Quenching of DNA:Platinum(II) Monoadducts as a Possible Mechanism of Resistance to cis-Diamminedichloroplatinum(II) in L1210 Cells

Kenneth Micetich, Leonard A. Zwelling, and Kurt W. Kohn

ABSTRACT

A line of mouse leukemia L1210 cells resistant to cis-diamminedichloroplatinum(II) (cis-DDP) was compared with its parent cell line in order to determine whether the sensitivity difference could be related to DNA interstrand cross-linking as measured by the alkaline elution technique. The study was stimulated by a previous finding that the magnitude of DNA interstrand cross-linking, although somewhat reduced in this resistant line, did not account for the relatively high degree of resistance. Therefore, the kinetics of cross-link formation and removal was studied. Cross-link removal rates were determined by the use of thiourea to stop the delayed formation of interstrand cross-links from cis-DDP:DNA monoadducts. There was no significant difference between the cross-link removal rates in the parent and resistant lines. Computer-analyzed kinetics was consistent with an enhanced cis-DDP:DNA monoadduct quenching mechanism in the resistant cells.

INTRODUCTION

cis-DDP is a bifunctional reagent that reacts with DNA intracellularly to form interstrand cross-links and DNA:protein cross-links, as well as other DNA adducts (4, 9). Bifunctionality is required for potent cytotoxic and antitumor effects. Resistance to cis-DDP has in some cases been found to be in proportion to a reduction in the peak of interstrand cross-linking (11). Even if such bifunctional lesions are the major source of cytotoxicity, however, one would expect other factors to contribute to the efficiency with which a given number and type of lesion is expressed as cytotoxicity. Among such factors is the kinetics of lesion formation and removal. The role of such factors could be brought to light by examining cases in which the magnitude of a sensitivity difference deviates from the magnitude of an observed difference in lesion frequency. We have therefore studied a pair of L1210 lines which had been observed to differ in cytotoxic sensitivity to a greater extent than expected from the difference in peak interstrand cross-linking (11). The results are consistent with the possibility that the resistance and the observed difference in interstrand cross-linking kinetics are due to enhanced quenching of DNA:platinum(II) monoadducts.

MATERIALS AND METHODS

Cell Lines. The history of the cell lines used in this work is as follows. Mouse leukemia L1210 from the Tumor Bank of the National Cancer Institute was used by Dr. F. Schabel, Southern Research Institute, Birmingham, Ala., to derive a tumor line resistant to L-phenylalanine mustard. This tumor line, designated L1210/PAM, was found to be cross-resistant to cis-DDP (5). We obtained the L1210/PAM tumor from Dr. D. Vistica, National Cancer Institute, who had obtained it from Dr. Schabel. Tumors were originally passaged weekly in C57BL/2F mice. Resistance was maintained in the mice bearing the L1210/PAM line by treatment with phenylalanine mustard (7.5 mg/kg i.p.) on Day 2 following transplantation. Ascites cells from the L1210/PAM tumor and from its parent tumor line (which we designate L1210/NCI) were explanted to soft agar tubes with RPMI Medium 1630 plus 15% heat-inactivated fetal calf serum and 50 μM 2-mercaptoethanol. An individual clone from the parent sensitive line and the resistant line were picked, expanded in suspension culture, and frozen down in liquid nitrogen. Cells for the studies described herein were grown for no more than 6 weeks before going back to a fresh ampul. During this interval, resistance was retained, as judged by 24-hr growth curves.

Cell Treatments. cis-DDP was obtained through the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. One hr prior to use, a 500 μM solution of cis-DDP was prepared in sterile 0.15 M NaCl. Exponentially growing cells in RPMI Medium 1630 containing 15% heat-inactivated fetal calf serum and 50 μM 2-mercaptoethanol were labeled with 24 hr with [2-14C]thymidine (51 mCi/mmol; 0.01 μCi/ml). The labeled cells were washed twice with RPMI Medium 1630 containing 1% serum and not containing 2-mercaptoethanol. The cells were then treated with cis-DDP in this medium. At the end of 1-hr drug treatment, cells were washed and suspended in RPMI Medium 1630 with 15% serum and 50 μM 2-mercaptoethanol. Between 106 and 108 cells were suspended in 3 ml of medium containing 0.1% Noble agar (Difco Laboratories, Inc., Detroit, Mich.) in closed tubes, and colony formation was scored after 10 to 14 days of incubation.

