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2,6-Dithiopurine blocks toxicity and mutagenesis in human skin cells exposed to sulfur mustard analogs, 2-chloroethyl ethyl sulfide and 2-chloroethyl methyl sulfide

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Abstract

Sulfur mustard (bis-(2-chloroethyl)sulfide) is a well known chemical warfare agent that induces debilitating cutaneous toxicity in exposed individuals. It is also known to be carcinogenic and mutagenic due to its ability to damage DNA via electrophilic attack. We previously showed that a nucleophilic scavenger, 2,6-dithiopurine (DTP), reacts chemically with several electrophilic carcinogens, blocking DNA damage in vitro and in vivo and abolishing tumor formation in a two-stage mouse skin carcinogenesis model. To assess the potential of DTP as an antagonist of sulfur mustard, we have utilized monofunctional chemical analogs of sulfur mustard, 2-chloroethyl ethyl sulfide (CEES) and 2-chloroethyl methyl sulfide (CEMS), to induce toxicity and mutagenesis in a cell line, NCTC2544, derived from a human skin tumor. We show that DTP blocks cytotoxicity in CEMS- and CEES-treated cells when present at approximately equimolar concentration. A related thiopurine, 9-methyl-6-mercaptopurine, is similarly effective. Correlated with this, we find that DTP is transported into these cells, and that adducts between DTP and CEES are found intracellularly. Using a shuttle vector-based mutagenesis system, which allows enumeration of mutations induced in the skin cells by a blue/white colony screen, we find that DTP completely abolishes mutagenesis induced by CEMS and CEES in the human cells.

Introduction

Sulfur mustard (SM¹, bis(2-chloroethyl)-sulfide) is well-known as a chemical warfare agent that causes acute cutaneous toxicity, as well as ocular and pulmonary toxicity (1). Chemically, SM reacts via an electrophilic episulfonium intermediate (2-4) and directly damages DNA and other macromolecules. The major identified DNA adducts are N7-(2-hydroxyethylthioethyl)-guanine, N3-(2-hydroxyethylthioethyl)-adenine and a crosslinked product, di-(2-guanin-7-yl) ethyl sulfide, which accounts for only 10-20% of the total adducts (5,6). A monofunctional

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Supporting Information **Available:** Two Supplementary Figures are available free of charge via the Internet at <http://pubs.acs.org>.

¹Non-standard abbreviations used are: SM, bis-(2-chloroethyl)sulfide; CEMS, 2-chloroethyl methyl sulfide; CEES, 2-chloroethyl ethyl sulfide; DTP, 2,6-dithiopurine; MMP, 9-methyl-6-mercaptopurine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

analog of SM, 2-chloroethylethylsulfide (CEES) forms analogous N7-guanine and N3-adenine adducts, but does not form cross-links with DNA (7). *In vitro* studies utilizing cells that genetically lack the ability to repair these adducts have provided strong evidence that DNA damage is the major determinant of cytotoxicity due to these sulfur mustards. Both nucleotide excision repair and base excision repair have been implicated in the repair of the monoadducts (8,9), while repair of the crosslink also involves homologous recombination.

There is a general correlation between the ability of toxic agents to damage DNA, and their ability to induce mutations and cause cancer. Indeed, epidemiological studies of mustard gas workers in the UK found significantly elevated risk of cancer of the upper aerodigestive tract (10). Cancers of the larynx, pharynx and oral cavity were two-five times more common in the mustard gas exposed population than expected, and lung cancer was also significantly elevated. Similarly, in studies of former mustard gas workers in Japan, deaths from cancers of the respiratory tract were over 30-fold higher than expected (11).

