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2,6-Dithiopurine, a Nucleophilic Scavenger, Protects Against Mutagenesis in Mouse Skin Treated *in vivo* with 2-(chloroethyl) ethyl sulfide, a Mustard Gas Analog

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Abstract

Sulfur mustard [bis(2-chloroethyl)sulfide, SM] is a well-known DNA-damaging agent that has been used in chemical warfare since World War I, and is a weapon that could potentially be used in a terrorist attack on a civilian population. Dermal exposure to high concentrations of SM produces severe, long-lasting burns. Topical exposure to high concentrations of 2-(chloroethyl) ethyl sulfide (CEES), a monofunctional analog of SM, also produces severe skin lesions in mice. Utilizing a genetically engineered mouse strain, Big Blue, that allows measurement of mutation frequencies in mouse tissues, we now show that topical treatment with much lower concentrations of CEES induces significant dose- and time-dependent increases in mutation frequency in mouse skin; the mutagenic exposures produce minimal toxicity as determined by standard histopathology and immunohistochemical analysis for cytokeratin 6 and the DNA-damage induced phosphorylation of histone H2AX (γ -H2AX). We attempted to develop a therapeutic that would inhibit the CEES-induced increase in mutation frequency in the skin. We **observe** that multi-dose, topical treatment with 2,6-dithiopurine (DTP), a known chemical scavenger of CEES, beginning 1 hour post-exposure to CEES, completely abolishes the CEES-induced increase in mutation frequency. These findings suggest the possibility that DTP, previously shown to be non-toxic in mice, may be useful as a therapeutic agent in accidental or malicious human exposures to SM.

Keywords

sulfur mustard; electrophile; carcinogen

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Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

Introduction

Sulfur mustard [bis(2-chloroethyl)sulfide, SM] has been used as a chemical warfare agent since WWI, and is known to be toxic to the skin, eyes and respiratory tract. In particular, dermal exposure leads to blistering and ulcerative lesions that resist treatment, and heal slowly and with extensive scarring (Papirmeister *et al.*, 1991). In several rodent models, topical SM treatment induces full-thickness burns that heal slowly (Vogt *et al.*, 1984; Casillas *et al.*, 1997; Smith *et al.*, 1997). Similar lesions are also induced in mice by treatment with the less potent half-mustard analog 2-(chloroethyl) ethyl sulfide (CEES) (Isidore *et al.*, 2007). In both humans and mice, lesions develop slowly and are not apparent for at least 12 hrs post-treatment.

Although both SM (Ogston *et al.*, 1946) and CEES (Liu *et al.*, 2010) hydrolyze readily in aqueous solution, several lines of evidence point to a more prolonged stability in biological systems. In an experimental model, macromolecular damage in response to mustard (globin adduct) has been shown to increase over a period of many days (Noort *et al.*, 2002). In an accidental SM exposure incident, excretion of hydrolysis products of SM was significantly above background 7 days post exposure (Barr *et al.*, 2008). The persistence of SM in skin tissue has been documented in a combat-related human exposure (Draschet *et al.*, 1987), and in experiments with cadaver skin (Hattersley *et al.*, 2008), leading to the concept that mustards survive in treated skin for extended periods of time in a “reservoir” where they are protected from aqueous hydrolysis and detoxification. If this long-lived, tissue reservoir of active mustard contributes to the toxic effects in skin, then it is conceivable that post-exposure application of treatments that detoxify this reservoir represents a window of therapeutic opportunity.

It is thought that direct damage to tissue macromolecules underlies the toxic effects of mustards. Indeed, both protein and nucleic acid adducts of mustards have been described. In particular, mustard-DNA adducts are highly mutagenic, and are likely responsible for the association of chronic mustard exposure with development of respiratory tract cancers seen in several epidemiological studies (Wada *et al.*, 1968; Easton *et al.*, 1988; Hu *et al.*, 2002). Low millimolar concentrations of CEES are mutagenic in human skin cell cultures (Powell *et al.*, 2010).

