Accumulation of *cis*-Diamminedichloroplatinum(II) and Platinum Analogues by Platinum-resistant Murine Leukemia Cells *in Vitro*¹

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**ABSTRACT**

Three murine leukemia lines resistant to *cis*-diamminedichloroplatinum(II) and one line resistant to diaminocyclohexane (DACH) platinum(II) complexes were compared to their platinum-sensitive parent lines to determine whether differences in net platinum accumulation were related to the resistant phenotype. The *cis*-diamminedichloroplatinum(II)-resistant lines (L1210PtR4, L1210DDP5, P388PtR4) and the DACH-resistant line (L1210DACH) were incubated in *vitro* with *cis*-diamminedichloroplatinum(II), [sp-4-2-(1R,2R)-1,2-cyclohexanediamine-N,N']dichloroplatinum(II), [sp-4-2-(1R,2R)-1,2-cyclohexanediamine-N,N'](ethanedioato(2-)-O,O')platinum(II), and diaminocyclobutane dicarboxylato platinum(II) and the time-dependent cellular platinum levels determined by flameless atomic absorption spectrophotometry. Cell lines resistant to a given platinum complex showed a reduction in the rate of platinum accumulation when compared to the sensitive line at 37°C. Intracellular levels of *cis*-diamminedichloroplatinum(II) were too low to confidently measure under the conditions of this study. Our data suggest that the mechanism of platinum resistance in these cell lines may be related to a reduced accumulation of the platinum-containing drug, although patterns of cross-resistance suggest other mechanisms may be operative as well.

**INTRODUCTION**

The development of resistance to chemotherapeutic agents used in the treatment of neoplastic disease often limits the efficacy of drugs resulting in subsequent failure of the treatment. Mechanisms of drug resistance have been the subject of numerous reports and for some drug and tumor types are beginning to be well characterized (2, 3). One of the mechanisms of drug resistance to emerge from these studies appears to be differences in drug transport between sensitive and resistant cells (4–6).

Aspects of resistance to *cis*-DDP² have been fruitfully studied in murine leukemia models originally described by Burchenal and colleagues (7, 8), Most studies of resistance in *cis*-DDP-resistant tumor systems have focused on descriptions of cross-resistance, differences in patterns of alklylation, differences in glutathione content, and differences in DNA cross-linking between sensitive and resistant cell lines (9–17). The kinetics of platinum accumulation in resistant cell lines has received little attention although some reports of platinum levels in sensitive and resistant cells have shown reduced amounts of platinum present in resistant cells (19–21).

To determine whether differences in intracellular platinum levels are involved in platinum resistance, we investigated the accumulation of platinum from four platinum-containing drugs including a compound undergoing clinical trial (22) in two sensitive and four resistant murine leukemia lines *in vitro*. To assess the contribution of glutathione to the resistant phenotype, we also measured the reduced glutathione levels in sensitive and resistant cells. Our results suggest that reduced accumulation of platinum in the resistant cell lines is related to the mechanism of resistance to these platinum compounds.

**MATERIALS AND METHODS**

**Drugs and Chemicals.** *cis*-DDP, DACH Cl₂, DACH oxalate, and CBDCA were obtained from J. D. Hoeschele (Warner-Lambert Co.). *cis*-DDP was dissolved at 1 mg/ml in 0.9% NaCl and stored at −20°C until diluted at time of use. DACH Cl₂ was dissolved in N,N-dimethylformamide at 2 mg/ml and stored at −20°C until dilution in 0.9% NaCl immediately prior to use. Solutions of the two carboxylate compounds were made immediately prior to use in 0.9% NaCl. N,N-dihydroxyethylpropanoic-N'-2-ethanesulfonic acid, reduced glutathione, one IV glutathione reductase, and 5,5'-dithiobis(2-nitrobenzoic acid) were from Sigma (St. Louis, MO). Doxorubicin and L-phenylalanine mustard (melphalan) were also obtained from Sigma Chemical Co. and were dissolved immediately prior to use in 0.9% NaCl and 55 mM HCl in 75% ethanol, respectively. 1,3-Bis(2-chlorethyl)-1-nitrosourea was a gift from J. Shilliss (Warner-Lambert Co.) and was dissolved in 50% dimethylsulfoxide. Nitric acid and dimethylsulfoxide were from Fisher Scientific (Fair Lawn, NJ). Perchloric acid and N,N-dimethylformamide were from MCB (Cincinnati, OH).

