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Cytotoxicity and DNA Cross-Linking Activity of 4-Sulfidocyclophosphamides in Mouse Leukemia Cells *in Vitro*[1]

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ABSTRACT

Two sulfido derivatives of cyclophosphamide (CP), 4-*S*-(hexane-6-ol)-sulfidocyclophosphamide and 4-*S*-(propionic acid)-sulfidocyclophosphamide, were studied for their *in vitro* cytotoxicity against L1210 cells and for their DNA-damaging effects in these cells. These derivatives spontaneously hydrolyze under physiological conditions to form 4-hydroxycyclophosphamide, the active metabolite of cyclophosphamide. The two derivatives were compared with phosphoramide mustard, the presumed alkylating species generated by 4-hydroxycyclophosphamide decomposition, for cytotoxic and DNA cross-linking actions. The three compounds yielded colony survival curves that were similar in shape, but the sulfido derivatives were 4 or 5 times as potent as was phosphoramide mustard. All three compounds produced DNA-protein cross-links as well as interstrand cross-links as measured by alkaline elution. The time course of cross-link formation and removal for the three compounds was similar. The sulfido compounds, however, were 4 to 5 times as potent as was the phosphoramide mustard in the formation of interstrand cross-links, in agreement with the cytotoxicity findings. The higher potency of the sulfido compounds was not attributable to the generation of acrolein. These findings indicate that sulfido derivatives of CP can act directly (without metabolic activation) on cells, probably through spontaneous stepwise conversion to phosphoramide mustard, the presumed proximal alkylating agent. The cell-killing effect may be mediated by phosphoramide mustard-induced DNA interstrand cross-linking. Sulfidocyclophosphamide CP derivatives appear to be suitable for *in vitro* studies of the mechanism of action of CP. Sulfidocyclophosphamide CP derivatives may also have therapeutic potential as CP-like drugs that do not require metabolic activation.

INTRODUCTION

Since its development as a potential antitumor agent in 1958 (1), CP[3] has proven to be an important chemotherapeutic agent against animal and human neoplasms. (For extensive reviews, see Refs. 4 and 8.) However, since CP requires activation by hepatic enzymes before demonstrating antitumor activity *in vivo*, studies on the mechanism of action of this compound at the cellular level have been difficult (2). Furthermore, the need for hepatic activation may be a disadvantage in the clinical use of CP because of variability of the activity of hepatic drug-metabolizing enzymes among patients. The effective dose of pharmacologically active products in a given patient depends on the rate of hepatic activation (hydroxylation of the CP 4-position) relative to the rate of CP excretion plus the rate of conversion to inactive products. Variability among patients due to differences in these rates might be reduced by the use of a CP derivative that does not require metabolic activation.

It has been shown that phosphoramide mustard, believed to be the ultimate alkylating moiety following CP activation, causes DNA interstrand cross-links as expected for a nitrogen mustard derivative (21). Phosphoramide mustard, however, is less active and less potent than is CP *in vivo* and therefore cannot be the major transport species (3, 15).

Several 4-alkylsulfido derivatives which spontaneously hydrolyze to the primary active intermediate of CP (4-OH-CP) have been synthesized (9, 10, 16, 17), and the stability of these compounds in crystalline form is substantially higher than is that of 4-OH-CP. Recently, we reported that the 2 sulfido-CP derivatives used in the present study showed comparable antitumor activity to CP if administered i.p. to early ascites L1210 tumor in mice (18).

Because 4-alkylsulfido derivatives of CP can bypass enzymatic activation, they appeared to be ideal for studies of the *in vitro* cytotoxicity and mechanism of 4-OH-CP. In this report, we show that 2 sulfido derivatives of CP have high cytotoxicity and DNA cross-linking activity in cell culture, as expected for compounds capable of spontaneous conversion to bifunctional alkylating agents.

