Effect of Thiourea on Survival and DNA Cross-Link Formation in Cells Treated with Platinum(II) Complexes, L-Phenylalanine Mustard, and Bis(2-chloroethyl)methylamine

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ABSTRACT

This study examines the mechanisms by which thiourea may protect cells against toxic actions produced by platinum complexes or nitrogen mustards. Mouse leukemia L1210 cells were assayed for survival by colony formation in soft agar and for DNA interstrand cross-linking using the alkaline elution method.

The survival of L1210 cells treated with cis- and trans-Pt(II)diamminedichloride was enhanced by posttreatment incubation with thiourea. This enhancement was lost over a period of 6 to 12 hr after drug exposure, suggesting that the cytotoxicity resulted from a delayed effect which was susceptible to blockade by thiourea.

DNA interstrand cross-link formation was prevented when thiourea was added immediately after cis-Pt(II)diamminedichloride treatment but was not reversed once cross-links had formed. Survival enhancement and cross-link prevention by thiourea depended in a similar manner on thiourea concentration and on the interval between platinum treatment and thiourea addition. Cytotoxicity by nitrogen mustards was also prevented by thiourea, but this protectability was lost much more rapidly than in the case of Pt(II) complexes. In contrast to cis-Pt(II)diamminedichloride, delayed interstrand cross-link formation by L-phenylalanine mustard was not prevented by thiourea.

The results suggest that thiourea can react with cis-Pt(II)-DNA monoadducts to prevent their conversion to potentially lethal cross-links. Thiourea may also react with nitrogen mustards as well as Pt(II) complexes to directly inactivate free drug.

INTRODUCTION

Although the mechanism by which Pt(II) complexes kill cells is not completely understood, the ability of these compounds to form bifunctional adducts with cellular DNA appears crucial to their cytotoxic potential (13, 19, 27, 28, 30). A possibly important class of bifunctional lesions produced by Pt(II) complexes is DNA interstrand cross-links which form in a delayed 2-step reaction over a period of 6 to 12 hr following treatment (27, 28). In the type of lesion produced and in the delay in its formation, Pt(II) complexes resemble bifunctional alkylating agents (5, 11, 23).

Thiourea reacts rapidly with Pt(II) complexes and can replace various ligands from such complexes (2, 10). Burchenal et al. (3) have recently reported that thiourea can counter the effects of cis-PDD in animals and cell culture when thiourea and cis-PDD were administered concurrently. In these experiments, thiourea may have reacted directly with the cis-PDD, preventing its initial reaction with intracellular targets. Thiourea also could have protected cells by reacting with monofunctionally bound cis-PDD, preventing its conversion to potentially lethal bifunctional adducts. Another possibility is that thiourea could have reversed platinum adducts as has been described in phage λ DNA treated with cis-PDD (8). In either of these latter 2 possibilities, thiourea would be expected to be effective even after removal of Pt(II) complex. We show that thiourea applied to cells after treatment with cis-PDD can protect cells from cis-PDD lethality as well as prevent the formation of DNA interstrand cross-links, although reversal of DNA cross-linking was not detected.

MATERIALS AND METHODS

Cells and Radioactive Labeling. L1210 mouse leukemia cells were grown in suspension culture in RPMI Medium 1630 supplemented with 20% fetal calf serum. Stock cultures were maintained in static bottles without antibiotics and were used to initiate suspension cultures containing penicillin and streptomycin. Cultures used to assess drug effects were in exponential growth phase with a doubling time of 12 hr. DNA was labeled by incubating the cells for 20 hr in the presence of [2-14C]thymidine (0.01 μCi/ml).

Drug Treatment. cis-PDD (NSC 119875), trans-PDD (NSC 131558), L-PAM, and HN2 were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute.

Pt(II) complexes were dissolved in RPMI Medium 1630 plus 1% fetal calf serum and used within 1 hr. L-PAM and HN2, 0.01 M, were dissolved in 0.1 N HCl and stored frozen. Prior to drug treatment, cells in exponential growth phase were centrifuged and resuspended in fresh RPMI Medium 1630 plus 1% fetal calf serum. Treatment was performed in this medium, after which cells were centrifuged and resuspended twice in drug-free medium containing 20% fetal calf serum.