It has long been appreciated that the unifying aspect of most chemical carcinogens is their ability to either act directly as electrophiles, or be metabolized to electrophilic intermediates. Thus, chemical strategies for scavenging electrophilic carcinogens may be expected to prevent the induction of DNA damage by the sulfur mustards and thereby block toxicity. One such successful strategy utilized thiopurines as nucleophilic trapping agents for the electrophilic ultimate carcinogen BPDE (12,13). Initial studies in CHO cells demonstrated that pretreatment with 6-mercaptopurine (6MP) could completely block the ability of BPDE to form covalent adducts in cells, with a corresponding reduction of BPDE-induced toxicity and mutation frequency (14). This was correlated with the intracellular formation of the expected adduct between 6MP and BPDE. Part of the reason for this exceptional activity is that thiopurines are substrates for the cellular purine transport system (15), allowing rapid accumulation of the scavenging agent intracellularly. However, 6MP is a cytotoxic anti-cancer agent; toxicity is due to its incorporation into DNA as a purine base. Other thiopurines, in particular 2,6-dithiopurine (DTP), are not converted into nucleotides in mammalian cells and therefore do not have this cytotoxic activity.

Chemically, DTP, thiopurinol, 9-methyl-6-mercaptopurine (MMP), 6-thioxanthine (6TX) and 2,6-dithiouric acid (DUA) were shown to react facily with BPDE and several other electrophilic carcinogens (13,16). These studies were extended in a mouse model of skin carcinogenesis in which the carcinogenic process was initiated with topical application of an initiating dose of BPDE to the shaved dorsal skin, followed by twice weekly application of the tumor-promoting agent TPA. This results in the formation of multiple papillomas per mouse over the course of ~20 weeks, and the ultimate conversion of a fraction of the lesions to squamous cell carcinomas. Topical application of DTP to the dorsal skin 15 min prior to BPDE treatment resulted in a dose-dependent reduction in both papilloma incidence and multiplicity, and in carcinoma incidence (17). The extent of reduction in tumor formation closely matched the reduction in the formation of BPDE-DNA adducts in the treated epidermis, with 90-95% reduction of all parameters at the higher dose of DTP.

We have recently found that DTP reacts facily with two monofunctional analogs of sulfur mustard, 2-chloroethyl ethyl sulfide (CEES), and 2-chloroethyl methyl sulfide (CEMS) (accompanying ms.). In those *in vitro* studies, DNA was not able to compete effectively with DTP for CEMS reaction. Since there is good reason to expect that preventing DNA damage should block both cytotoxicity and mutation induction, we hypothesized that DTP might provide protection from CEES- and CEMS-induced cytotoxicity in cells by scavenging the reactive toxicant before any cellular damage is produced. In the present study we show that DTP can block the cytotoxic and mutagenic effects of CEES and CEMS in human skin cells.

Experimental Procedures

Chemicals

CEMS and CEES (>97% purity) were obtained from Aldrich Chemicals (St. Louis, MO) and used as supplied. Working stocks were prepared in anhydrous ethanol (Shelton Scientific, Peosta, IA) at 200 mM and stored at -20° C. The integrity of the stock solutions (lack of hydrolysis) was verified before use by a spectrophotometric assay for reactivity with 6-mercaptopurine (accompanying ms.). Stocks with less than 90% of maximal reactivity were discarded.

DTP obtained from Aldrich Chemical Co. at a stated purity of >95% was found to be ~50-60 % pure, based on HPLC analysis. The major contaminant was removed by extraction with boiling H₂O, followed by lyophilization, yielding a brownish solid that gave a single peak on HPLC analysis. MMP was synthesized by Chemsyn Laboratories (Lenexa, KS) based on published methods (18,19). Stocks of 10 mM DTP were prepared in either 0.05 N NaOH or 0.1 M K₂HPO₄, and stored at -20° C until use. CEMS and CEES are toxic compounds with the potential to damage DNA and must be handled with caution. All solutions containing these chemicals were treated with bleach prior to disposal, and solid waste was treated as biohazardous. All cellular exposures were carried out in class IIB biological safety cabinets.

