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# Effect of Acrolein on Phosphoramide Mustard-induced Sister Chromatid Exchanges in Cultured Human Lymphocytes[1]

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## ABSTRACT

**Although phosphoramide mustard (PM) is generally recognized as being the most genotoxic metabolite of cyclophosphamide (CP), the contribution of acrolein to the cytogenetic toxicity of CP is unclear. Besides covalently binding to DNA, acrolein can inactivate critical proteins necessary for replicative DNA synthesis, RNA transcription, cell membrane integrity, and metabolism of xenobiotic and endogenous substrates. Because enzymatic processes are involved in sister chromatid exchange (SCE) formation and DNA excision repair, we hypothesized that acrolein might modulate SCE induction by PM due to acrolein's high binding affinity for proteins and low molecular weight sulfhydryl compounds. Human mononuclear leukocytes were isolated on a Ficoll-Hypaque density gradient, and $10^6$ cells were inoculated into 1.9 ml of complete medium. T-cells were stimulated to grow with 4 μg concanavalin A/ml, and 5-bromo-2′-deoxyuridine (5 μM) was added 24 h later. The cultures were then treated with PM (0.069 μM) in the absence or presence of diethyl-4′-hydroperoxy-CP (DEHP-CP), an activated acrolein-generating compound, at concentrations of 0.1, 1, or 10 μM for 48 h. Demecolcine (1.35 μM) was added for the final 4 h of culture. PM alone induced about a 2-fold increase in the SCE frequency (PM, 14.1 ± 0.5 (SD) *versus* control, 7.7 ± 0.4) without cell cycle inhibition or reduced mitotic activity. DEHP-CP induced a concentration-related increase in the SCE frequency of up to 1.6-fold without any significant cell cycle inhibition or lowered mitotic activity. When PM and DEHP-CP were combined, SCE induction was additive for all three DEHP-CP concentrations. Except at the highest molar ratio of DEHP-CP:PM (145:1), there was no evidence of cytotoxicity in the other treatment groups. These results suggest that acrolein has a diminished role in mediating the cytogenetic and cytotoxic effects of CP. In addition, enzymes associated with SCE formation and, by inference, DNA excision repair may not be particularly susceptible to acrolein-induced inactivation.**

## INTRODUCTION

CP[5] is an important chemotherapeutic alkylating agent used in the treatment of malignant tumors and autoimmune diseases (1, 2). The metabolism and pharmacokinetics of CP in mammals have been extensively characterized (1-4), and there is a growing concensus that the anti-tumor specificity and selectivity of CP *in vivo* is closely related to the activated oxazaphosphorine ring, the transport of activated CP (*e.g.*, 4′-OH-CP or aldophosphamide) to the neoplasm, the selective uptake of activated CP by the neoplastic cells, the compartmentalization of the primary active metabolites, and finally the liberation of PM and acrolein from decomposing aldophosphamide within the cells. While acrolein was postulated initially to be a significant mediator of CP's anti-tumor activity (5, 6), acrolein has been repeatedly demonstrated to be without significant tumoricidal activity in a number of bioassays (2, 3, 7-9).

Although PM is generally considered to be the ultimate alkylating, genotoxic, and cytotoxic metabolite when released inside cells based on the activity of PM in *in vitro* and *in vivo* bioassays (1-4, 9-13), there are still uncertainties about the role of acrolein in the cytogenetic toxicity and, ultimately, the therapeutic efficacy of CP. Acrolein has been shown to react with a large number of compounds containing active hydrogens, including alcohols, thiols, amines, and amides, principally by nucleophilic addition at the terminal ethylenic carbon atom (14). Thus, it is not surprising that acrolein can react with nucleotides (15), polynucleotides (16), and native DNA (17-19) *in vitro*. In addition, acrolein can induce single-stranded DNA breakage (13, 16, 20), mutation (15, 21, 22), and SCEs (10, 12) *in vitro*. Yet, some investigators have proposed that acrolein, apart from its ability to bind directly to DNA and induce genetic damage, might modulate the toxicity of PM or other electrophilic agents due to the high affinity of acrolein for reduced sulfhydryl groups on proteins and low molecular weight sulfhydryl compounds such as glutathione and cysteine (12, 13, 18, 23-26).

Those proteins known to be inactivated or bound by acrolein are listed in Table 1. It is important to note that DNA polymerase α, an enzyme involved in the semiconservative synthesis of DNA as well as in DNA proofreading, can be inactivated by acrolein (27). $O^6$-AGT, a nuclear enzyme involved in the non-excision removal of alkyl adducts from $O^6$-guanine, has a reduced sulfhydryl on a cysteine residue at the alkyl-acceptor site which is vulnerable to acrolein binding and inactivation (24, 25). In addition, a recent study has shown that DNA methylase is particularly susceptible to inhibition by acrolein (19), thus raising the possibility of altering DNA methylation patterns and subsequent gene expression *in vivo* in cells exposed to activated CP.