DNA Interstrand Cross-Linking. Alkaline elution assays for DNA interstrand cross-linking were performed as described previously (3, 11). 14C-Labeled experimental cells, 5 × 106, were mixed with 5 × 106 3H-labeled internal standard L1210 cells for alkaline elution assay. The internal standard cells were labeled with [methyl-3H]thymidine (20 Ci/ mmol; 0.1 μCi/ml) and were subjected to 300 rads of X-ray at ice temperature. The cells were deposited on polyvinyl chloride filters that were 25 mm in diameter, with a 2-μm pore size (Millipore Corp., Bedford, Mass.; Type BS). The cells were lysed with 2% sodium dodecyl sulfate (BDH Biochemicals Ltd., Poole, England), 0.1 M glycine, and 0.025 M EDTA (pH 10). Proteolytic digestion of the lysate was accomplished by the addition of proteinase K (0.5 mg/ml; EM Biochemicals, Cincinnati, Ohio) dissolved in the lysis solution. The eluting solution was 0.1 M tetrapropylammonium hydroxide:0.02 M EDTA:0.1% sodium dodecyl sulfate (pH 12.1) and was pumped at a rate of 2 ml/hr. Fractions were collected for scintillation counting at 3-hr intervals for 15 hr. Radioactivity of the eluted fractions, lysate, filter, and filter holder were determined.

Cross-linking was determined by:

\[
K_c = \left( \frac{1 - r_0}{1 - r} \right)^N - 1 \times (300 \text{ rads})
\]

where \( r \) and \( r_0 \) are the fraction of DNA retained on the filter for drug-treated and untreated cells, respectively. \( r \) and \( r_0 \) were determined utilizing as an end point the point at which 35% of the internal standard
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(3H radioactivity) is retained on the filter. $K_\text{s}$, the interstrand cross-linking coefficient, is given in rad-equivalents.

**DNA:Protein Cross-Linking.** DNA:protein cross-linking was determined by alkaline elution as described previously (3, 11). The cells were exposed to 3000 rads of X-ray at ice temperature. The lysing solution was the same as in the assay for DNA: interstrand cross-links but was followed by a wash of 5 ml of 0.02 M EDTA (pH 10), and proteinase K was omitted. Alkaline elution was carried out as above, except that the sodium dodecyl sulfate was omitted from the eluting solution.

DNA:protein cross-linking was calculated by:

$$P_s = \left[ (1 - r)^{-1} - (1 - r_0)^{-1} \right] \times (3000 \text{ rads})$$  \hspace{1cm} (B)

where $r$ and $r_0$ are the fractions of DNA in the slow eluting component for drug-treated and untreated cells, respectively. $P_s$, the DNA:protein cross-linking coefficient, is given in rad-equivalents.

**Thiouria Experiments.** Thiouria (Fisher Scientific Co., Pittsburgh, Pa.) was dissolved to a final concentration of 0.1 M in RPMI Medium 1630 containing 15% serum. The resulting solution was then sterilized by filtration through a 20-$\mu$m Nalgene Filter unit (Syrcon Corp., Rochester, N. Y.). Five hr after cis-DDP removal, cells were suspended in the above solution with thiouria, kept for 1 hr, and washed back into RPMI Medium 1630 containing 15% serum and 50 $\mu$M 2-mercaptoethanol. Alkaline elution was then performed at the appropriate time intervals.

**RESULTS**

The sensitivities of the parent (L1210/NCI) and resistant (L1210/PAM) cell lines to cis-DDP were measured by colony formation in soft agar after 1-hr drug treatments (Chart 1). The parent line exhibited a 2-component survival curve, with the second component constituting about 10% of the total cells; the latter might be due to a component of resistant cells in the population. The survival curve of the resistant line exhibited a shoulder, thus giving a curvature in the opposite direction compared to the parent line. At high drug concentrations, the slopes of the survival curves for the 2 cell lines were similar.

In relating survival to DNA damage, it is the first log of cell kill that is important, in that it represents 90% of the cell mass. The resistant component in the L1210/NCI line would constitute only about 10% of the cells, and the contribution by this component to the DNA measurements would be small. The dose modification factor for equal survival over the first log of cell kill was approximately 4.

DNA interstrand cross-linking was measured over the same range of cis-DDP dosage as in the survival studies (Chart 2). The measurement was made 6 hr after a 1-hr exposure to drug, so as to allow enough time for DNA:platinum(II) monoadducts to react further to form interstrand cross-links (9). Interstrand cross-linking was nearly proportional to drug concentration. The slopes of the interstrand cross-linking versus concentration lines for the parent and resistant cells were in a proportion of 1.7:1. This was significantly less than the ratio of 4:1 which might have been expected from the dose-modification factor for survival (11).

cis-platinum(II) also produces DNA:protein cross-links (9). Since DNA:protein cross-link formation is relatively rapid, this measurement was made immediately after drug treatment (Table 1). Three comparative determinations of DNA:protein cross-linking were made. In no case was DNA:protein cross-linking in the resistant cells significantly less than in the sensitive cells. Hence, the resistance cannot be attributed to reduced drug uptake or to generally reduced intracellular reactivity.