Cell culture and treatment

NCTC2544, a cell line derived from a human skin tumor, was obtained from Interlab Cell Line Collection (Genoa, Italy) and routinely grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). NCTC2544 cells are known to have inducible Phase I and Phase II detoxication activities (20,21) and have previously been used for studies of sulfur mustard toxicity (22). The medium used for exposure to the alkyl mustards was modified to accommodate relatively high concentrations of the potential scavenger, DTP, which is not readily soluble in water, phosphate buffered saline or DMEM. Initially, DTP was dissolved at 10 mM in 0.05 N NaOH. Labeling medium 1 (LM1) included, in addition to the standard components of DMEM/10% FBS, .0075 N NaOH and .0075 N HCl, and was used to achieve final concentrations of DTP up to 1.5 mM. We subsequently found that DTP could also be dissolved at 10 mM in 0.1 M K₂HPO₄. LM2 contained, in addition to the standard components of DMEM/10% FBS, 0.03 M K₂HPO₄, and was used to achieve final DTP concentrations up to 3.0 mM.

For cytotoxicity measurements, subconfluent cell cultures were treated for 5 min in the presence of 10% FBS with various doses of CEMS or CEES at a final ethanol concentration of 1.0%. To achieve this, the medium on cell cultures to be treated was replaced with either LM1 or LM2, and appropriate ethanolic stocks of CEMS or CEES were added directly (1:100 dilution) to the medium on the culture dishes. Preliminary studies (data not shown) indicated that no increase in toxicity was seen with either longer exposure periods or in the absence of FBS. Treated cells were rinsed with phosphate buffered saline, refed with DMEM/10% FBS and returned to the incubator. Viable cell numbers were determined either 48 or 72 h post-treatment by harvesting the cells with trypsin/EDTA and counting in a Coulter counter. Each treatment group comprised four independent cultures, and differences between treatment groups were analyzed for statistical significance using Student's t-test. Experiments were repeated three times.

DTP uptake, adduct formation

Cells growing in 60 mm dishes were exposed to various concentrations of DTP in LM1 for times ranging from one to 20 min. After rinsing with phosphate-buffered saline, cells were digested overnight at 37° with nuclear lysis buffer (Promega, Madison, WI) containing

proteinase K, under conditions utilized for the preparation of cellular DNA. To reduce viscosity due to released DNA, lysates were sheared by 5 passages through a 19 gauge needle. The amount of DTP taken up by the cells was determined by spectrophotometry of the lysate, utilizing the previously measured extinction coefficient of DTP, $\epsilon_{348} = 12,700$. The number of cells per dish was directly determined by cell count with a Coulter counter in companion dishes. To convert the measured uptake to intracellular concentration, a measured number of cells (between $6-8 \times 10^7$ in replicate experiments) were harvested, pelleted by centrifugation, and the volume of the pellet determined by visual comparison to identical centrifuge tubes containing known volumes of water. The conversion factor so obtained was $45 \pm 2 \mu\text{L}$ per 1×10^7 cells.

Mutagenesis assay

For mutagenesis assays, NCTC2544 cells were grown under the same conditions in 96-well plates. Twenty-four h after plating, the shuttle vector pSupFG1 (23) was transfected into the cells by GenePorter (Genlantis, San Diego, CA). Four h post-transfection, cells were treated with DTP at various concentrations in LM1 for 45 min, followed by treatment with various concentrations of CEMS for 5 min. For treatments with CEES, LM2 was used. Treated cultures were refed with DMEM/10% FBS and allowed to grow for 72 h. Shuttle vector DNA was isolated from the treated cultures by an alkaline lysis method (24). Purified plasmid DNA was used to transform *E. coli* MB7070 cells, which were then plated on X-gal containing plates. The *supF* gene in the plasmid suppresses an amber mutation in the *lacZ* gene of MB7070 cells, allowing the cells to metabolize X-gal to a blue product and therefore giving rise to a blue colony. If the *supF* gene is mutated during growth in the human cells, the amber codon is not suppressed and the colony is white. Nutrient media also contained isopropyl β -D-thiogalactopyranoside to induce the *lac* operon and ampicillin to select for bacteria that had taken up plasmid DNA. In each experiment, at least 75,000 colonies were counted, and all putative mutant colonies were picked and regrown on X-gal plates to verify the mutation. Mutation frequencies are calculated as mutant colonies/total colonies. Three to four independent experiments were performed at each dose. Plasmid DNA was purified from selected mutant colonies, and the *supF* gene was sequenced on an ABI 3730XL sequencer.

