due to its toxic effects on the eyes, lungs, and skin, and the relative ease with which it may be synthesized, mustard gas remains a potential chemical threat to the present day. SM exposed skin develops fluid filled bullae resulting from potent cytotoxicity of cells lining the basement membrane of the epidermis. Currently, there are no antidotes for SM exposure; therefore, chemopreventive measures for first responders following an SM attack are needed. Glutathione (GSH) is known to have a protective effect against SM toxicity, and detoxification of SM is believed to occur, in part, via GSH conjugation. Therefore, we screened 6 potential chemopreventive agents for ability to induce GSH synthesis and protect cultured human keratinocytes against the SM analog, 2-chloroethyl ethyl sulfide (CEES). Using NCTC2544 human keratinocytes, we found that both sulforaphane and methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me) stimulated nuclear localization of Nrf2 and induced expression of the GSH synthesis gene, GCLM. Additionally, we found that treatment with CDDO-Me elevated reduced GSH content of NCTC2544 cells and preserved their viability by ~3-fold following exposure to CEES. Our data also suggested that CDDO-Me may act additively with 2,6-dithiopurine (DTP), a nucleophilic scavenging agent, to increase the viability of keratinocytes exposed to CEES. These results suggest that CDDO-Me is a promising chemopreventive agent for SM toxicity in the skin.
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Introduction

Sulfur mustard (SM) is an extremely reactive bifunctional alkylating agent capable of inducing DNA damage and necrosis of epithelial cells of the lung, cornea and skin (reviewed in Balali-Mood and Hefazi, 2005; Shakarjian et al., 2010). Following cutaneous exposure to the aerosolized liquid, cytotoxicity occurs primarily in the basal layer of the epidermis resulting in epidermal–dermal separation and blistering (Smith et al., 1998; Kehe et al., 2009). For this reason SM is categorized as a vesicant. Recovery requires weeks to months, often with permanent changes in skin pigmentation (reviewed in Balali-Mood and Hefazi, 2005; Shakarjian et al., 2010). Due to these incapacitating effects as well as effects on vision and respiratory function following exposure, mustards have been used as warfare agents since World War I. More recent exposure incidents have occurred in the Iran–Iraq war (United Nations Security Council, 1984) and among fishermen who encountered sunken stockpiles of SM at sea (Wulf et al., 1985; Aasted et al., 1987). Currently, no clinically-validated antidotes are available. Because of the potential for use of SM as a chemical terrorism agent, there is renewed interest in developing effective chemopreventive and therapeutic measures.

SM undergoes intramolecular cyclization to form an electrophilic episulfonium ion, which can adduct cellular macromolecules such as DNA (reviewed in Balali-Mood and Hefazi, 2005). Analyses of urinary metabolites in rats and humans have suggested that SM (presumably in the cyclized form) either participates in a spontaneous reaction with nucleophilic glutathione (GSH) or is a substrate for GSH *S*-transferase (GST)-mediated metabolism (Black et al., 1992; Black and Read, 1995). The cysteinyl sulfur atom of GSH is predicted to provide electrons for a nucleophilic attack on the episulfonium ring, rendering the compound less reactive; therefore, GSH-conjugation may represent a major detoxification pathway for SM. In previous work, a spontaneous reaction between CEES and GSH was detected only when the pH was raised above the pK_a of the sulfhydryl moiety of GSH (Liu

Abbreviations: SM, bis-(2-chloroethyl)sulfide; CEES, 2-chloroethyl ethyl sulfide; DTP, 2,6-dithiopurine; GSH, glutathione; GST, glutathione S-transferase; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modifier subunit; DTT, dithiothreitol; CDNB, 1-chloro-2,4-dinitrobenzene; NEM, N-ethylmorpholine; NDA, naphthalene dicarboxaldehyde; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; SSA, sulfosalicylic acid; CDDO-Me, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate; CDDO-Im, 2-Cyano-3, 12-dioxooleana-1,9-dien-28-imidazolide.

* Corresponding author at: Division of Pharmacology & Toxicology, College of Pharmacy, The University of Texas at Austin, 1400 Barbara Jordan Blvd, Austin, Texas 78723, USA. Fax: +1 512 495 4947.
E-mail address: john.digiovanni@austin.utexas.edu (J. DiGiovanni).

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et al., 2010); however, GST-mediated conjugation of structurally-similar nitrogen mustards has been previously demonstrated and is predicted to occur for SM (Dulik et al., 1986; Bolton et al., 1991).

Exposure to SM or its monofunctional analog, CEES, has been shown to deplete GSH stores in vitro and in vivo (Vijayaraghavan et al., 1991; Gross et al., 1993; Atkins et al., 2000; Kumar et al., 2001; Han et al., 2004; Gautam and Vijayaraghavan, 2007). GSH depletion may inhibit further clearance of SM, and, importantly, elicit oxidative stress, lipid peroxidation and macromolecular damage (Pal et al., 2009; Laskin et al., 2010). In line with these ideas, supplementation with GSH or GSH analogs protected against CEES- or SM-induced cytotoxicity in a number of cell lines (Amir et al., 1998; Andrew and Lindsay, 1998; Lindsay and Hambrook, 1998; Han et al., 2004), whereas blocking GSH synthesis rendered cells more sensitive to mustard toxicity (Gross et al., 1993; Atkins et al., 2000). Recently, pre- and post-treatment with GSH was shown to increase the viability of keratinocytes exposed to CEES (Tewari-Singh et al., 2011). Taken together, these findings suggest that agents capable of elevating levels of GSH and/or GST for extended periods of time may inhibit the toxic effects of SM and serve as effective and long-lasting chemopreventive agents in skin.