MATERIALS AND METHODS

Cell Culture. Mouse L1210 leukemia cells were grown in spinner culture in RPMI Medium 1630 supplemented with 20% heat-inactivated (56°, 30 min) fetal calf serum (Flow Laboratories, Rockville, Md.), 1 mM L-glutamine, penicillin, and streptomycin. Stock cultures were maintained in exponential phase at a density of 0.3 to 1.8 × 10^6 cells/ml. Colony-forming ability of drug-treated cells was assayed by seeding the cells in polystyrene culture tubes (Falcon Plastics, Oxnard, Calif.) containing RPMI Medium 1630 and 0.1% agar. The colony-forming ability of untreated control cells was >80% in this system.

The DNA of L1210 cells used in alkaline elution assays was radioactively labeled by growing the cells in either [^{14}C]thymidine, 0.02 μCi/ml (>56 mCi/mmol; New England Nuclear, Boston, Mass.), or [^{3}H]thymidine, 0.05 μCi/ml (20 Ci/mmol; New England Nuclear), and 10^{-6} M cold thymidine.

Drug Treatment. The drugs used in this study were the gift of Dr. H. Ringsdorf, W. Klesse, and Dr. T. Hirano, Institute of

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[3] The abbreviations used are: CP, cyclophosphamide (NSC 26271); 4-OH-CP, 4-hydroxycyclophosphamide; sulfido-CP, sulfidocyclophosphamide; RPMI, Roswell Park Memorial Institute; C-1, 4-*S*-(hexane-6-ol)-sulfidocyclophosphamide; C-2, 4-*S*-(propionic acid)-sulfidocyclophosphamide.

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Organic Chemistry, Mainz University, Mainz, Federal Republic of Germany (9, 10). C-1 was dissolved in sterile dimethyl sulfoxide and diluted 1:10 with 0.1 M sodium phosphate buffer (pH 7.9) immediately before treatment of cell cultures. C-2 was prepared immediately before use by dissolving in 0.1 M sodium phosphate buffer (pH 7.9). Phosphoramide mustard (NSC 69945) was supplied by the Drug Synthesis and Chemistry Branch, National Cancer Institute, and was dissolved in sterile distilled water immediately before use. L1210 cells were centrifuged and resuspended in fresh RPMI Medium 1630 30 min before treatment at a density of 1.0×10^6 cells/ml. Following drug exposure, the cells were washed 3 times by centrifugation and resuspension in fresh medium. Following wash, cells were either assayed by alkaline elution or incubated at 37° for 3 to 24 hr.

Alkaline Elution. The alkaline elution procedure used in these experiments has been described in detail (12) and recently reviewed (14). L1210 cells used in this study were analyzed by both the direct alkaline elution method and the proteinase modification (14). In the direct elution assay, 5×10^5 drug-treated ^{14}C-labeled cells were mixed with 5×10^5 ^{3}H-labeled control cells in ice-cold phosphate-buffered saline (0.15 M NaCl:0.014 M KH_2PO_4:0.086 M K_2HPO_4). The tube containing the cells was placed in ice and irradiated with 300 R of X-ray. X-ray was delivered by 2 vertically opposed Phillips RT-250 X-ray tubes operating at 250 keV, 15 ma, equipped with 0.55-mm aluminum and 0.25-mm copper filters. The cells in the phosphate-buffered saline were layered onto a 2-μm pore-size polyvinyl chloride filter (Type BSWP; Millipore Corp., Bedford, Mass.) using mild suction. The cells were immediately lysed with 5 ml of a solution containing 2% sodium lauryl sulfate:0.1 M glycine:0.02 M EDTA, pH 10.0. Following lysis, the filter and lysate were washed with 3 ml of 0.02 M disodium EDTA:0.04 M NaOH, pH 10.0. Tetrapropylammonium hydroxide:0.02 M EDTA, pH 12.1, was pumped through the filter at 0.035 ml/min, and 1.5-hr fractions were collected. Fractions and filters were processed as described previously (14).

