

## Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host cell reactivation assay

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**DNA damage is thought to be the initial event that causes sulfur mustard (SM) toxicity, while the ability of cells to repair this damage is thought to provide a degree of natural protection. To investigate the repair process, we have damaged plasmids containing the firefly luciferase gene with either SM or its monofunctional analog, 2-chloroethyl ethyl sulfide (CEES). Damaged plasmids were transfected into wild-type and nucleotide excision repair (NER) deficient Chinese hamster ovary cells; these cells were also transfected with a second reporter plasmid containing *Renilla* luciferase as an internal control on the efficiency of transfection. Transfected cells were incubated at 37°C for 27 h and then both firefly and *Renilla* luciferase intensities were measured on the same samples with the dual luciferase reporter assay. Bioluminescence in lysates from cells transfected with damaged plasmid, expressed as a percentage of the bioluminescence from cells transfected with undamaged plasmid, is increased by host cell repair activity. The results show that NER-competent cells have a higher reactivation capacity than NER-deficient cells for plasmids damaged by either SM or CEES. Significantly, NER-competent cells are also more resistant to the toxic effects of SM and CEES, indicating that NER is not only proficient in repairing DNA damage caused by either agent but also in decreasing their toxicity. This host cell repair assay can now be used to determine what other cellular mechanisms protect cells from mustard toxicity and under what conditions these mechanisms are most effective.**

### Introduction

Sulfur mustard, bis-(2-chloroethyl) sulfide (SM), is a bifunctional alkylating agent that has cytotoxic, mutagenic and vesicant properties, and is considered carcinogenic by the IARC (1). Sulfur mustard interacts with cellular DNA to form the cross-link, di-(2-guanin-7-yl-ethyl)-sulfide, and two monoadducts, 7-(2-hydroxyethylthioethyl) guanine (HETEG) and 3-(2-hydroxyethylthioethyl) adenine (HETEA) (reviewed in ref. 2). DNA modification by SM has been shown to interfere with replication and transcription and is probably responsible for its various toxicities (3–5).

Since the resistance of *Escherichia coli* cells to the lethal effect of SM correlates with their ability to remove cross-links, it has generally been assumed that the formation of DNA cross-links is a major cause of SM toxicity (3,6).

**Abbreviations:** CEES, 2-chloroethyl ethyl sulfide; HETEA, 3-(2-hydroxyethylthioethyl) adenine; HETEG, 7-(2-hydroxyethylthioethyl) guanine; NER, nucleotide excision repair; SM, sulfur mustard, bis-(2-chloroethyl) sulfide.

However, the toxicity and vesicating properties of monofunctional derivatives of sulfur mustard, such as 2-chloroethyl ethyl sulfide (CEES), which generate similar monoadducts but do not form cross-links (2), suggest that monoadducts also contribute to the biological effects of sulfur mustard.

Previously, the cellular repair of SM-damaged DNA has been demonstrated either by measuring the disappearance of alkyl groups from DNA (7,8) or by monitoring the occurrence of non-semiconservative DNA synthesis ('repair synthesis') in cells exposed to sulfur mustard (9). The removal of DNA cross-links specifically has been demonstrated by several investigators in both *E.coli* and mammalian cells (3,8,10–12).

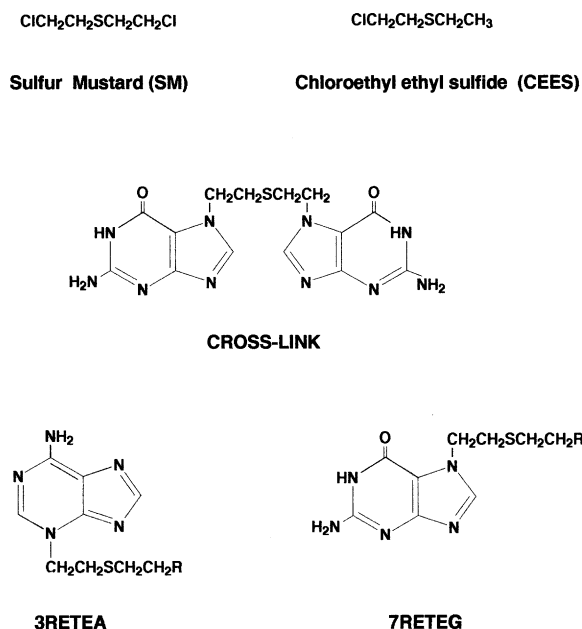
However, the specific cellular repair pathways and enzymes that act on SM-induced DNA adducts have not been completely established. *In vitro* studies have shown that bacterial 3-alkyl adenine DNA glycosylase II releases both of the SM monoadducts, 7HETEG and 3HETEA, from SM-modified DNA indicating that base excision repair may play a role in repairing sulfur mustard lesions (13). The involvement of another repair pathway in eukaryotic organisms, nucleotide excision repair (NER), has been suggested by the study of Kircher *et al.* (14) who have shown that yeast mutants deficient in nucleotide excision repair are much more sensitive to sulfur mustard than wild-type cells.