We have previously shown that the non-toxic thiopurine 2,6-dithiopurine (DTP) is biologically active in mouse skin as a scavenger for an electrophilic carcinogen, 7r,8t-dihydroxy-9,10t-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene, completely abolishing both DNA adduct formation and the initiation of carcinogenesis (MacLeod *et al.*, 1991). DTP reacts facilely with a wide variety of electrophilic compounds at neutral pH *in vitro* (MacLeod *et al.*, 1993; Qing *et al.*, 1996), including CEES and 2-(chloroethyl) methyl sulfide (Liu *et al.*, 2010). Competition experiments demonstrated that DTP was almost an order of magnitude more active in detoxifying CEES than common anti-oxidants such as glutathione and N-acetyl-cysteine (Liu *et al.*, 2010). Correlated with this activity, we found that DTP could effectively block both cytotoxicity and mutagenicity of CEES in human skin cell cultures (Powell *et al.*, 2010). These findings suggest the possibility that the scavenging activity of DTP may be effective in blocking the mutagenic effects of mustards *in vivo*. We now present evidence in a mouse skin model that moderate doses of CEES induce mutations *in vivo*, and that therapeutic treatment with DTP following CEES-exposure completely abolishes this mutagenic activity.

Materials and Methods

Animal treatments

Big Blue mice were obtained from Stratagene (La Jolla, CA), and a breeding colony, homozygous for the recombinant chromosome, was maintained in a temperature and humidity controlled, AAALAC-approved facility with a 12 hr light-dark cycle. C57BL/6 mice were obtained from the NIH. All experiments were carried out under protocols approved by the IACUC of The University of Texas MD Anderson Cancer Center. Initial experiments indicated that female and male animals responded similarly to topical treatment with CEES, and all further experiments were conducted with females, 8-10 weeks of age. Two-three days prior to treatment, mice were shaved dorsally with clippers. On the day of treatment, mice were anesthetized with ketamine. All treatments were performed in a class IIB biological safety cabinet, and the treated mice were held in disposable cages in the safety cabinet for the duration of the experiment. During treatment and for the first 24 h post-treatment laboratory personnel were further protected by the use of powered air-purifying respirators.

CEES (Aldrich Chemicals) was diluted just before use in anhydrous ethanol to concentrations in the range of 100-400 mM. Anesthetized mice were treated with 20 μ L of CEES stock, applied by micropipet to the shaved dorsum approximately 3.5 cm rostral to the base of the tail on the midline. The applied dose spread across the skin to form irregular regions that could be approximated as a circle with a diameter of $\sim$ 1.5 cm. The mice were held for approximately 25 sec, allowing the ethanol to be absorbed or evaporated, and then returned to disposable holding cages as above. For routine treatments, 200 mM CEES was used, resulting in an applied dose of 4.0 μ mol, at a dose density of $\sim$ 2.3 μ mol/cm². Control mice received ethanol only.

Mutagenesis measurements

The genome of Big Blue mice (on a C57BL/6 background) contains $\sim$ 100 copies of a modified lambda phage genome that includes a mutagenesis target, the *lacI* gene, that allows the scoring of mutations induced by mutagenic treatments *in vivo* (Kohler *et al.*, 1991). This is done by isolating genomic DNA from the appropriate target organ of the treated mice, in this case dorsal skin, excising the lambda genomes, packaging the DNA *in vitro* into lambda phage, and enumerating white (wild type) and blue (mutant) plaques after infection of a lawn of reporter bacteria in the presence of the beta-galactosidase substrate X-gal.

To determine mutation frequencies in the treated skin, mice treated as described above were sacrificed by CO₂ asphyxiation, the dorsal skin was dissected and trimmed to a small piece containing the treated area, and frozen in liquid nitrogen. Genomic DNA was purified from the frozen skin by an overnight digestion with Proteinase K and RNase A in the presence of 1% SDS, followed by phenol:chloroform extraction and ethanol precipitation. Phage genomic sequences were excised from purified genomic DNA and packaged into lambda phage utilizing Transpack Kits obtained from Stratagene (La Jolla, CA). The *lacI* mutant screen was performed as suggested by the manufacturer with minimal modifications. Indicator *E. coli* cultures were transduced with packaged phage from each skin sample, and plated to determine the transduction efficiency. For each skin tissue sample at least 18,000 pfu (generally $>70,000$ pfu) were plated at an average of 300-400 pfu/15 cm Petri dish and incubated overnight at 37°C. The plates containing the transduced *E. coli* were left at room temperature for a period of two days to allow detection of all mutants. Mutation frequencies were calculated as the number of total blue plaques divided by the overall pfu counted per skin sample.

Immunohistochemistry

All immunohistochemistries were performed by the Histology and Tissue Processing Facility Core of the Center for Research on Environmental Disease. Detection of cytokeratin 6 was performed as previously described (Wang *et al.*, 2007), using an antibody from Covance (Berkeley, CA) that does not cross-react with other epidermal cytokeratins. Anti-phospho γ H2AX antibody was obtained from Cell Signaling (Danvers, MA; #2577).