The proteinase modification of the alkaline elution technique minimizes the effect of DNA-protein cross-linking on the elution of DNA. The procedure was performed similarly to the direct assay except that 2-μm pore-size polycarbonate filters (Nucleopore Corp., Pleasanton, Calif.) were used, and following lysis, a solution containing 2% sodium lauryl sulfate, 0.1 M glycine, 0.02 M EDTA, and 0.5 proteinase K per ml, pH 10.0, was pumped through the filter for approximately 1 hr. Following the proteinase treatment, tetrapropylammonium hydroxide: 0.02 M EDTA, pH 12.1, containing 0.1% sodium lauryl sulfate, was pumped through the filter at 0.035 ml/min, and fractions were collected as above.

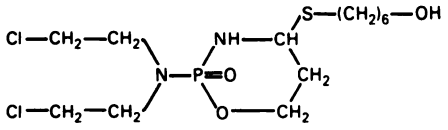
Apparent cross-link frequencies (in rad equivalents) were computed using the formula:

$$(\sqrt{(1 - r_0)/(1 - r)} - 1) \times \text{(X-ray dose in rads)}$$

where r_0 and r are the fractions of the ^{3}H-labeled and ^{14}C-labeled DNA's remaining on the filter after approximately 10 hr of elution (6, 14).

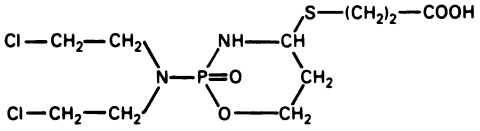
DNA-protein cross-links were assayed by means of the high-dose X-ray alkaline elution method (13). A mixture of ^{14}C-labeled drug-treated cells and ^{3}H-labeled control cells was irradiated with 3000 R on ice and subjected to alkaline elution from polyvinyl chloride filters without the use of proteinase K.

C-1



4-S-(hexane-6-ol)-sulfidocyclophosphamide

C-2



4-S-(propionic acid)-sulfidocyclophosphamide

Chart 1. Structures of C-1 and C-2.

The slowly eluting components were extrapolated to zero time, and DNA-protein cross-link frequencies were calculated by the formula:

$$\left(\frac{1}{\sqrt{1 - r}} - \frac{1}{\sqrt{1 - r_0}}\right) \times \text{(X-ray dose in rads)}$$

where r and r_0 are the extrapolated fractions of slowly eluting DNA in drug-treated and control cells, respectively (14).

RESULTS

The chemical structures of C-1 and C-2 are shown in Chart 1. The half-lives of hydrolysis of C-1 and C-2 to 4-OH-CP are 9 and 8 min, respectively (10). Because these compounds hydrolyze to 4-OH-CP, which has a half-life of hydrolysis to phosphoramide mustard of about 300 min (1), we chose 2-hr exposures to the drugs.

The *in vitro* cytotoxicities of C-1, C-2, and phosphoramide mustard following a 2-hr exposure of L1210 cells to various concentrations of the drugs are shown in Chart 2. It can be seen that the survival curves for the 2 sulfido-CP derivatives are quite similar and that both compounds are much more potent than is phosphoramide mustard. The survival curve parameters for the 3 compounds are shown in Table 1. The D_0 values for the sulfido-CP derivatives were 20 to 25% of the values for phosphoramide mustard. The shapes of the survival curves, as indicated by the D_Q:D_0 ratios, were similar for all 3 compounds. The sulfido compounds thus were 4 or 5 times as cytotoxic as was phosphoramide mustard.

Alkaline elution assays of L1210 cells exposed to 20 μM C-1 or C-2 for 2 hr and assayed immediately after treatment are shown in Chart 3. As has been seen with a variety of alkylating agents (*e.g.*, nitrogen mustard and melphalan)[4] *cis*-platinum (22, 23), and chloroethylnitrosoureas (6, 7), the CP derivatives produced a combination of DNA-protein cross-links (measured by alkaline elution without proteinase digestion) and DNA interstrand cross-links (measured by alkaline elution with proteinase digestion).

[4] W. E. Ross, L. A. Zwelling, and K. W. Kohn, personal communications.