Kinetic studies were then carried out in order to determine whether the discrepancy between the dose modification factors for survival and interstrand cross-linking might be due to differences in interstrand cross-link formation or removal rates. Initial experiments at first suggested that interstrand cross-links were removed significantly faster in the resistant cells than in the parent cells, especially noted after 18 hr (Chart 3). This apparent result was confirmed over a range of drug concentrations (Chart 4).

The number of interstrand cross-links present at any one time, however, depends on the net result of cross-link formation and removal. These 2 processes can be separated by means of thiouria, which can quench any remaining reactive DNA:platinum(II) monoadducts (1, 10). Cells were treated with cis-DDP for 1 hr, postincubated for 5 hr, and then exposed to 0.1 M thiouria for 1 hr. Previous work had shown that this thiouria treatment is by itself nontoxic and improves the survival of cis-DDP-treated cells (10). The thiouria treatment stopped any

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**Table 1**

<table>
<thead>
<tr>
<th>cis-DDP ($\mu$M)</th>
<th>L1210/NCI (parent)</th>
<th>L1210/PAM (resistant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>200</td>
<td>180</td>
</tr>
<tr>
<td>30</td>
<td>250</td>
<td>265</td>
</tr>
<tr>
<td>40</td>
<td>280</td>
<td>360</td>
</tr>
</tbody>
</table>

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**Chart 1**

Survival of colony-forming ability of parent (L1210/NCI) and resistant (L1210/PAM) cell lines treated with cis-DDP for 1 hr.

**Chart 2**

DNA:protein cross-linking (in rad-equivalents) after treatment of cells with cis-DDP for 1 hr.
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Further cross-link formation and allowed the true rate of removal of interstrand cross-links to be measured. It was assumed that thiourea does not itself remove cross-links (1). The first-order rate constants for interstrand cross-link removal were 0.156 ± 0.016 (S.D.)/hr for the parent line treated with 20 μM cis-platinum and 0.151 ± 0.008/hr for the resistant line treated with 40 μM cis-platinum (Chart 5). The 2 cell lines thus did not differ with respect to interstrand cross-link removal rate.

The alternative possibility was then considered that the difference between the 2 cell lines might reside in a difference in the ability to quench or repair monoadducts that otherwise could go on to form interstrand cross-links. This can be represented by Scheme 1:

DNA:platinum(ll) monoadduct → interstrand cross-link → repair

This simplified model assumes that the number of potentially cross-linkable monoadducts formed initially (M₀) is proportional to the concentration of drug added (c). Monoadducts are assumed to be converted to interstrand cross-links (X) at a rate governed by a first-order rate constant, k₁, and interstrand cross-links are assumed to be repaired according to a first-order rate constant, k₂. DNA:platinum(ll) monoadducts are assumed to be quenched or repaired in a pseudo-first-order process, given by k₃, which may differ in the 2 cell types. k₁, k₂, and M₀ are assumed to be the same in the 2 cell lines. The question posed is whether a difference in ability to quench (or repair) monoadducts (k₃) could account simultaneously for the observed differences in peak interstrand cross-linking and in apparent interstrand cross-link removal rate (Charts 3 and 4).

The number of interstrand cross-links as a function of time, according to Scheme 1, is governed by the differential equations

\[ \frac{dM}{dt} = -(k₁ + k₃) M \]  

and

\[ \frac{dX}{dt} = k₁ M - k₂ X \]

Equation C represents the decay of monoadducts; its solution, under the initial condition M = M₀ at t = 0, is

\[ M(t) = M₀ e^{-k₁ t} \]

Substitution of Equation C' into Equation D, which represents the balance between formation and repair of cross-links, gives

\[ \frac{dX}{dt} = k₁ M₀ e^{-k₁ t} - k₂ X \]

The solution to Equation E, under the initial condition that there are no cross-links (X = 0) at t = 0, is

\[ X(t) = \frac{k₁ M₀}{k₁ - k₂ + k₃} [e^{-k₁ t} - e^{-k₂ t}] \]