Thiourea Treatment. Thiourea, H2N—CS—NH2 (Eastman Organic Chemicals, Rochester, N. Y.), was dissolved in 0.1 N HCl and stored frozen. Prior to drug treatment, cells in exponential growth phase were centrifuged and resuspended in fresh RPMI Medium 1630 plus 1% fetal calf serum. Treatment was performed in this medium, after which cells were centrifuged and resuspended twice in drug-free medium containing 20% fetal calf serum.

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1 The abbreviations used are: PDD, Pt(II)diamminedichloride; RPMI, Roswell Park Memorial Institute; L-PAM, L-phenylalanine mustard (melphalan); HN2, bis(2-chloroethyl)methylamine (nitrogen mustard).
Survival Experiments. Cells were treated as described above and colony-forming ability in soft agar was assessed by the method of Chu and Fisher (4).

Alkaline Elution Analysis. Alkaline elution with proteinase K was performed and quantified as previously described (7, 27, 28) except that lysis solution consisted of 2% sodium lauryl sulfate (BDH Biochemicals Ltd., Poole, England), 0.1 M glycine, and 0.025 M disodium EDTA, pH 10 (17). This change from the previous procedure reduces the amount of protein remaining adsorbed to the filter after proteinase K treatment and more effectively eliminates the effects of DNA-protein cross-links. In addition, 0.1% sodium lauryl sulfate was included in the eluting solution (tetrapropylammonium hydroxide and EDTA) to further decrease protein adsorption. The procedure therefore provides a more reliable measure of protein-independent retardation of DNA elution which we consider to be due to interstrand cross-linking (7, 16, 17, 24, 27, 28).

As shown in our previous work (27, 28, 31), the accuracy and reproducibility of elution data are greatly increased by plotting the retention of [14C]DNA on the filter against the retention of [3H]DNA from internal standard Li210 irradiated with 150 R prior to elution, which can be considered a corrected time scale. The point when 60% of the internal standard DNA is retained on the filter is the end point at which to analyze DNA elution. The fraction of [14C]DNA retained on the filter at this end point is designated the "relative retention." The relative retention of DNA from cells treated with a cross-linking agent (R) exceeds the relative retention of DNA from control cells (Ro) when both cells have received 300 R of X-irradiation to introduce random single-strand breaks. If $P_a$ is set equal to 300 R, as was used in these experiments, the number calculated for $P_x$ is said to be in "rad-equivalents."

RESULTS

The relationship between drug dose and survival of cells treated with cis-PDD, trans-PDD, and L-PAM for 1 hr is shown in Chart 1. Posttreatment incubation of platinum-treated cells with 100 mM thiourea for 1 hr increased their survival when compared to that of platinum-treated cells which received no thiourea. The dependence of enhanced survival on thiourea concentration for cis-PDD-treated cells is shown in Chart 2a. The survival enhancement was limited, becoming maximum above a thiourea concentration of 5 to 10 mM. The midresponse point was about 0.5 mM.

The cytotoxicity of L-PAM, another bifunctional agent that produces delayed DNA cross-linking (23), however, was not affected by thiourea (Chart 1). Because this drug may react more rapidly than does cis-PDD, the experiments were repeated using 0.5-hr treatments (Table 1). There was, nevertheless, no enhancement of survival by subsequent exposure to 100 mM thiourea for 1 hr. Thiourea also failed to significantly enhance survival after treatment with HN2. Thus, the effect of thiourea was specific for platinum complexes.