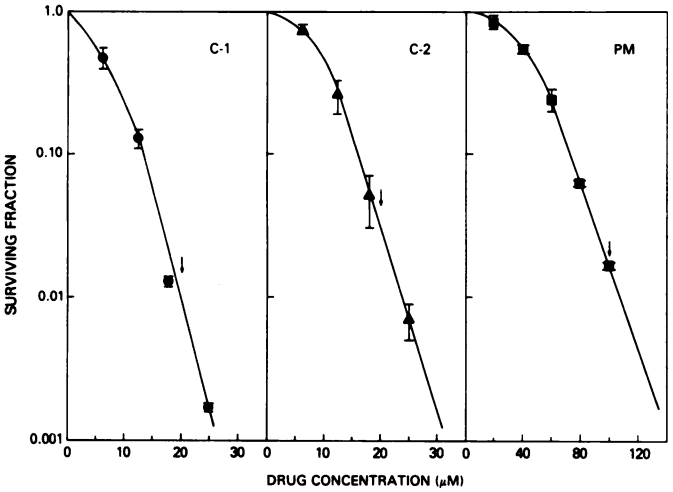


Chart 2. Survival of L1210 cells following 2-hr treatment with C-1, C-2, or phosphoramide mustard (*PM*). *Arrows*, drug dose used in alkaline elution studies. (Note changes in phosphoramide mustard *abscissa* scale in panel.) *Bars*, S.D.

Table 1

Colony survival parameters for L1210 cells treated with C-1, C-2, or phosphoramide mustard for 2 hr (Chart 2)

Compound	D_0[a] (μM)	D_Q (μM)	D_Q:D_0
C-1	3.0	6.4	2.1
C-2	3.5	8.0	2.3
Phosphoramide mustard	15	39	2.6

[a] D_0, Dose of drug required to reduce survival by $1/e$; D_Q, intercept with the abscissa of the exponential phase of the survival curve.

In Chart 4, DNA interstrand cross-linking produced by 20 μM C-1 or C-2 is shown in alkaline elution assays performed at different times following a 2-hr drug exposure. These data show that the 2 sulfido compounds produce similar levels of DNA interstrand cross-links.

The kinetics of interstrand cross-link formation and removal are shown in Chart 5. Two experiments are presented, one based on 2-hr drug exposures (Chart 5*A*) and the other based on 0.5-hr exposures to higher drug concentrations (Chart 5*B*). In all experiments, the concentration of phosphoramide mustard was 5 times the concentration of the sulfido-CP derivatives. In both experiments, interstrand cross-linking peaked after approximately 6 hr. Since comparable levels of cross-linking were observed despite 5-fold increase in phosphoramide mustard, the 2 sulfido-CP compounds appear to be 4 to 5 times as potent as is phosphoramide mustard. The kinetics of cross-link removal, presumably by repair processes, was similar for all 3 compounds.

DNA-protein cross-links were assayed by means of the high-X-ray-dose alkaline elution method ("Materials and Methods"; Chart 6). All 3 compounds again behaved in a similar fashion. The apparent DNA-protein cross-link frequencies peaked at 6 hr after drug removal. Although this assay is not affected by interstrand cross-linking as much as is the usual low-X-ray-dose alkaline elution method, some contribution by interstrand cross-linking is still possible. Therefore, the apparent reduction in cross-linking between 6 and 18 hr (Chart 6) could reflect the elimination of interstrand cross-links during this time interval. However, at 18 hr, when most interstrand cross-links had been removed, high levels of DNA-protein cross-links were still observed. The concentration ratios producing equal DNA-protein cross-linking for phosphoramide mustard relative to C-1 or C-2 ranged from 2.5 to 4.0 in different experiments. The results suggested that these ratios may be lower for DNA-protein cross-linking than for interstrand cross-linking.