As stated above, it is reasonable to assume that the effective initial concentration of monoadducts is proportional to the concentration of added drug, c; thus, M₀ = m₀c, where m₀ is the proportionality constant.
The value for $k_2$ in Equation F was obtained from the independent experiments using thiourea (Chart 5) and was fixed at 0.154/hr. Additional independent experiments were performed and combined with the data represented in Charts 3 and 4 to produce a set of kinetic data for several drug concentrations for the 2 cell lines. A curve-fitting computation was first carried out on the assumption that monoadduct quenching or repair in the parent cell line was negligible ($k_2^{m_0} = 0$). The best fits for the parameters, $m_0$, $k_1$, and $k_2^{m_0}$ for this case, are shown in Table 2. The calculated kinetic curves and the corresponding data points are shown in Chart 6. A similar computation was also carried out under the assumption that $k_2^{m_0} = 0.05$/hr, and the best fit of parameters for this case is shown in Table 2. The fit of the kinetic curves to the experimental data was equally as good in this case as in the case of $k_2^{m_0} = 0$.

**DISCUSSION**

The resistant cell line (L1210/PAM) that was the subject of our investigation was originally selected for resistance to L-phenylalanine mustard and was found to be cross-resistant to cis-DDP (5). The line exhibited peaks of interstrand cross-linking following treatment with L-phenylalanine mustard or with cis-DDP than did its parent line (L1210/NCI) (11). In the case of L-phenylalanine mustard, the decrease in peak interstrand cross-linking had been found to be commensurate with the magnitude of the resistance. For cis-DDP, however, the decrease in interstrand cross-linking was less than expected from the magnitude of the resistance. The foregoing results had been obtained using ascites cells grown in mice.

The current work investigated the possibility that the poor correspondence between interstrand cross-linking and cell survival could be due to a difference in the kinetics of cross-link formation or repair. For the current work, cell culture lines cloned from the original ascites cells were used. The lack of correspondence between interstrand cross-linking and cell survival following treatment with cis-DDP was similar to that which had been observed in the ascites cells, in that the ratio between the cross-linking peaks (which occurred 6 hr after 1-hr drug exposures in both cell lines) was less than expected from the sensitivity difference measured by colony formation. Thus, survival of colony-forming ability was not a function of peak interstrand cross-linking alone. The evidence obtained argued against the possibility of the discrepancy being due to either a reduced drug uptake or to an enhanced interstrand cross-link repair rate in the resistant cells. The evidence was, however, consistent with the possibility that the resistant cells have an enhanced ability to quench or repair DNA: platinum(II) monoadducts that otherwise could react or form interstrand cross-links. The reasoning that leads to this conclusion will next be examined.

The finding that the resistant cell line did not form significantly less DNA:protein cross-linking than did the sensitive line (Table 1) argues against the possibility that a difference in drug uptake is a major factor. (This is not inconsistent with the hypothesis that the cell lines differ in monoadduct quenching rates, because DNA: protein cross-link formation is rapid enough to go to completion before there is enough time for significant quenching of monoadducts to take place.)

The formation of DNA interstrand cross-links by cis-DDP occurs in a sequence of 2 steps, an initial production of monoadducts followed by the conversion of some of these monoadducts to interstrand cross-links (9). The time required for the second step of this reaction is shown by the continued rise in interstrand cross-linking to a peak 6 hr after washing of the cells with drug-free medium. It is unlikely that the delayed rise in interstrand cross-linking is mainly due to unreacted free platinum species retained in the washed cells, because platinum species such as aquated forms, which might not efflux readily from the cells, would probably react rapidly with thiol or amino groups within the cell. Thiol groups are the most reactive platinum binders within the cell. Some of these groups may also have the ability to bind to and thereby quench the second reactive position of platinum species bound to DNA as monoadducts. As we will see, our kinetic data are consistent with the possibility that the L1210/PAM line that we studied has an increased content of a thiol that is capable of quenching DNA: platinum(II) monoadducts.

The possibility that a thiol is responsible for the quenching is suggested by the finding of Suzukake et al. (7, 8) that this cell line has an increased glutathione content that could be responsible for its resistance to L-phenylalanine mustard.

A nonphysiological example of a compound that rapidly quenches DNA: platinum(II) monoadducts is thiourea (1, 10). The ability of thiourea to stop further conversion of monoadducts to interstrand cross-links was utilized to allow the measurement of true interstrand cross-link removal rates. We found in this way that the resistant and parent cell lines removed interstrand cross-links at similar rates.

The kinetic model for resistance based on enhanced quenching of monoadducts (Scheme 1) yielded a fit to the experimental data, as shown in Chart 6. The parameters for best fit to the kinetic equation (Equation C) are given in Table 2.