We hypothesized that acrolein, when released from the activated precursor DEHP-CP, might modulate SCE induction in human lymphocytes concurrently exposed to PM. SCEs are thought to arise by enzymatic processes controlling the apparent homologous exchange of DNA duplexes, whether at the sites of the advancing replication forks (39) or at the junctions of replicon clusters (40). Also, because DNA repair processes are known to modify the extent of cytogenetic or mutagenic damage expressed in mammalian cells (22, 24, 25, 41-43), the inactivation of critical repair enzymes by acrolein might influence SCE induction and the degree of cytotoxicity as measured by mitotic activity and cell cycle progression.

## MATERIALS AND METHODS

**Chemicals.** PM (as the cyclohexylammonium salt) was a gift from Leonard H. Kedda (Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD) and was prepared in Dulbecco's PBS (pH 7.3). DEHP-CP (Asta 7037) was a gift from Dr. Norbert Brock (Asta-Werke AG, Degussa Pharma Gruppe, Bielefeld, Federal Republic

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[5] The abbreviations used are: CP, cyclophosphamide; DEHP-CP, diethyl-4′-hydroperoxycyclophosphamide; PM, phosphoramide mustard; SCE, sister chromatid exchange; $O^6$-AGT, $O^6$-alkylguanine-DNA alkyltransferase; PBS, phosphate-buffered saline; MNL, mononuclear leukocyte.



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Table 1 *Proteins inhibited or covalently bound by acrolein*

| | Ref. |
|---|---|
| Eukaryotic proteins | |
| DNA polymerase α (polymerizing site) | 27 |
| RNA polymerase | 28 |
| $O^6$-Alkylguanine-DNA alkyltransferase | 24, 25 |
| DNA methylase (deoxycytidine) | 19 |
| Cytochrome P-450 | 23, 29 |
| NADPH-cytochrome *c* reductase | 30 |
| Aldehyde dehydrogenases (rat liver) | 31 |
| Alcohol dehydrogenase (yeast) | 32 |
| S-Adenosylmethionine decarboxylase | 33 |
| Deoxyribose-5-phosphate aldolase | 34 |
| Prostaglandin endoperoxide E isomerase | 35 |
| 15-Hydroxyprostaglandin dehydrogenase | 36 |
| Spectrin (structural protein; binding detected only) | 37 |
| Prokaryotic enzyme | |
| DNA polymerase I (*Escherichia coli*) | 38 |

of Germany) and was dissolved in ice-cold Dulbecco's PBS (pH 7.4). DEHP-CP undergoes spontaneous hydrolysis or enzymatic reduction to diethyl-4'-OH-CP (44), which then can release acrolein and *N,N*-bis(ethyl)phosphorodiamidic acid, a decomposition product lacking the bifunctional alkylating activity associated with the 2-chloroethyl side chains of PM (6). The test chemical stock solutions were prepared from 5–10 min before addition to the lymphocyte cultures and sterilized using 0.22-μm Millex-GS filter units (Millipore Corp., Bedford, MA). All test chemicals and PBS were added to the cultures in 20-μl aliquots, and the final culture volumes were the same.

**Blood Processing, Lymphocyte Culture Technique, and Chemical Exposure.** Whole blood (~22 ml) was obtained by venipuncture from a healthy adult male (34 years old) into a heparinized syringe. MNLs were separated on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradients and cultured as described previously (12, 45). The cultures were established by inoculating $10^6$ MNLs into 1.9 ml of complete medium composed of RPMI 1640 plus 25 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer, 10% heat-inactivated fetal bovine serum, 100 units of penicillin and 100 μg of streptomycin sulfate/ml, and an additional 292 μg L-glutamine/ml. Three MNL cultures were used for each treatment group. T-lymphocytes were stimulated to grow with concanavalin A (4 μg/ml). The cultures were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. At 24 h post-culture initiation, 5-bromo-2'-deoxyuridine (5 μM) was added, and then the test compounds were added separately to the cultures in the following concentrations: PM (0.069 μM) and DEHP-CP (0.1, 1.0, and 10.0 μM). The molar ratios between DEHP-CP and PM, when combined in the same treatment group, were therefore 1.45:1, 14.5:1, and 145:1 as compared to the 1:1 stoichiometric release of acrolein and PM from decomposing aldophosphamide. The PM concentration was chosen based on previous data (12) which showed that a 2-fold increase in the SCE frequency could be achieved without a concomitant decrease in the mitotic index or slowing of the cell cycle. The DEHP-CP concentrations were chosen for their ability to induce a modest increase in the SCE frequency without substantial cytostatic activity. The cultures were harvested 72 h following a 4-h exposure to demecolcine (1.35 μM). The procedure for cell harvest was as described previously (45).