The decomposition of the sulfido-CP compounds to active alkylating products would be expected to release acrolein as

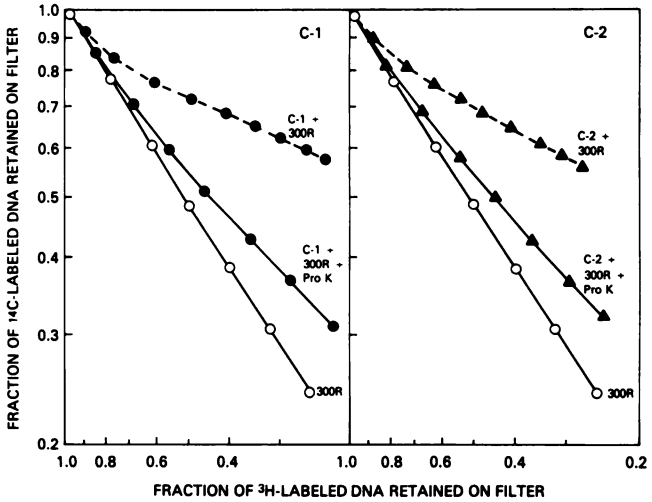


Chart 3. DNA-protein cross-linking and DNA interstrand cross-linking in L1210 cells treated with 20 μM C-1 or 20 μM C-2 for 2 hr. All cells received 300 R of X-ray prior to alkaline elution. Assays were performed with (——) or without (- - - -) proteinase-K (*Pro-K*) immediately after drug treatment.

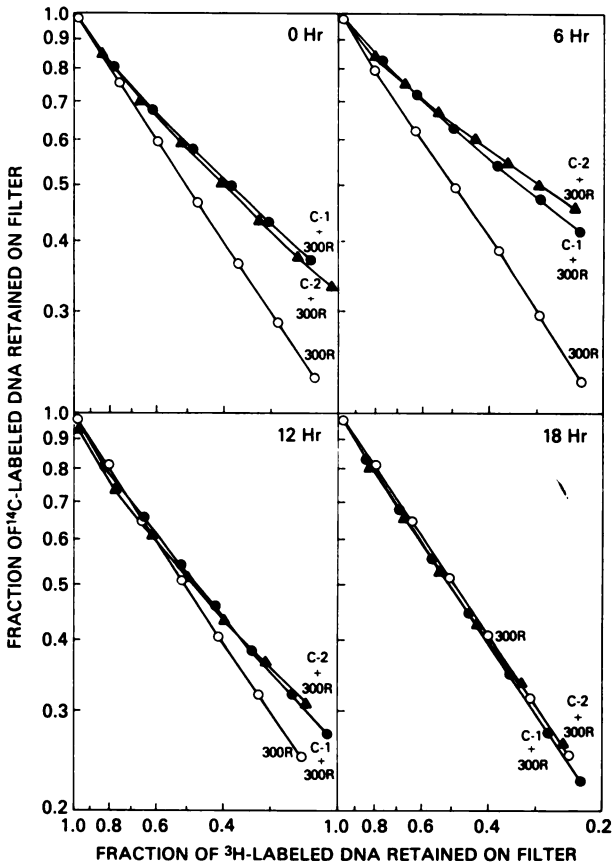


Chart 4. DNA interstrand cross-linking in L1210 cells incubated in drug-free medium for 0, 6, 12, or 18 hr after a 2-hr exposure to 20 μM C-1 or 20 μM C-2. All cells received 300 R X-ray prior to alkaline elution and were eluted after proteinase-K treatment.

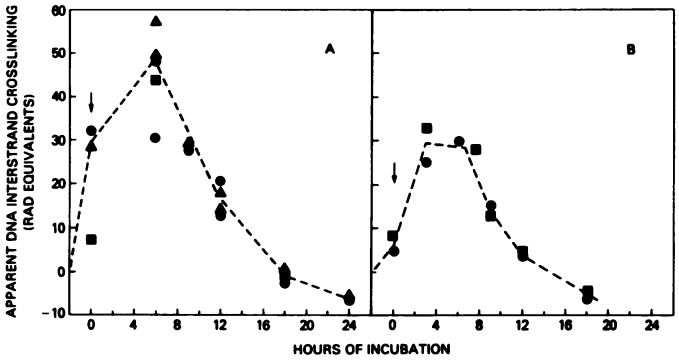


Chart 5. Apparent DNA interstrand cross-linking in L1210 cells exposed to 20 μM (*A*) or 30 μM (*B*) C-1 (●), 20 μM C-2 (▲), or 100 μM (*A*) or 150 μM (*B*) phosphoramide mustard (■), following a 2-hr exposure (*A*) or following a 20-min exposure (*B*). *Arrows*, drug removal time.