If thiourea treatment was delayed after the removal of cis-PDD (Chart 3), survival enhancement decreased as the interval between treatments with the 2 agents increased. After an interval of 6 to 12 hr, thiourea no longer enhanced survival to a detectable degree. In the case of trans-PDD, the ability to enhance survival with thiourea was lost more slowly. The survival of cells treated with L-PAM was not enhanced by thiourea at any time after treatment. Thiourea thus appeared to interfere with a delayed event responsible for cell killing by platinum complexes.
Thiourea Prevents cis-PDD Cytotoxicity and DNA Cross-Linking

Chart 2. Effect of thiourea concentration on cell survival and DNA interstrand cross-linking. Cells were treated with 20 μM cis-PDD for 1 hr followed, after drug removal, by thiourea for 1 hr. a, survival fraction. Each point is mean of at least 3 replicate tubes; bars, S.D. of at least 3 independent experiments. If less than 3 experiments, individual observations are shown. b, proteinase-resistant DNA cross-linking measured by alkaline elution 6 or 12 hr after cis-PDD removal; bars, S.D. of 3 experiments. If less than 3 experiments, individual observations are shown. There was no significant difference between values obtained 6 or 12 hr after drug removal.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (μM)</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-PDD</td>
<td>35</td>
<td>1.64</td>
</tr>
<tr>
<td>L-PAM</td>
<td>100 mm</td>
<td>2.04</td>
</tr>
<tr>
<td>HN2</td>
<td>2</td>
<td>2.99</td>
</tr>
</tbody>
</table>

* Cells incubated in medium without thiourea.

The delayed event in question could be the reaction of the second arm of monofunctionally bound drug to form an interstrand cross-link. A platinum complex bound at one site on a DNA strand would, in this reaction, link to a nucleophilic site on the opposite strand. Interstrand cross-linking is thought to be a delayed effect of both bifunctional alkylating agents (5, 11, 23) and Pt(II) complexes (19, 27, 28). We therefore examined the effect of thiourea on cross-link formation by cis-PDD as well as L-PAM. DNA interstrand cross-linking was measured by alkaline elution using proteinase K and sodium lauryl sulfate to eliminate the effects of DNA-protein cross-links (7, 16, 17, 24, 27, 28).

In Chart 4, DNA elution kinetics are shown for cells treated with 20 μM cis-PDD for 1 hr followed by a 1-hr incubation with various concentrations of thiourea. Cells were harvested 12 hr after cis-PDD treatment at which time cross-linking was maxi-
Chart 5. Effect of thiourea on DNA interstrand cross-linking following 1-hr treatments with 20 μM cis-PDD or L-PAM. Cells were harvested for assay at 0, 6, or 12 hr following removal of cis-PDD or L-PAM. 

Table 2. Effects of thiourea on cross-link formation in cells treated with cis-PDD or L-PAM for 0.5 hr

<table>
<thead>
<tr>
<th>Time after drug removal (hr)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No thiourea</td>
<td>Thiourea</td>
<td>No thiourea</td>
<td>Thiourea</td>
</tr>
<tr>
<td>1</td>
<td>23.5</td>
<td>14.3</td>
<td>43.5</td>
<td>59.1</td>
</tr>
<tr>
<td>6</td>
<td>63.9</td>
<td>33.1</td>
<td>101.3</td>
<td>83.2</td>
</tr>
</tbody>
</table>

DISCUSSION

Thiourea is an avid binder of Pt(II) complexes, capable of displacing otherwise stable ligands from coordination com-
plexes (2, 10) and enhancing the survival of cells treated with cis- and trans-PDD. The ability of thiourea to counter the biological effects of Pt(II) complexes could be due to: (a) binding of Pt(II) complexes outside or inside the cell preventing their reaction with critical target macromolecules; (b) binding to the second arm of a bifunctional Pt(II) complex the first arm of which has already become linked to a critical macromolecule, thereby preventing the formation of a lethal cross-link; and (c) displacement of Pt(II) species which have bound through one or both arms to critical macromolecular sites. Each of these factors may play a significant role under various conditions.

Binding of unreacted Pt(II) complexes is most consequential when thiourea is already present at the time of drug addition. In this case, thiourea concentrations as low as 1 mM can prevent the cytotoxicity of cis-PDD (3). When thiourea is added at the end of a 1-hr cis-PDD treatment, concentrations of 10 to 100 mM are required to counter cytotoxicity (Chart 2). This requirement for higher concentrations suggests that a different chemical process is involved.