Therapeutic treatments with DTP

DTP was synthesized as described previously (Ozola and Mikstais, 1978) or repurified from a commercially available source as described (Liu *et al.*, 2010). Purity was at least 97% as determined by HPLC (Liu *et al.*, 2010). For topical application, a cream commonly used for formulation of pharmaceuticals for human use, Lipoderm (LD; PCCA USA, Houston, TX) was utilized. DTP was pre-wet with propylene glycol, and thoroughly mixed with LD in an ointment mill to obtain a final concentration of 20% DTP (wt/wt). Concentrations were verified by extracting DTP from a measured amount of the cream with 0.1 M K_2HPO_4 , and determining the amount of DTP extracted spectrophotometrically.

To determine the approximate lifetime of applied DTP in the skin of mice, 0.1cc of 20% DTP was applied to the shaved backs of C57BL/6 mice, and gently rubbed in for about 30 sec. Control mice received 0.1 cc of LD only. After various times, mice were sacrificed and the dorsal skin dissected. A dermatome was used to excise 4 samples of skin (0.4 cm diameter circles) from each mouse. Each sample was digested overnight at 37° C in 134 μ L of Nuclei Lysis Solution (Promega, Madison WI) containing 90 mM EDTA and 1.5 mg/mL proteinase K, then diluted with 1000 μ L 0.1 M K_2HPO_4 . After centrifugation at 12,000 rpm for 5 min, the clear supernate was carefully removed with a Pasteur pipet, and an aliquot diluted and assayed spectrophotometrically, utilizing the characteristic 348 nm absorption band of DTP (Qing *et al.*, 1995).

Statistics

In an experiment in which a Big Blue mouse is treated with a given concentration of a mutagen, the distribution of mutants between DNA molecules in the treated skin is expected to be random, and therefore, can be described by Poisson statistics. The probability that the mutation frequency observed at a particular dose is significantly different than the background frequency can then be calculated from the Poisson distribution.

In experiments where multiple mice were used in each experimental group, the significance of a difference between groups was evaluated by Student's t-test. In both cases, $p < 0.05$ was taken as the cutoff for statistical significance.

Results

Histopathology of CEES-induced lesions

When liquid CEES (~8.59 M) is applied without dilution to the dorsal skin of C57BL6 mice, severe lesions are obtained within **24 h, with severe necrosis** of the epidermal cells (Isidore *et al.*, 2007). By 3-4 days, no intact interfollicular epidermal cells can be observed, and the epidermis has been replaced by eschar tissue (data not shown). To test the effects of lower, potentially mutagenic doses, we diluted CEES to either 200 or 400 mM in EtOH and applied 20 μ L to the shaved dorsum of female C57BL6 mice; control mice received ethanol only. Mice were sacrificed three days later, and the dorsal skin prepared for histological analysis. Representative microscopic images of H&E-stained sections of the dorsal skin 3 days post-treatment are shown in Figure 1. At 200 mM CEES (Figure 1B) histological analysis revealed little or no change from control (Figure 1A). In particular, we did not observe

significant thickening of the epidermis, inflammation, or ulcerative lesions. In contrast, 400 mM CEES (Figure 1C) induced severe hyperplasia over the entire treated region, and focal lesions characterized by severe acute inflammation, the presence of thick eschar tissue and a breakdown of the integrity of the interfollicular epidermis (IFE). In some cases, frank ulcers were seen (Figure 1D).