Although the biochemical studies mentioned above indicate that DNA repair processes act on SM-modified DNA, they do not demonstrate whether or not the damaged DNA has been restored to a functional state. To address this issue, other investigators have used a variety of host cell reactivation assays to demonstrate functional repair of DNA after damage by antitumor agents (15–18). In this manuscript, we describe the use of a dual luciferase reporter assay to demonstrate cellular repair of mustard-induced DNA damage.

We have first established that NER-competent Chinese hamster ovary cells are more able to withstand the toxic effects of SM than are NER-deficient cells. Then, using the dual luciferase host cell reactivation assay, we have shown that NER-competent cells are able to repair SM-damaged reporter plasmid and bring luciferase expression from the plasmid to higher levels than in NER-deficient cells, thus correlating DNA repair with SM toxicity.

We have also used the host cell reactivation assay to examine cellular repair of the damage caused by the single armed mustard, CEES. As shown in Figure 1, CEES forms monoadducts that are very similar to those formed by SM, thus allowing the effects of monoadducts on survival and repair to be examined independently from the effects of cross-links. These studies have shown that the toxicity of CEES is also decreased in NER-competent cells in comparison with NER-deficient cells. Luciferase expression from plasmid damaged by CEES is enhanced in NER-competent cells compared with NER-deficient cells, indicating that the monofunctional mustard adducts formed by CEES are cytotoxic and are also substrates for NER. From these results, we conclude that this

## SULFUR MUSTARDS and their DNA ADDUCTS



**Fig. 1.** Structures of SM and CEES and the adducts they form with DNA. Sulfur mustard forms the indicated cross-link and two monoadducts with R = OH; CEES forms only monoadducts with R = H.

relatively simple host cell reactivation assay can be used to determine what repair mechanisms restore mustard-damaged DNA to a functional state and under what conditions they are most effective.

## Materials and methods

## Materials

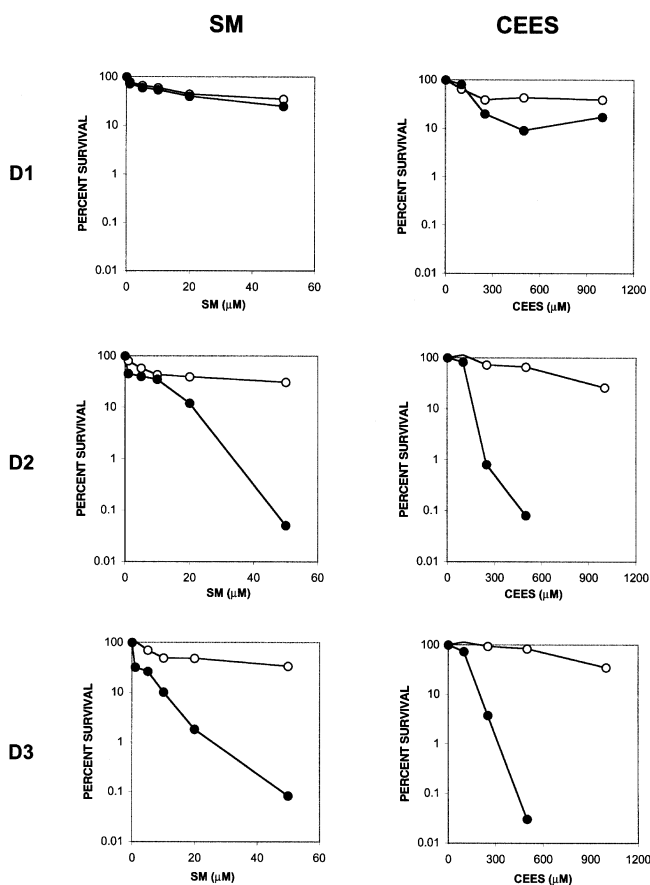
Bis-(2-chloroethyl)sulfide (SM) was supplied by the US Army Institute of Chemical Defense (Aberdeen Proving Ground, MD) and 2-chloroethyl ethyl sulfide (CEES) was purchased from Aldrich (Milwaukee, WI). The luciferase reporter vectors pGL3-Control (containing the firefly luciferase gene) and pRL-TK (containing the *Renilla* luciferase gene), the transfection reagent TransFast and the Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). Plasmid vectors were propagated in *Escherichia coli* strain JM109 and plasmid DNAs were purified by using the EndoFree Plasmid Mega kit (Qiagen, Chatsworth, CA). Chinese hamster ovary (CHO) cell lines AA8 (wild type) and UV41 (NER group 4) were obtained from the American Type Culture Collection. These cells were grown at 37°C in monolayer culture in  $\alpha$ -modified minimum essential medium supplemented with 10% fetal bovine serum and antibiotics.