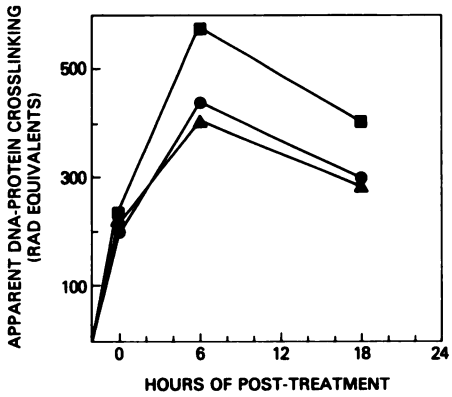


Chart 6. Apparent DNA-protein cross-linking in L1210 cells exposed to 20 μM C-1 (●), 20 μM C-2 (▲), or 75 μM phosphoramide mustard (■).

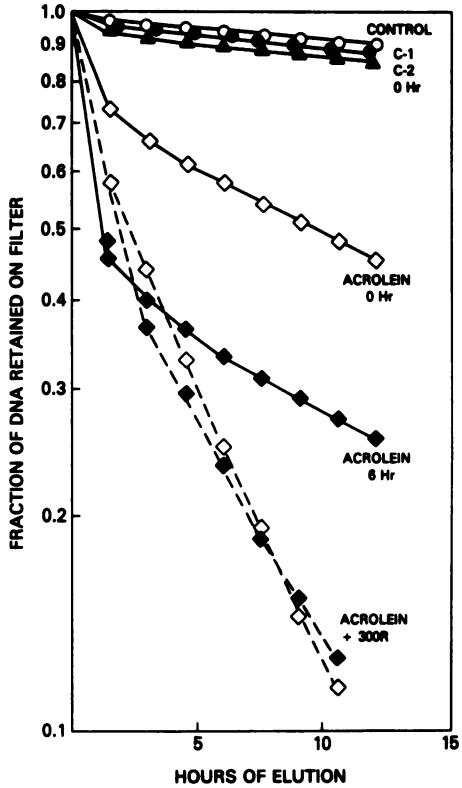


Chart 7. Measurement of DNA single-strand breaks following exposure of L1210 cells to sulfido-CP derivatives or acrolein. ○——○, untreated control; ●——●, 20 μM C-1, 2 hr; ▲——▲, 20 μM C-2, 2 hr; ◇——◇, 20 μM acrolein, 2 hr; ◆——◆, 20 μM acrolein, 2 hr (6-hr incubation in drug free medium); ◇- - -◇, 20 μM acrolein, 2 hr (cells irradiated with 300 R prior to alkaline elution); ◆- - -◆, 20 μM acrolein, 2 hr (6-hr incubation in drug-free medium, cells irradiated with 300 R prior to alkaline elution); *0 hr*, 0-hr incubation in drug-free medium.

a byproduct (2). Phosphoramide mustard, however, does not generate acrolein. This raises the question of whether acrolein, which is known to be highly reactive and toxic, is responsible for the higher potencies of the sulfido-CP compounds. The effect of acrolein on DNA in L1210 cells was therefore examined (Chart 7). The effect of acrolein on DNA was entirely different from that of the sulfido-CP compounds. Instead of DNA cross-linking, the predominant effect of acrolein was strand breakage and/or production of alkali-labile sites. Since the sulfido-CP compounds failed to show strand breakage, we conclude that only a small fraction of the sulfido-CP molecules yielded active alkylating species and acrolein during the 2-hr period of cell exposure.