The presence in nuclei of a specific, phosphorylated form of histone H2A, called γ -H2AX, is often used as a biomarker of DNA damage (Dickey *et al.*, 2009). The formation of γ -H2AX 1-3 days after treatment of mouse skin with SM has been reported (Joseph *et al.*, 2011). We therefore also examined skin sections for γ -H2AX by immunohistochemistry (IHC). In vehicle control-treated skin, strongly labeled, γ -H2AX positive nuclei were extremely rare in the epidermis ($\ll 1\%$ of the IFE cell nuclei). After treatment with 200 mM CEES the presence of nuclei stained positively for γ -H2AX in the IFE increased (Figure 2A), although the incidence was still only $\sim 1\text{--}2\%$ of the interfollicular nuclei. In the skin treated at 400 mM CEES, positive staining for γ -H2AX was widespread, with many positively stained nuclei seen in both the basal and suprabasal layers of the IFE (Figure 2B). At higher magnification, staining for γ -H2AX was clearly punctate as expected for DNA damage repair foci (Figure 2C; (Sedelnikova *et al.*, 2003)). Positive nuclei were also seen in the hair follicles and in fibroblast nuclei in the dermis and hypodermis (Figure 2D; arrows and arrowheads, respectively). Cytokeratin 6 (CK6) expression in IFE is often associated with hyperplasia and epidermal irritation (Kopan and Fuchs, 1989; Rothnagel *et al.*, 1999), whereas hair follicles normally express CK6. When examined for CK6 expression by IHC, most of the **interfollicular** epidermis in mice treated with 200 mM CEES was negative; normal staining of hair follicles was seen throughout. Occasional groups of keratinocytes exhibited cytoplasmic staining for CK6 (Figure 2E). No CK6 expression was observed in the interfollicular epidermis of control ethanol-treated mice (not shown). At 400 mM CEES, cytoplasmic expression of CK6 was ubiquitous in the treated, hyperplastic IFE (panel 2F). Thus, the extent of expression of these two markers was relatively modest in mice treated with 200 mM CEES, in agreement with the relative normality of the histopathology, and provided no evidence for severe DNA and tissue damage.

***In vivo* CEES-induced mutagenesis**

To optimize conditions for estimating induced mutation frequencies, we applied various concentrations of CEES to the shaved dorsal skin, and isolated genomic DNA from the treated skin 1 or 2 days following treatment. Preliminary measurements made after control treatment with ethanol only, led to an average background mutation frequency of 4×10^{-5} (range $2\text{--}6 \times 10^{-5}$). This is similar to previous background measurements in Big Blue skin (de Boer *et al.*, 1998). As shown in Figure 3, mutation frequencies measured 24 h after CEES-treatment were above background at doses of 300 and 400 mM CEES, but this increase was not statistically significant ($p > 0.05$, Poisson distribution). In contrast, mutation frequencies measured 48 h after treatment were significantly higher than background at all doses greater than or equal to 200 mM CEES, ranging from about $11\text{--}13 \times 10^{-5}$, an increase of about 3-fold above background (**$p < 0.005$, Poisson distribution**). This was higher than the maximal frequencies measured at 24 h, suggesting that 24 h is not sufficient time for maximal mutation induction in keratinocytes. The conversion of CEES-induced DNA damage to mutations is thought to involve trans-lesion synthesis and is known to require DNA replication and cell division (Provost *et al.*, 1993; Lange *et al.*, 2011). Indeed, further experiments **with a dose of 200 mM CEES** showed a higher mutation frequency at 4 days following treatment (data not shown), and the rest of the study was carried out using 200 mM CEES, with genomic DNA isolation 4 days following treatment.

Development of DTP therapeutic regimen

We wished to determine whether DTP, a known nucleophilic scavenger of CEES (Liu *et al.*, 2010), could modify mutagenicity in this model. For initial experiments, we chose to deliver DTP by topical treatment of the CEES-exposed skin with a commercial cream used for trans-dermal delivery of pharmaceuticals in humans. The delivery vehicle used for these experiments, Lipoderm (LD), was chosen for its ability to penetrate the skin, as described by the manufacturer. Indeed, a single topically applied dose of DTP in LD rapidly entered the skin, and was cleared from the skin with a half-life of about 4 hr (Figure 4A). Since as noted above the persistence of active mustard in skin is expected to be considerably longer than 4 h, it seemed likely that multiple treatments with DTP would be needed to inhibit DNA adduct formation and mutagenesis.

Based on the preliminary pharmacokinetics, we simulated the expected levels of DTP in skin after multiple treatments. This allowed us to develop a schedule of DTP treatments that could be expected to maintain a high level of DTP in the skin. The first treatment was applied 1 hr post exposure to CEES to model the delay between exposure and treatment to be expected in a mass casualty scenario. Further treatments were given 2 hr later, then 4hr later, and then every 6 hr for a total of 13 treatments. Figure 4B presents a simulation of the relative tissue concentration of DTP for the first 9 treatments, assuming a constant half-life of 4 hrs throughout this regimen. The predicted tissue concentration of DTP remained fairly high for the duration of the treatment regime (Figure 4B); the average level of skin DTP over the 48 h simulation shown was about 98% of the maximal level seen following the initial dose.