## Sulfur mustard exposure and cytotoxicity determinations

Cells were plated in 12-well plates at a density of  $2 \times 10^4$  cells/cm<sup>2</sup>. After 24 h, the medium was replaced with fresh medium containing the indicated concentrations of SM or CEES; dilute solutions of these compounds in absolute alcohol were prepared immediately before treatment. Cells were exposed to SM or CEES for 1 h at room temperature in a SterilchemGard hood and then incubated in fresh medium at 37°C. At the indicated times, cell viability was determined by the trypan blue exclusion assay.

## Alkylation of plasmid DNA

Purified pGL3 DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at a concentration of 1  $\mu$ g/ $\mu$ l and incubated with SM or CEES in a SterilchemGard hood at room temperature for 1 h. DNA was precipitated with ethanol, dissolved in TE buffer and stored at -20°C until used for transfection. Aliquots were analyzed by gel electrophoresis on 1% agarose gel. The percent conversion of supercoiled DNA (Form I) into the nicked circular form (Form II) after exposure to SM or CEES was determined



**Fig. 2.** Survival of CHO cells after exposure to SM or CEES. Viable cell numbers were determined by trypan blue exclusion and are expressed as a percent of control on day 1 (D1), day 2 (D2) and day 3 (D3). Wild-type cells (○); NER-deficient cells (●).

densitometrically from a UV photograph of the ethidium bromide-stained agarose gel.

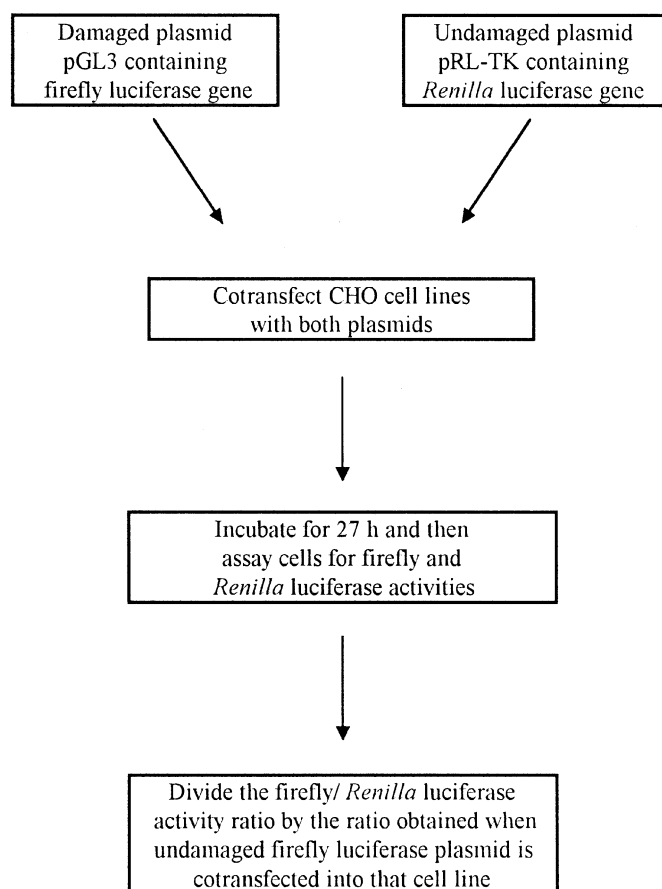
## Transfection conditions

For transfection experiments, cells were plated in 24-well plates at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> and incubated for 24 h at 37°C. Transfection was performed using the liposome-based transfection reagent, TransFast. Optimal conditions for transfection were established as 0.5  $\mu$ g plasmid DNA per well, at a charge ratio of transfection reagent to DNA of 1:1, and a ratio of pGL3 to pRL-TK of 10:1. Twenty-four hours after plating, the growth medium was replaced with 0.2 ml serum-free medium containing the transfection mixture. After the cells were incubated for 1.5 h at 37°C, the transfection reagent was diluted by the addition of 1 ml complete medium and cells were further incubated for luciferase expression.