Results

Cytotoxicity of CEMS and CEES in NCTC2544 cells

Previous work has demonstrated the cytotoxicity of CEES in mammalian cells (25); apoptosis is the major death pathway observed (26). Exposure of NCTC2544 cells to increasing doses of either CEES (Figure 1, open circles) or CEMS (Figure 1, closed triangles) resulted in dose-dependent toxicity as determined by a decrease in viable cell number 72 h post-exposure. The approximate LC50's determined in this way were 0.75 mM for CEMS and 1.0 mM for CEES.

Uptake of DTP by NCTC2544 cells

DTP is thought to be actively transported into mammalian cells via the purine transport mechanism (15), and was previously found to be active intracellularly against DNA adduct formation by the carcinogen BPDE (17). NCTC2544 cells rapidly took up DTP as determined spectrophotometrically (Figure 2, solid diamonds). In a series of experiments in which cells were exposed for various periods of time to LM2 containing 2.6 mM DTP, the amount of DTP associated with the cells was found to be independent of the exposure time between one and twenty minutes of exposure (ANOVA, $p=0.682$). Under the conditions of this assay, uptake increased linearly with external concentration (Supplementary Figure S1). The amount of DTP taken up led to an apparent intracellular concentration of 4.3 mM, almost twice the concentration of the external medium, suggesting that an active transport process may have been responsible for the uptake.

Inhibition of CEMS- and CEES-induced cytotoxicity by DTP

To determine whether DTP may protect cells from mustard-induced toxicity, NCTC2544 cells were exposed for 5 min at room temperature to CEMS in LM1 at 0.75 and 1.5 mM (approximately one- and two- times the LC_{50}), in the presence or absence of 1.3 mM DTP. After 72 h, cells were harvested and counted. In the absence of CEMS treatment (ethanol controls), the number of cells per plate was not significantly different in cultures treated with or without DTP ($p > 0.05$, t-test). Thus, cell numbers were normalized to the mean of all ethanol controls to calculate the surviving fraction. Figure 3 shows results of a representative experiment. At both 0.75 and 1.5 mM CEMS, the surviving fraction was significantly higher in the presence of 1.3 mM DTP than in its absence ($p < .001$). Results from both CEMS doses and from three replicate experiments were averaged to calculate the protection ratio (number of cells in DTP-treated cultures / number of cells in control cultures), which was 2.15.

Similar experiments were conducted with CEES at 1.0 and 2.0 mM (approximately one- and two-times the LC_{50}). However, because of the higher concentration of electrophile, we increased the concentration of DTP to 3.0 mM in LM2. As can be seen in Figure 4, under these conditions DTP was also effective in protecting cells from cytotoxicity at both doses of CEES. Again, the surviving fraction was significantly higher in the presence of DTP than in its absence ($p < .001$); the overall protection ratio in three replicate experiments was 2.73. Thus, DTP appears to have broad protective effects against the cytotoxicity associated with sulfur mustard analog exposure. Similar protection from the toxic effects of CEES was also obtained with another reactive thiopurine, 9-methyl-6-mercaptopurine (Supplementary Figure S2).