GSH is a tripeptide antioxidant composed of glutamate, cysteine and glycine residues. The rate-limiting step in GSH synthesis is catalyzed by glutamate cysteine ligase (GCL) (reviewed in Franklin et al., 2009; Lu, 2009). GCL is composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM), which are encoded by separate genes in humans. GCLC catalyzes the reaction between glutamate and cysteine. GCLM regulates the activity of GCLC by lowering the K_m for glutamate and raising the K_i for GSH; therefore, GSH synthesis is more efficient in the presence of GCLM (Huang et al., 1993a; Huang et al., 1993b; Chen et al., 2005). Transcriptional regulation of GSTs and GSH synthesis enzymes can occur through activation of the transcription factor, Nrf2 (reviewed in Lu, 2009). Under basal conditions, Nrf2 complexes with Keap1 in the cytosol where it is targeted for ubiquitination and proteolysis (reviewed in Kensler et al., 2007). Upon stimulation by oxidative stress signals or electrophilic agents, Nrf2 is released from the Keap-1 mediated ubiquitination allowing for newly translated Nrf2 to translocate to the nucleus and elicit transcription of its target genes via binding to antioxidant response elements. A number of cancer chemopreventive agents have been shown to activate signaling through Nrf2 (Zhang and Hannink, 2003; Iida et al., 2004; Liby et al., 2005; Yates et al., 2009).

The goal of this study was to identify agents that could induce the GSH conjugation detoxification pathway and protect against CEES-induced cytotoxicity in human keratinocytes. Six potential chemopreventive agents were screened for their ability to induce expression of GSTs and/or elevate reduced GSH content in the human keratinocyte cell line, NCTC2544. Our studies suggest that the synthetic triterpenoid, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), can stimulate Nrf2 translocation to the nucleus, elevate GSH content, and provide protection against CEES-induced cytotoxicity.

Materials and methods

Chemicals

2-Chloroethylethyl sulfide (CEES) was obtained from Aldrich Chemicals (St. Louis, MO). Working stocks of CEES were prepared in ethanol at 200 or 600 mM and stored at −20 °C. Stocks were verified for alkylating ability just prior to use by a spectrophotometric assay (Liu et al., 2010). CEES is a toxic, vesicating agent, which may potentially damage DNA; therefore, CEES and CEES-containing samples were handled with gloves in a chemical fume hood. CEES containing solutions were decontaminated with bleach prior to disposal, and all items that came into contact with CEES were treated as solid biohazardous waste.

DTP was obtained and purified as previously described (Liu et al., 2010). CDDO-Me and 2-Cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) were synthesized as previously described (Samudio et al., 2005; Ling et al., 2007). *R*-Sulforaphane and oltipraz were purchased from LKT Laboratories (St. Paul, MN). Reduced GSH, ebselen, silibinin, 1-chloro-2,4-dinitrobenzene (CDNB), *N*-ethylmorpholine (NEM), naphthalene dicarboxaldehyde (NDA), and dithiothreitol (DTT) were obtained from Sigma-Aldrich (St. Louis, MO); all were used without further purification. All other chemicals were purchased from standard sources and were analytical reagent grade or higher.

Cells and cell culture

NCTC2544 cells were obtained from Interlab Cell Line Collection (Genoa, Italy) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 2 mM L-glutamine. The chemopreventive agents were delivered in dimethyl sulfoxide (DMSO) at either 0.5% or 0.1% final concentration in the media, depending upon the solubility of the compound. The medium for exposure to the scavenger, DTP, which is not readily soluble in water, phosphate buffered saline, or DMEM, was modified as previously described (Powell et al., 2010). In addition to the standard components of DMEM/10% FBS, the modified media (referred to as LM2) contained 0.0075 N NaOH and 0.0075 N HCl. DTP was dissolved at 10 mM in 0.1 M K_2HPO_4 and used to achieve a final DTP concentration of 1.5 mM in the modified media. Duplicate or triplicate plates (or wells) were analyzed for each treatment group.

MTT-based cell viability assay

To determine the maximum tolerated dose of the various chemopreventive agents in NCTC2544 cells, cells were plated in 96 well plates to achieve ~70% confluency at the time of agent application on the following day. The cells were treated with vehicle only (0.1% or 0.5% DMSO) or chemopreventive agent at a range of concentrations spanning at least one order of magnitude. Following a 24, 48 or 72 hour exposure, cell viability was assessed using the MTT-based Cell Titer 96 Non-radioactive Cell Proliferation Assay kit (Promega, Madison, WI) in a 96-well plate format according to the manufacturer's protocol. Absorbance values were normalized to the respective control values and expressed as percentage of cells viable at each time point. Initial experiments confirmed a lack of significant toxicity associated with either 0.1% or 0.5% DMSO in the media as compared to no treatment.

Generation of cellular lysates

To examine the effects of chemopreventive agent on expression of Nrf2, GSH synthesis enzymes, and GSTs in NCTC2544 cells, cells were plated and allowed to achieve approximately 70% confluency. Subsequently, the plates were refreshed with DMEM/10% FBS containing the chemopreventive agent at the appropriate concentration (or DMSO only). At the indicated time points, the cells were washed with PBS and harvested by scraping into 0.3 mL ice cold Homogenization Buffer (80 mM Tris, 0.2 mM DTT, 1 mM ethylenediaminetetraacetic acid solution (EDTA), pH 7.4). Cells were lysed by 5 s sonication using a Fisher Scientific 60 Sonic Dismembrator handheld sonicator. Cell lysates were spun at 14,000×*g* for 15 min at 4 °C in a tabletop centrifuge, and the supernatant fraction was collected and stored at −80 °C. Protein content of the supernatant samples was determined using a Bradford-based assay (Bio-Rad, Hercules, CA) in a 96 well format according to manufacturer's protocol. The lysates were

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