Preliminary experiments with undamaged pGL3 and pRL-TK plasmids have shown that the difference in genetic background between the AA8 and UV41 cells does not affect the kinetics of expression of either firefly or *Renilla* luciferase. The level of expression of both luciferases increases during the first 25 h of incubation at 37°C and then reaches a plateau. Therefore in repair experiments, cells were incubated for 27 h after transfection to allow a maximal level of luciferase expression. At that time, cells were approximately 90% confluent.

## Cell lysis and assay for luciferase activity

Cells were lysed in multi-well plates with 150  $\mu$ l/well passive lysis buffer provided with the Dual Luciferase Reporter Assay kit; lysates were stored frozen at -20°C until they were assayed. The assays for firefly luciferase activity and *Renilla* luciferase activity were performed sequentially in one reaction tube using 20  $\mu$ l aliquots of cell lysates. The supplier's standard protocol for the dual luciferase assay was followed. The luminescent signal from the luciferase reaction was monitored by a single-sample luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, CA) with spectral sensitivity over the range 360–620 nm. The values shown are means  $\pm$  SD from three to five separate assays.



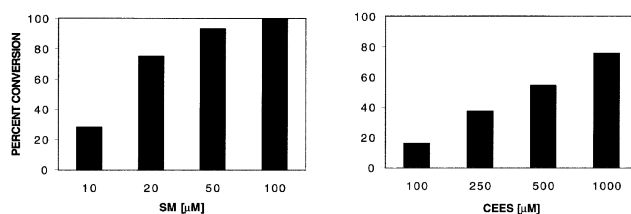
**Fig 3.** Scheme for monitoring DNA repair using damaged luciferase reporter gene in CHO cells. The level of firefly luciferase activity relative to *Renilla* luciferase activity is a measure of how efficiently the damaged firefly luciferase gene has been repaired.

## Results

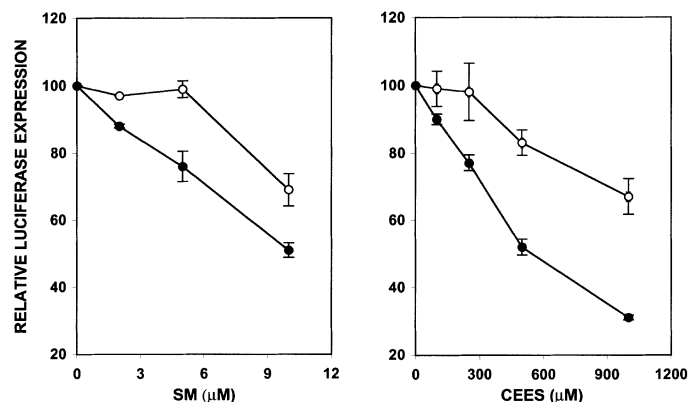
The data in Figure 2 show that CHO cells exposed to either SM or CEES are protected from cytotoxicity by the NER mechanism. Referring to data on day 3 (D3), >50% of NER-competent cells survive exposure to 20  $\mu$ M SM while fewer than 2% of the NER-deficient cells survive. A similar difference is noted after exposure to CEES except that concentrations of CEES >10-fold greater than those of SM are required to produce the same level of cytotoxicity. Again at day 3, survival for wild-type cells is close to 100% after exposure to 300  $\mu$ M CEES, but only ~5% for NER-deficient cells.

Based on the assumption that unrepaired DNA damage is responsible for SM cytotoxicity, we would expect that the increased survival shown in Figure 2 would be accompanied by an increase in cellular repair of DNA. This has been confirmed by measuring cellular DNA repair directly with the host cell repair assay as described below. In this assay, a plasmid that contains a gene for firefly luciferase is damaged by SM or CEES and then transfected into the cells that are to be evaluated for repair. Cells that can repair the DNA damage will express the luciferase gene at a higher level (i.e. the cells will 'reactivate' the gene). This results in an increased intensity of firefly luciferase bioluminescence that can be used to determine the extent of repair.