The mechanism by which DTP is expected to block cytotoxicity of CEES is via scavenging the active electrophilic episulfonium ion, thus preventing its reaction with critical cellular macromolecules. Consistent with this mechanism, we have shown facile reaction of DTP with CEES in aqueous solution by combined application of spectrophotometric, HPLC and mass spectrometric methods (accompanying ms.). The chemical reaction of CEES with one or both thiols of DTP results in quantifiable shifts in the absorbance spectrum of DTP. In particular, the local absorbance maximum shifts from 348 nm for DTP (Figure 5C, dotted line) to about 310 nm for the monoadduct formed with CEMS (Figure 5C, dashed line) or with CEES (Figure 5C, solid line). As shown in Figure 5, after treatment of NCTC2544 cells with LM2 containing 2.6 mM DTP and 2.0 mM CEES, a similar shift in the absorbance spectrum of DTP (dotted line) in the medium (panel A, solid line) and in the cell lysate (panel B, solid line), consistent with adduct formation, is easily demonstrable. Indeed, comparison of the relative strengths of the absorbance signals at 310-320 compared to 348 nm suggests that the putative DTP-CEES adducts are preferentially found intracellularly. Analogous experiments with CEMS and DTP gave comparable results (Figure 5, dashed lines).

Inhibition of CEMS- and CEES-induced mutagenesis by DTP

One of the major determinants of carcinogen-induced toxicity in cell culture is DNA damage. Thus, under conditions where CEMS or CEES induce cytotoxicity it seems likely that cellular mutation rates are also increased. We therefore wished to determine whether DTP could be effective in blocking CEES- or CEMS-induced mutagenesis. For these studies we used a shuttle vector that could be introduced into NCTC2544 cells, treated with the toxic agents, and recovered after a period of growth and repair (Figure 6A). The shuttle vector, pSupFG1 (23), contains the bacterial *supF* gene which, when transformed into an indicator strain of *E. coli* and plated on X-gal medium, suppresses an amber mutation in the *lacZ* gene and gives rise to a blue colony. Mutations in the *supF* gene that result from treatment and growth in the human cells lead to white colonies. Determining the fraction of white colonies in such an experiment gives a measure of the mutation frequency in the mammalian cells.

As described in Experimental Procedures, we utilized this assay in NCTC2544 cells treated with either CEMS or CEES or mock-treated with vehicle only (1.0% ethanol). The background mutation frequency in the vehicle controls varied between 0.44×10^{-4} in the CEMS experiments and 1.05×10^{-4} in the CEES experiments; this is within the range expected for background mutation frequencies in mammalian cells using this system. Treatment of cells with 1.3 mM DTP did not significantly increase the mutation frequency above background levels (analysis of odds ratios, $p=0.8957$). However, treatment with 1.2 mM CEMS resulted in an increase in the mutation frequency to 12.6×10^{-4} (Figure 6B, gray bars); this difference is statistically significant (Chi-squared analysis of odds ratios, $p<0.00005$). Intermediate concentrations of CEMS gave intermediate values for the mutation frequency, and analysis of the trend in the odds ratios indicated that this trend was significant ($p<0.00005$, Chi-squared trend test).

To determine whether DTP can block the increase in mutation frequency induced by CEMS, cells were pretreated with 1.3 mM DTP, and then exposed to 1.2 mM CEMS (Figure 6B, white bars). The CEMS-induced mutation frequency dropped from 12.6×10^{-4} to 1.61×10^{-4} ; this mutation frequency was not significantly different than the background mutation frequency in the absence of CEMS ($p=.9114$). Pre-treatment with intermediate concentrations of DTP lowered the mutation frequency induced by 1.2 mM CEMS by intermediate amounts; this trend was significant ($p<0.00005$, Chi-squared trend test).

Analogous experiments were carried out with CEES as the mutation-inducing agent (Figure 6C), with very similar results. The induced mutation frequency at this concentration of CEES was 8.88×10^{-4} , almost nine-times the background mutation frequency; this was statistically significant ($p<0.00005$). Pre-treatment with DTP reduced this to 1.72×10^{-4} , which was not significantly different than background ($p=0.5629$). We conclude that DTP effectively abolishes CEMS-induced and CEES-induced mutation at a molar ratio slightly greater than 1.