DTP blocks CEES-induced mutagenesis

To test the effects of DTP on CEES-induced mutagenesis *in vivo*, mice were treated with either EtOH or 200 mM CEES, and one hour later the 13-dose regimen was begun with either LD only or LD containing 20% DTP. Genomic DNA was isolated from the treated skin 4 days after CEES treatment, and used to determine the mutation frequency. Three independent experiments are summarized in Figure 5. The average background mutation frequency (EtOH/LD) was 4.7×10^{-5} , similar to previous measurements without the LD treatment. Treatment with CEES followed by LD induced a mutation frequency of 13.0×10^{-5} , approximately 2.8-fold higher than the control; this difference was statistically significant (t-test: $p < 10^{-5}$). When treatment with CEES was followed by multiple treatments with DTP, the mutation frequency was reduced to 4.4×10^{-5} , not significantly different than the control (t-test: $p > 0.5$). Thus, this DTP treatment regimen completely abolished the mutagenesis induced by CEES in the skin of Big Blue mice.

Discussion

Induction of mutations by CEES was first measured in a bacterial system (Gilbert *et al.*, 1975), and we have previously reported induced mutation rates in a human keratinocyte cell line (Powell *et al.*, 2010). Mutagenicity of SM in rats has been inferred from dominant lethal analysis after exposure by inhalation (Rozmiarek *et al.*, 1973) and gavage (Sasser *et al.*, 1993). The results presented above clearly show that topical application of CEES induces a significant increase in mutation frequency in the skin, using a phage-based reporter system. This is the first quantitative measurement of the *in vivo* mutagenic action of a sulfur mustard in mammals, although such an effect was clearly expected based on measurements *in vitro* and in lower model organisms. Mutation frequency was close to maximal in these assays at a dose of CEES that was insufficient to induce major histopathological changes in the treated skin. Furthermore, the induced mutation frequency dropped to background levels when the CEES treatment was followed by a therapeutic regimen of 13 treatments with

DTP. Since we have previously shown facile adduct formation between CEES and DTP, both in a test tube (Liu *et al.*, 2010) and in cell culture (Powell *et al.*, 2010), this suggests that this scavenging action may be the primary mechanism by which DTP protects against mutagenicity in this model. For this to be the case, much of the mutagenic action of CEES must have taken place after the one hour lag between CEES exposure and the first DTP treatment. As noted in the Introduction, it is thought that sulfur mustards remain stably in the skin for periods of time much longer than one hour, and are able to continue to produce macromolecular adducts several days following treatment (Noort *et al.*, 2002).

The lack of overt pathological changes in mouse skin treated with 200 mM CEES was somewhat unexpected, given the severe toxicity shown previously with neat CEES in C57BL/6 mice (Isidore *et al.*, 2007). We have found histopathological lesions similar to those described by Isidore *et al.* at an intermediate dose of 430 mM (unpublished data), including loss of all epidermis, extensive damage to the dermis and massive inflammation. However, Tewari-Singh and colleagues reported similar “full thickness” lesions in SKH-1/ hairless mice after treatment with CEES at approximately 80 mM in acetone (Tewari-Singh *et al.*, 2009). This apparent difference could be a strain dependent difference in sensitivity, or a solvent effect. Indeed, when we treated C57BL/6 mice with 80 mM CEES in acetone we also observed extensive epidermal damage (unpublished results). This suggests that acetone somehow enhances the skin toxicity of CEES compared to effects with ethanol as supporting solvent.

Exposure of mouse skin to SM in the vapor phase has recently been shown to induce γ -H2AX formation, visible 1-3 days following treatment (Joseph *et al.*, 2011). This phosphorylation of H2AX is induced by DNA double strand breaks, mediated by the ATM/ATR/DNA-PK kinases, and plays a key role in the organization of multi-protein complexes in damaged chromatin that facilitate double strand break repair (Dickey *et al.*, 2009). Although classically induced by agents that directly create double strand breaks such as ionizing radiation, alkylating agents are also known to induce γ -H2AX formation (Dickey *et al.*, 2009). This is presumed to be due to the formation of double strand breaks during cellular attempts to repair the simple alkylation adducts in DNA. Thus, it is not surprising that the mono-functional alkylating agent CEES induces γ -H2AX foci (Figure 2, B-D). The presence of multiple labeled nuclei suggests that in many keratinocytes DNA repair is still not complete three days following exposure to 400 mM CEES.