**Table 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$k_2^{m_0}$ (hr$^{-1}$)</th>
<th>$k_1$ (hr$^{-1}$)</th>
<th>$m_0$ rad-equivalents</th>
<th>$k_2^{m_0} - k_2^{m_0}$ (hr$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First computation</td>
<td>0</td>
<td>0.108 ± 0.005$^a$</td>
<td>10.9 ± 0.3</td>
<td>0.109 ± 0.007</td>
</tr>
<tr>
<td>Parameter value</td>
<td>0.571</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dependency value$^a$</td>
<td></td>
<td></td>
<td></td>
<td>0.619</td>
</tr>
<tr>
<td>Second computation</td>
<td>0.05</td>
<td>0.058 ± 0.005</td>
<td>20.2 ± 1.2</td>
<td>0.109 ± 0.007</td>
</tr>
<tr>
<td>Parameter value</td>
<td>0.932</td>
<td></td>
<td></td>
<td>0.932</td>
</tr>
<tr>
<td>Dependency value$^a$</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

$^a$ Mean ± S.D.

$^a$ The dependency value is the fraction of the variance of the given parameter that can be attributed to uncertainty in the other parameters (2).
The kinetic equations applied to the parent and resistant cell lines include 5 parameters: $m_0$, $k_1$, $k_2$, $k_{\text{rem}}$, and $k_{\text{pen}}$. The hypothesis is that the difference in interstrand cross-link kinetics in the 2 cell lines is due to a difference in the pseudo-first-order rate constants for monoadduct quenching in the parent ($k_{\text{rem}}$) and resistant cells ($k_{\text{pen}}$). Five unknown parameters, however, would be too many to allow a test of the hypothesis. Fortunately, the value of $k_2$, the rate constant for interstrand cross-link removal, was obtained from independent experiments using thiourea, and was found to be the same in the 2 cell lines. The available data, however, were still inadequate to determine the remaining 4 parameters by direct-curve fitting.

The initial assumption was therefore made that the quenching rate in the parent cells, $k_{\text{rem}}$, equals 0. The best fit to the data under this assumption is given in Table 2 and in Chart 6. All of the data points in Chart 6, which includes data for 7 different kinetic curves for various drug concentrations applied to the 2 cell lines, were simultaneously fit to the 3 parameters, $m_0$, $k_1$, and $k_{\text{pen}}$. The fit between data points and theoretical curves in Chart 6 is not disappointing, in view of the simplifying assumptions.

The main deviation between theory and experiment was in data points on the ascending limb of the cross-linking curves for the resistant line. The weakest assumptions, both of which could influence this part of the kinetics, are: (a) neglect of possible continued monoadduct formation by reactive platinum species remaining in the cells despite washing of the cells; such species might be quenched more rapidly in the resistant cells; and (b) neglect of monoadduct quenching which may occur in the parent cell line (i.e., the assumption that $k_{\text{rem}} = 0$).

The theoretic interpretation was further tested by carrying out similar curve-fitting computations using several values of $k_{\text{rem}} > 0$. An example, for the case of $k_3^{\text{rem}} = 0.05/hr$, is shown in Table 2. The main point to be noted here is that, regardless of the exact value chosen for $k_3^{\text{rem}}$, the difference, $k_{\text{pen}} - k_3^{\text{rem}}$, was essentially unchanged. Furthermore, the low S.D. and dependency value for this parameter attest to the validity of these estimates. The data thus indicate a self-consistent value (0.109/hr) for the enhancement in a pseudo-first-order rate constant for monoadduct quenching in the resistant line.

The values of $m_0$ and $k_1$, which govern the height of the cross-linking peak, are not uniquely determined. It is interesting to note, however, that the product, $k_1m_0$, was little affected by the choice of $k_3^{\text{rem}}$. This further encourages us to believe that this analysis has captured an element of truth.

The greater-than-expected resistance of the L1210/PAM line may thus be due to an early quenching of DNA:platinum(II) monoadducts. The proposed mechanism is as follows. In the parent L1210/NCI cells, the conversion of monoadducts to cross-links continues over a period of 20 hr or more ($k_1 = 0.108/hr$). The quenching process in the resistant cells shortens the time period over which cross-links continue to form from monoadducts, and allows the cross-link repair mechanisms to clear these lesions from the DNA more quickly.

In a recent study of cis-DDP-resistant lines of L1210 cells, Strandberg et al. (6) also noted greater resistance than was expected on the basis of observable cross-linking differences. An interesting observation was that DNA:protein cross-links persisted for up to 72 hr in the sensitive but not in the resistant lines. The authors, however, still could not account for the high degree of resistance (dose modification factors of 20 to 50). The resistance of these lines might in part also be due to enhanced quenching of monoadducts.

REFERENCES

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