**Slide Preparation and Microscope Analysis.** The microscope slides were prepared as described previously, coded, and stained using a modified fluorescence-plus-Giemsa technique (46, 47). Twenty-five second division metaphases, 100 consecutive metaphases, and 1000 nuclei were scored from each culture for SCE frequency, cell cycle kinetics, and mitotic index, respectively. The replicative index (*RI*) was calculated from the cell cycle kinetics data by the formula:

$$RI = [(1 \times \%\ \text{first division cells}) + (2 \times \%\ \text{second division cells}) + (3 \times \%\ \text{third cells}) + (4 \times \%\ \text{fourth division cells})]\ (48)$$

**Statistical Analyses.** All cytogenetic data were tested for normality and homogeneity of variance prior to a one-way analysis of variance (49). A square root transformation of the data was done to equalize the variances (50). Comparison of the various treatment groups to the concurrent control was accomplished by either a one-tailed Dunnett's multiple comparison test or Student's *t* test (51).

## RESULTS

As shown in Table 2, PM induced a 1.83-fold increase in the SCE frequency without a significant reduction in the mitotic index or inhibition of cell cycle kinetics. DEHP-CP induced significant concentration-related increases in the SCE frequency also without evidence of cytotoxicity. When these two genotoxins were combined in lymphocyte cultures, a concentration-related SCE increase was observed again. A significant slowing of the cell cycle occurred only with the combination of 0.069 μM PM and 10 μM DEHP-CP.

A closer examination of the SCE data revealed that the observed SCE frequency in the cultures with PM and DEHP-CP combined was slightly less (0.6–1.9 SCEs/metaphase) than expected, based on their abilities to induce SCEs as single agents (Table 3). However, there is no significant difference from additivity.

## DISCUSSION

These results show that acrolein, when released from decomposing DEHP-CP, does not potentiate the SCE induction by PM even at molar ratios (up to 145:1) far exceeding the 1:1 stoichiometric release of acrolein and PM from aldophosphamide. The data suggest that the predominant antiproliferative activity of CP is unrelated to the combined genotoxicity of PM and acrolein but rather principally to the cytogenetic toxicity of PM alone. As additional support for this suggestion, PM

Table 2 *Potential cytogenetic interaction between acrolein released from DEHP-CP and PM in cultured human lymphocytes*

| Treatment group | PM (0.069 μM) | SCE frequency/cell | Mitotic index | Replicative index |
|---|---|---|---|---|
| Control | − | 7.7 ± 0.4[a] | 7.4 ± 1.7 | 2.6 ± 0.2 |
| PM (0.069 μM) | + | 14.1 ± 0.5[b] | 7.0 ± 0.5 | 2.5 ± 0.3 |
| DEHP-CP | | | | |
| 0.1 μM | − | 9.0 ± 0.4[c] | 7.0 ± 2.4 | 2.6 ± 0.1 |
| 1.0 μM | − | 9.6 ± 0.5[c] | 5.6 ± 0.3 | 2.5 ± 0.1 |
| 10.0 μM | − | 12.5 ± 0.5[c] | 5.1 ± 0.2 | 2.4 ± 0.1 |
| DEHP-CP | | | | |
| 0.1 μM | + | 13.8 ± 0.8[c] | 6.0 ± 0.4 | 2.6 ± 0.2 |
| 1.0 μM | + | 15.4 ± 1.0[c] | 6.7 ± 1.7 | 2.5 ± 0.1 |
| 10.0 μM | + | 17.0 ± 0.7[c, d] | 4.0 ± 1.0[e] | 2.1 ± 0.3[f] |

[a] Mean ± SD.
[b] Significant increase in the SCE frequency compared to the PBS control; Student's *t* test, $P < 0.001$.
[c] Significant concentration-related increase in the SCE frequency compared to the PBS control; Dunnett's multiple comparison *t* test, $P < 0.01$.
[d] Significant increase in the SCE frequency compared to PM; Dunnett's multiple comparison *t* test, $P < 0.03$.
[e] Significant decrease in the mitotic index compared to the PBS control and PM; Dunnett's multiple comparison *t* test, $P < 0.01$.
[f] Significant decrease in the replicative index compared to the PBS control and PM; Dunnett's multiple comparison *t* test, $P < 0.05$.