The cytotoxic effects of cis-PDD may result from reactions with intracellular DNA which are detectable within 1 to 2 hr of drug exposure (9, 19, 20, 27, 28). The ability of thiourea to counter the cytotoxicity produced by 1-hr cis-PDD treatments was greater immediately after cis-PDD removal and declined as the interval between cis-PDD and thiourea increased (Chart 3). This is as would be expected if thiourea reacted with monofunctionally bound cis-PDD, blocking its subsequent conversion to lethal cross-links.

This hypothesis was supported by measurements of DNA interstrand cross-linking (Charts 2b and 5). Thiourea prevented the delayed formation of cross-links which occurs after treatment with cis-PDD. The ability of thiourea to prevent the formation of these cross-links declined as the length of the interval between drug and thiourea treatments increased. Cross-link prevention and survival enhancement depend in a similar manner on the thiourea concentration and on the interval between platinum treatment and thiourea. The cytotoxic lesions, therefore, are likely to be bifunctional adducts having chemical characteristics similar to interstrand cross-links. The data do not exclude intrastrand cross-linking, for example, as the major cytotoxic lesion produced by cis-PDD (12, 13, 18, 19, 21).

Thiourea can also reverse lethal cross-links in DNA, at least, in chemical systems. This has been shown for the case of phage A DNA treated with cis-PDD and assayed for interstrand cross-linking and transfectivity (8). The thiourea concentration required for cross-link reversal in this system was on the order of 1 M, about 2 orders of magnitude larger than the concentrations that appeared to prevent the conversion of monoadducts to cross-links in cells (Chart 2). In the current experiments, there was no evidence of cross-link reversal, possibly because high enough concentrations of thiourea could not be achieved without significantly reducing cell viability.

In addition to having a high affinity for reaction with platinum, thiourea may behave as a nucleophile that could inactivate alkylating agents. This possibility was examined for the case of the cytotoxicity produced by L-PAM and HN2. Thiourea was able to prevent the cytotoxicity of these nitrogen mustards if it was present at the time of drug addition (Chart 6). Thiourea thus can inactivate unreacted nitrogen mustards as well as cis-PDD. The protective effect of thiourea, however, was lost much more rapidly in the case of the nitrogen mustards than in the case of cis-PDD. The difference is not simply related to the kinetics of cross-link formation, because L-PAM is, in this respect, more like cis-PDD than like HN2 which forms cross-links more rapidly than either of the other 2 compounds (6, 23, 27).

Reaction schemes for cis-PDD and the nitrogen mustards

are proposed in Chart 8. The 2 classes of bifunctional agents can undergo analogous reaction paths, involving sequential activation (Reactions 1 and 3) and DNA base addition (Reactions 2 and 4). Thiourea could react with free drug (Reactions 5 and 5') or with monoaducts (Reactions 6 and 6') to prevent cross-link formation. cis-PDD cross-links could be reversed by Reaction 7, and the monoadducts might be reversed by Reactions 8 or 8'. Such reversal reactions are unlikely for the nitrogen mustards (25).

In the case of cis-PDD, monoadduct inactivation would require that the rates of Reactions 3 and/or 4 be slow enough to permit the monoadducts to react with thiourea (Reactions 6 and 6'). With nitrogen mustards, the corresponding requirement is that Reaction 4 must be slow relative to Reaction 3 so as to allow a sufficient accumulation of the activated monoadduct to favor reaction with thiourea (Reaction 6). This condition is more likely to be met by nitrogen mustards, such as HN2, that have the kinetics of S_n2 reactions, than by those like L-PAM that have kinetics approximating S_n1 reactions (1, 22, 25, 26). This could account for the observation that thiourea, in some experiments, gave late protection against the cytotoxic effect of HN2 but not against that of L-PAM.

For cis-PDD and HN2, the time course for loss of protectability by thiourea and the kinetics of cross-link formation (6, 23, 27)* were consistent with the hypothesis that both cytotoxicity and cross-link formation are prevented by reaction of thiourea with DNA monoadducts. The corresponding data for L-PAM, however, are inconsistent with this hypothesis, so that the early protection conferred by thiourea is, in this case, attributable to direct reaction with free drug.

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