In these experiments, all mutations were verified by replating individual colonies to assure that the white (mutant) phenotype was maintained. To determine the nature of the induced mutations, DNA was purified from selected colonies and the region corresponding to the *supF* gene was sequenced. All mutant colonies analyzed ($n=55$) were found to contain sequence alterations affecting the *supF* target gene. Overall, 44% of the mutations identified by sequencing were single-base changes, 38% were short (≤ 4 base pairs) deletions or insertions, and the rest were longer and/or more complex rearrangements. Most deletions and insertions were in runs of G-C base pairs. Of the single base changes, about 47% occurred at G-C base pairs, and 53 % at A-T base pairs.

Discussion

The present results demonstrate partial protection from the cytotoxic effects of CEES and CEMS by DTP in NCTC2544 cells, derived from a human skin tumor. Significant protection levels of 2-3-fold were seen with concentrations of both nucleophile and electrophile in the 1-3 mM range, and a second nucleophilic thiopurine, MMP, was found to be similarly active. The best protection was seen when DTP was added to the medium shortly before the cells were exposed to the mustard. Measurements of intracellular DTP indicated rapid uptake, maximal by 2 minutes of exposure, and intracellular concentrations well in excess of the extracellular concentration were seen (Figure 2). Spectrophotometric analysis was consistent with the formation of adducts between DTP and CEES, demonstrable both in the extracellular medium and inside the cells, consistent with the hypothesized mechanism of action of DTP as a scavenger. Significantly, maximal protection was attained at close to equimolar ratios of nucleophile to electrophile. In contrast, previous reports of protection from SM-induced cytotoxicity by glutathione derivatives (27) and alkylamines (28) required nucleophile to electrophile ratios greater than 10:1.

Mustards have long been known to be mutagenic, and are suspected carcinogens. The ability of DTP to block mutagenesis induced by CEES and CEMS (Figure 6) was even more dramatic than the effect on cytotoxicity. Both electrophiles induced about 10-fold increases in the mutation frequency in a shuttle vector replicating in NCTC2544 cells, as detected by a blue/white colony assay in bacteria. This increase in mutation frequency was abolished by approximately equimolar DTP treatment for both CEMS and CEES. Lower doses of DTP gave graded decreases in mutation frequency induction by CEMS. Treatment of purified DNA with CEES has been reported to produce the N7-guanine and N3-adenine adducts in approximately a 6:1 ratio (29). DNA isolated from human cells treated for 3 h with SM has an even lower amount of the N3-adenine adduct (5). Thus, our finding of approximately a 1:1 ratio of mutations at G:C and A:T base pairs suggests that either the two major monoadducts have different intrinsic mutagenicity, or that the adducts are repaired at different rates and/or with different fidelity.

These results suggest that purinethiols may prove useful in protecting humans from the short-term toxicity of sulfur mustards. In a hypothetical terrorist incident against a civilian population using sulfur mustard, it might be several hours before the nature of the toxic agent became apparent. The clearest application would be to use purinethiols as a topically applied “antidote” to protect first responders, medical personnel and decontamination personnel from accidental exposure at the scene or through contact with exposed patients. However, data from animal studies and human case reports strongly suggest that sulfur mustard has an unexpectedly long biological half-life, possibly measured in days (30-32). Thus, it is also possible that purinethiols will prove to be therapeutic against skin toxicity if provided to exposed individuals at some time post-exposure. Furthermore, in such an incident a large number of individuals could receive lower doses of toxicant such that short-term toxicity was not apparent, but that significant mutation induction occurred and could lead to longer-term effects. The present results suggest the possibility that purinethiols might block the induction of mutations in these individuals.