Table 3 *Observed and expected changes in the SCE frequency during the interaction between acrolein (released from DEHP-CP) and PM*

| Chemical exposure | Observed change in SCE/metaphase | Expected change in SCE/metaphase | Difference: observed − expected |
|---|---|---|---|
| 0.069 μM PM − control | 6.4 | | |
| 0.1 μM DEHP-CP − control | 1.3 | | |
| 1.0 μM DEHP-CP − control | 1.9 | | |
| 10 μM DEHP-CP − control | 4.8 | | |
| (0.069 μM PM + 0.1 μM DEHP-CP) − control | 6.1 | 7.7 | −1.6 |
| (0.069 μM PM + 1.0 μM DEHP-CP) − control | 7.7 | 8.3 | −0.6 |
| (0.069 μM PM + 10 μM DEHP-CP) − control | 9.3 | 11.2 | −1.9 |



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has been shown previously to be about 130 and 193 times more effective on a molar basis than DEHP-CP and acrolein, respectively, at inducing SCEs in cultured human lymphocytes, and only PM was clastogenic (12). Therefore, acrolein appears to have a diminished role in mediating genotoxic and cytotoxic effects in proliferating cells during CP exposure.

As listed in Table 1, acrolein can bind and inactivate at least 12 critical enzymes as well as binding to a major cytoskeletal protein, spectrin. Four of these enzymes are associated with nucleic acid metabolism including the functions of DNA and RNA polymerization, DNA methylation, and non-excision repair of $O^6$-alkylguanine. It seemed unlikely that inactivation of $O^6$-AGT in lymphocytes would have an effect on potentiating SCE induction by PM. PM reacts predominantly at the $N^7$-position of guanine, and it is thought that the initial ethyl monoadduct at $N^7$-guanine or the subsequent depurination stimulates DNA excision repair (11). This is in contrast to the non-excision repair of ethyl monoadducts from $O^6$-guanine after cells are exposed to chloroethylnitrosoureas (42, 52). Activated CP and nitrogen mustard, a direct-acting analog of PM, are known to induce unscheduled DNA synthesis in human leukocytes cultured in the presence of hydroxyurea (53–55). It seemed possible that, if acrolein inactivated nuclear enzymes involved in DNA excision repair, then more ethyl monoadducts might persist from exposure to PM and stimulate SCE induction during S-phase. Similarly, because SCE formation probably involves enzymatic processes governing the apparent homologous exchange of DNA duplexes within a chromosome (39, 40) regardless of the actual site of exchange, acrolein might bind and inhibit a protein(s) mediating DNA duplex exchange and, consequently, modulate the SCE frequency or the extent of cytotoxicity. With the exception of a moderate, but statistically significant, slowing of cell cycle progression and reduction of mitotic activity at the highest DEHP-CP:PM molar ratio of 145:1 (Table 2), there was no significant evidence of a potentiation of the PM-induced SCE frequency by acrolein. The resulting SCE frequencies were additive when DEHP-CP was combined with PM (Table 3). These data suggest that enzymes involved in SCE formation and, by inference, DNA excision repair are not particularly susceptible to acrolein-induced inactivation. These enzymes may not have a reduced sulfhydryl group on a cysteine residue at an active catalytic site as do $O^6$-AGT (24, 25) and DNA polymerase $\alpha$ (27).

Glutathione depletion by acrolein might also be another important factor in the modulation of PM-induced cytogenetic damage, because the reduced sulfhydryl group on cysteine could be a target for PM binding and subsequently reduce the overall amount of DNA alkylation. Sulfhydryl compounds such as 2-mercaptoethanesulfonic acid can clearly reduce the cytogenetic toxicity of PM in human lymphocytes *in vitro*, but there is only partial protection even with extremely high molar ratios of 2-mercaptoethanesulfonic acid:PM (12). In a similar manner, other investigators have shown that PM does not deplete glutathione significantly in cultured human leukemia cells during a 4-h exposure, in sharp contrast to acrolein (1–20 $\mu$M) which caused a concentration-related loss of glutathione to almost undetectable levels during a 1-h exposure (26). These investigators concluded that glutathione is a significant factor in detoxifying 4′-OH-CP through a direct condensation reaction and by protecting acrolein-sensitive aldehyde dehydrogenases, which are capable of metabolizing aldophosphamide to the detoxified product, carboxyphosphamide; however, PM is not detoxified appreciably by glutathione (26). Thus, it seems likely that acrolein-mediated glutathione depletion would have a minor effect on the potentiation of PM-induced DNA damage.

Finally, the results of the present study support the conclusions of Wrabetz *et al.* (9), who demonstrated that the high cytotoxic activity and specificity of 4′-hydroperoxy-CP does not result from the purely additive cytotoxicities exerted by the alkylating moiety and acrolein. By treating L1210 ascites cells *in vitro* with a series of activated CP analogs and acrolein, they showed that 4′-hydroperoxy-CP was the only effective antitumor agent as measured by long-term survival of mice given i.p. injections of the treated L1210 cells. They also suggested that alkylation was the ultimate cytotoxic event. Thus, cytogenetic analyses in two studies (Ref. 12 and present study) have demonstrated that PM is the far more potent genotoxic and cytotoxic metabolite of CP and acrolein does not substantially potentiate PM-induced SCEs in cultured human lymphocytes even when present in excess concentrations.

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