Concerns have been expressed that SM could be utilized by terrorists to harm civilian populations (Saladi *et al.*, 2006). In a terrorist attack on an urban population, it is likely that many people would be exposed to amounts of liquid sulfur mustard that produce obvious, albeit delayed skin toxicity. These patients would require immediate treatment, and extended hospitalization. However, it seems likely that a large number of people might receive lower doses that do not cause overt toxicity, and that this exposure could go undetected and untreated. Because CEES and SM react with nucleophiles by a common reaction mechanism, the present results suggest that some of these victims would receive mutagenic doses. It is well known that most mutagens are also carcinogens, and that chronic low-dose mustard exposure is associated with increased risk of cancer (Wada *et al.*, 1968; Easton *et al.*, 1988; Hu *et al.*, 2002). In experimental animals, acute treatment with SM has been shown to be carcinogenic (Heston, 1953; Fox and Scott, 1980). Taken together, these results suggest the possibility that civilian victims receiving a single, low dose of SM would be at increased risk for cancer development later in life. Thus, a therapeutic intervention with DTP based on our mouse model would be likely to benefit that fraction of the victims receiving a sub-toxic dose.

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Abbreviations

SM	Sulfur mustard [bis(2-chloroethyl)sulfide]
CEES	2-(chloroethyl) ethyl sulfide
DTP	2,6-dithiopurine
IFE	Interfollicular epidermis
CK6	Cytokeratin 6
LD	Lipoderm
IHC	Immunohistochemistry

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Highlights

- 200 mM 2-(chloroethyl) ethyl sulfide (CEES) induces mutations in mouse skin
- This dose of CEES is not overtly toxic, as assayed by histopathology
- 2,6-dithiopurine (DTP), applied after CEES-treatment, abolishes CEES-mutagenesis
- This supports the idea that sulfur mustards exhibit long biological half-lives

Figure 1. Histopathology of CEES-treated skin

C57BL/6 mice were treated as described in Materials and Methods with A. ethanol only; B. 200 mM CEES in ethanol; or C,D. 400 mM CEES in ethanol. The treated dorsal skin was harvested at necropsy 72 hrs later, and prepared for histopathology; representative H&E-stained sections are shown. Scale bar in panel C also applies to panels A, B, and D in this figure.

Figure 2. Immunohistochemical analysis of CEES-treated skin

Dorsal skin sections from the experiment described in Figure 1 were analyzed by IHC for γ -H2AX (panels A-D) or CK6 (panels E,F). Treatments were with 200 mM CEES in ethanol (panels A,E) or 400 mM CEES in ethanol (panels B-D,F). In panel A, **arrow points** to IFE nuclei positive for γ H2AX. In panel D, arrows point to γ H2AX-positive nuclei in a hair follicle, arrowheads to positive fibroblasts.

Figure 3. Effects of CEES treatment time and dose on mutation frequency

Big Blue mice were treated with the indicated concentration of CEES as described in Materials and Methods, and sacrificed after 24, 48 h. At least 80,00 plaques were enumerated with DNA obtained from one mouse for each treatment. Asterisks indicate treatments that gave an estimated mutation frequency significantly higher than the background of 4×10^{-5} ($p < 0.005$, Poisson distribution).

Figure 4. Persistence of DTP in mouse skin

A. 0.1 cc of DTP/LD was applied to the shaved dorsal surface of Big Blue mice (4 per group) and gently rubbed in for 30 sec. At intervals, mice were sacrificed, the dorsal skin was dissected and four dermatome samples (4 mm in diameter) were taken from within the treated area. As described in Materials and Methods, the amount of DTP remaining in the skin was determined spectrophotometrically. The log of the DTP recovered is plotted as a function of time. The half-life determined by linear least squares fit is about four h.

B. The expected relative amount of DTP present in skin after multiple treatments is calculated assuming a constant half-life of four h, and assuming that subsequent treatments increase the amount in a strictly additive manner. Large closed circles indicate the average DTP amount during each cycle between treatments. The final 4 cycles (not shown) are similar to the last cycle in the graph.

Figure 5. Effect of therapeutic DTP treatment on CEES-induced mutation frequency

Groups of Big Blue mice (n= 4 to 6) were treated with either ethanol or 200 mM CEES. One h later, the standard protective regimen of 13 treatments of DTP/LD was begun; control mice were similarly treated with LD only. All mice were sacrificed 96 h after the initial treatment, and skin mutation frequencies were determined. The mean and standard deviation of the mutation frequency ($\times 10^{-5}$) is plotted for each of the designated groups.