To compensate for variations in the efficiency of transfection and other experimental variables, luciferase reporter gene assays are usually run using dual transfection with DNA from



**Fig 4.** DNA damage caused by SM and CEES. The percent conversion of the firefly luciferase plasmid to Form II is plotted versus the concentration of mustard to which it was exposed.



**Fig 5.** Relative luciferase expression in wild type (○) and NER-deficient cells (●); values are means  $\pm$  SD from three to five separate assays. See text for details.

two plasmids, one containing the damaged firefly luciferase gene and the other (pRL) containing an undamaged *Renilla* luciferase gene. The intensity of firefly luciferase enzyme activity can then be compared with the level of *Renilla* luciferase activity as a control. This protocol is shown in Figure 3.

The firefly luciferase gene was damaged as described in the Materials and methods by exposing plasmid pGL3 to either SM or CEES *in vitro*. Damaged plasmid was then recovered and used in the host cell reactivation assays as shown in Figure 3.

Damage to plasmid DNA can be detected by measuring the conversion of the supercoiled form of the plasmid (Form I) to the nicked circular form (Form II). We assume that the extent of DNA damage that causes this conversion parallels the DNA damage that interferes with expression of the luciferase gene. Accordingly, we have measured conversion of the plasmid to Form II as an indication of the DNA damage caused to the firefly luciferase gene by SM and CEES. The level of conversion of Form I to Form II is shown in Figure 4. As expected, both SM and CEES convert Form I to Form II, but it takes an ~10-fold higher concentration of CEES to cause as much damage as is caused by a given concentration of SM.

The host cell reactivation data in Figure 5 show the extent to which this damage was repaired in the two different cell lines. In these experiments, plasmid containing damaged firefly luciferase gene was transfected separately into either wild-type or NER-deficient CHO cells. As shown in Figure 5, wild-type cells can return luciferase expression to normal if the plasmids are damaged with low concentrations of SM or CEES. In contrast, NER-deficient cells show much less repair, and levels of firefly luciferase expression fall off as the concentrations of SM or CEES are raised.

It is possible that some of the damage to the luciferase gene

could be repaired by mechanisms other than NER in the NER-deficient cells, but the lack of a shoulder on the curve for NER-deficient cells at low concentrations of damaging agent suggests that NER is needed to restore full expression. In any case, the data in Figure 5 indicate that the host cell reactivation assay can be used to examine repair of SM- or CEES-induced DNA damage within the cell. It is also apparent from the right hand panel of Figure 5 that NER removes the monofunctional adducts caused by CEES and, by analogy, the monofunctional adducts caused by SM as well.

## Discussion

The survival curves in Figure 2 show that the difunctional agent SM is ~10-fold more cytotoxic than CEES for both cell lines, in agreement with previous observations (2). Since CEES forms monofunctional adducts that are similar to those formed by SM, this difference in cytotoxicity between SM and CEES has been assumed to be the result of cross-link formation (2). The data (Figure 2, left panel) show that NER competent cells are more resistant to the cytotoxic action of SM than are NER deficient cells suggesting that the cytotoxic cross-link is removed by the NER repair mechanism. Again, this would agree with previous observations that the NER pathway recognizes bulky adducts like DNA cross-links.

Since the data (Figure 2, right panel) show that NER competent cells are also more resistant to the cytotoxic action of CEES, we can conclude that NER repairs the monoadducts formed by CEES and, presumably, the similar monoadducts formed by SM as well. These adducts, as well as the cross-link, would probably be classified as bulky because of the size of their adducted groups.

Host cell reactivation data provide direct information on cellular repair of DNA damage, and the data in Figure 5 support the role of the NER mechanism in protecting cells from mustard toxicity since firefly luciferase gene damaged by either SM or CEES is restored to a higher level of expression in the NER-competent CHO cells. This reinforces the belief that DNA repair mechanisms protect against SM toxicity.

Thus the host cell reactivation assay described in this manuscript demonstrates that there is a direct relationship between resistance to SM and CEES toxicity and the cellular repair of DNA damage caused by these agents. This not only validates the hypothesis that DNA damage is the root cause of mustard toxicity, but provides a method of testing environmental conditions such as hypothermia that may increase the extent of DNA repair.

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