These results in NCTC2544 skin cells confirm that DTP, MMP and presumably other reactive thiopurines, can protect biological systems from the toxic consequences of exposure to mustards. Similar preliminary results have been obtained in A549 lung cells treated with CEES², suggesting that this is a general phenomenon for mammalian cells in culture. However, SM is a bifunctional mustard, and may be more toxic than the monofunctional analogs. The presence of two moles of reactive 2-chloroethyl- per mole of compound may in essence double the effective concentration of the toxicant. In addition, although the majority of the DNA adducts are similar, SM produces DNA cross-links that may be more difficult to repair than the monoadducts. Thus, it remains to be seen whether DTP or MMP may provide *in vivo* protection from the toxic and/or mutagenic effects of either CEES or SM in exposed mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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²Unpublished data.

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Figure 1. Cytotoxicity of CEES and CEMS in NCTC2544 cells

NCTC2544 were exposed to increasing concentrations of CEES (open circles) or CEMS (solid triangles) as described in Materials and Methods. Cytotoxicity was determined 72 h later by counting viable cells, and normalizing the cell number in treated cultures to the cell number in ethanol-treated controls. In this and all Figures, error bars indicate the standard deviation of the mean; if no error bars are visible, the standard deviation was smaller than the size of the data point.

Figure 2. Uptake of DTP by NCTC2544 cells

Cells were exposed for varying periods of time to 2.6 mM DTP, then rinsed with PBS and lysed as described in Experimental Procedures. The amount of DTP present in the lysate was determined spectrophotometrically, and converted to intracellular concentration (solid triangles) by dividing by the volume expected for the number of cells present in the culture; this was determined in replicate cultures. Extracellular concentrations were also determined (solid circles). The difference between the extracellular and intracellular concentrations of DTP was statistically significant (t-test, $p < 1 \times 10^{-6}$).

Figure 3. Protection from CEMS-toxicity by DTP

Cells were exposed to 0, 0.75 or 1.5 mM CEMS in the presence (white bars) or absence (gray bars) of 1.3 mM DTP, and cytotoxicity was determined as in Figure 1. Asterisks indicate significant differences between control and DTP treatments (t-test, $p < 0.05$).

Figure 4. Protection from CEES-toxicity by DTP

Cells were exposed to 0, 1.0 or 2.0 mM CEES in the presence (white bars) or absence (gray bars) of 3.0 mM DTP, and cytotoxicity was determined as in Figure 1. Asterisks indicate significant differences between control and DTP treatments (t-test, $p < 0.05$).

Figure 5. Apparent formation of mustard-DTP adducts in cells

Cells were treated with DTP (dotted lines), DTP + CEES (solid lines), or DTP + CEMS (dashed lines) for 5 min. **A.** The spent culture medium was collected and assayed for DTP and/or adducts spectrophotometrically. **B.** Cells were rinsed, then lysed and assayed for DTP and/or adducts spectrophotometrically. Absorbance spectra shown are the means of three independent cultures, with the background absorbance from untreated cells subtracted. **C.** DTP (2 mM) was allowed to react to completion with either CEMS or CEES (2 mM), the reaction mixture was acidified and the precipitate was collected by centrifugation. The precipitated monoadduct was redissolved in dilute base, diluted 40-fold in 12.5 mM PB, pH 7.5, 25 mM NaCl and UV

absorbance spectra were collected. Dashed line, CEMS monoadduct; solid line, CEES monoadduct; dotted line, DTP only.

Figure 6. Protection from CEMS- and CEES-induced mutagenesis

A. Diagram of the mutagenesis assay used. **B.** Mutation frequencies induced by increasing concentrations of CEMS in the presence (white bars) or absence (gray bars) of 1.3 mM DTP. **C.** Mutation frequencies induced by CEES in the presence (white bars) or absence (gray bars) of 1.3 mM DTP. Asterisks indicate values that are significantly different than background. Error bars represent the standard deviation of mutation frequency measured in three-four independent experiments.