Also arguing against a major contribution to cytotoxicity by acrolein was the finding that 20 μM acrolein yielded a 10-fold higher survival of colony-forming ability than did equivalent concentrations of the sulfido-CP compounds (data not shown).

DISCUSSION

In this report, we have shown that 2 recently synthesized sulfido derivatives of CP (C-1 and C-2), which spontaneously hydrolyze to 4-OH-CP under physiological conditions, are cytotoxic to cultured L1210 cells incubated with the compounds *in vitro*. These drugs were 4 to 5 times as potent as was phosphoramide mustard in reducing colony-forming ability of treated L1210 cells. Using the alkaline elution technique, it was shown that C-1, C-2, and phosphoramide mustard produce DNA-protein cross-links and DNA interstrand cross-links. Following drug exposure and removal of drug by washing the cells, DNA interstrand cross-linking increased gradually, reaching a maximum at about 6 hr for all 3 drugs. Interstrand cross-linking then declined to very low levels during the 6- to 18-hr interval following drug treatment.

The concentration ratios giving equal interstrand cross-linking for phosphoramide mustard relative to C-1 or C-2 were similar to the concentration ratios giving equal survival of colony formation. This is consistent with the possibility that the cell killing by all 3 compounds is due to phosphoramide mustard-induced interstrand cross-links.

Phosphoramide mustard has been reported previously to cause DNA interstrand cross-linking in L1210 cells, measured by dextran:polyethylene glycol partitioning of double-stranded and single-stranded DNA (21). We have confirmed this finding by an independent method and have measured the kinetics of cross-link formation and removal. Although phosphoramide mustard may be the ultimate alkylating moiety generated from CP *in vivo*, recent evidence suggests that 4-OH-CP may be the transport form of CP once it is activated in the liver (5). Since C-1 and C-2 are hydrolyzed to 4-OH-CP with half-lives of 9 and 8 min, respectively (10), and the half-life of 4-OH-CP degradation to phosphoramide mustard is 312 min (11), our experiments may be modeling the process of the interaction of the 4-OH-CP intermediate and the tumor cell.

It is likely that 4-OH-CP and/or aldophosphamide, with which

it is interconvertible, can penetrate readily through cell membranes. The phosphoramide mustard product, however, is a charged molecule which may be impeded in entry or exit from the cell. The cell-washing procedure which followed drug exposure in our experiments therefore may not have removed phosphoramide mustard species from intracellular compartments. The remaining phosphoramide mustard could have reacted with cell DNA after the cell wash and could have contributed to the observed delay in cross-link formation. However, since similar delays have been seen with chloroethylnitrosoureas (6, 7) and *cis*-platinum (22, 23), it is possible that a major contributor to the delay is the time required for conversion of DNA monoadducts to interstrand cross-links. This type of delay has also been seen with melphalan but not with nitrogen mustard (20).[4]

Once inside the cell, the sulfido-CP compounds and 4-OH-CP:aldophosphamide would be expected to decompose slowly to equimolar amounts of phosphoramide mustard and acrolein. Since acrolein produced extensive DNA strand breakage, whereas the sulfido-CP compounds did not, it appears that there was much less than stoichiometric conversion of sulfido-CP to acrolein. This implies that there was also much less than stoichiometric formation of phosphoramide mustard and that intracellular phosphoramide mustard efficiently produces cross-links but that extracellular phosphoramide mustard penetrates into cells to only a small extent.

In this study, we have shown the usefulness of 2 sulfido derivatives of CP for modeling the mechanism of action of CP. Since these drugs are stable in crystalline form and spontaneously hydrolyze to 4-OH-CP *in vitro* without requiring metabolic activation, they should be useful for studying the effects of 4-OH-CP on other cellular functions. The sulfido-CP compounds produced the cytotoxic and intracellular DNA effects expected for the active products of cyclophosphamide metabolism. This provides a basis for the development of a sulfido-CP drug that would not require metabolic activation. Since C-1 and C-2 have been noted to produce severe hepatotoxicity in mice (18), it may be necessary to develop other derivatives.

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