**Cell Lines.** Two murine lines, L1210PtR4 and P388PtR4, were established *in vitro* from two *in vivo* cis-DDP-resistant lines from W. R. Leopold (Warner-Lambert Co.) which had been previously selected *in vivo* with cis-DDP. Two other murine lines, L1210DDP5 and L1210DACH, were obtained from A. Eastman (University of Nebraska, Omaha, NE). The L1210PtR4 and P388PtR4 lines were cultured in increasing concentrations of *cis*-DDP until a level of 4 µg/ml drug was reached and the doubling times were roughly equivalent to those of the L1210S and P388S lines. The L1210PtR4 and P388PtR4 lines were routinely passed in media containing 4 µg/ml cis-DDP; the L1210DDP5 lines was maintained in 5 µg/ml cis-DDP. DACH Cl₂ at 5 µg/ml was present in the passage medium of the L1210DACH line. Cells were removed from drug-containing media and passed for 1 wk prior to experimental use in the appropriate medium containing no platinum complex to assure drug-free cells. The L1210 lines were grown in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (KC Biologicals, Kansas City, KS) and 50 µg/ml gentamycin sulfate (Sigma, St. Louis, MO). The P388 lines were cultured in Fischer's medium (Gibco) supplemented with 10% donor equine serum (HyClone, Logan, UT) and 50 µg/ml gentamicin sulfate. Additionally, for routine passage, the P388PtR4 line was grown in the presence of 2.5 µM 2-mercaptoethanol. All cells used were cultured in humidified 95% air–5% CO₂ at 37°C and were maintained in exponential phase growth.

**Relative cell volume** was determined with a Becton-Dickinson FACS analyzer (Sunnyvale, CA).

**Drug Treatments and Accumulation Studies.** Determination of the ID₅₀ values of the drugs in the sensitive and resistant lines was accomplished by incubating cells with drug in duplicate wells of 24-well plates and counting the cells after 72 h using an electronic particle counter (Coulter Electronics, Hialeah, FL). For accumulation studies, exponentially growing cells at 1 x 10⁶ cells/ml in appropriate fresh medium (RPMI 1640 or Fischer's with serum) were incubated with 20 µg/ml drug at 37°C in a shaking water bath. Aliquots of 2.00 ml were

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³The abbreviations used arc: *cis*-DDP, *cis*-diamminedichloroplatinum(II); DACH Cl₂, [sp-4-2-(1R,2R)-1,2-cyclohexanediamine-N,N']dichloroplatinum(II); DACH oxalate, [sp-4-2-(1R,2R)-1,2-cyclohexanediamine-N,N'](ethanedioato(2-)-O,O')platinum(II); CBDCA, diammine[1,1-cyclobutatedicarboxylato(2-)-1,2]-O,O'platinum(II); ID₅₀, concentration that inhibits 72-h cell growth by 50%.
taken at intervals, centrifuged at 1000 × g for 0.8 min, resuspended in ice-cold phosphate-buffered saline, pH 7.2, recentrifuged, and stored at \(-20\)°C. Initial rates of uptake were also initially determined in Earle’s balanced salt solution (Gibco) or a salts-glucose medium (0.05 M N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid, pH 7.2, 0.1 M NaCl, 2 mM CaCl₂, 5 mM glucose, 5 mM KCl). For rates of platinum accumulation, time points were taken over the course of 70 or 80 min.

Glutathione Determination. The glutathione contents of five cell lines were determined by an enzymatic assay utilizing glutathione reductase (23).

Platinum Determinations. Cell pellets were digested in nitric/perchloric acid (24) and platinum content was determined by graphite furnace atomic absorption spectrophotometry using a model AA1475 with a GTA-95 furnace (Varian Associates, Sunnyvale, CA). Values for each sample were determined in triplicate.

RESULTS The resistance and cross-resistance of four murine leukemia cell lines to cis-DDP, three analogues, and three non-platinum drugs are shown in Table 1. The L1210 cis-DDP-resistant lines (L1210PtR4 and L1210DDP5) were found to be sensitive to the two DACH-containing complexes while the DACH-resistant L1210 line was relatively sensitive to cis-DDP as expected (11) as well as to the second generation platinum complex, CBDCA. The L1210PtR4 cell line and to a lesser extent the L1210DDP5 line were collaterally sensitive to DACH oxalate. The resistant P388PtR4 line was not sensitive to either of the DACH compounds. None of the cell lines showed appreciable resistance to melphalan or doxorubicin while the P388PtR4 line was 3-fold cross-resistant to 1-3-bis(2 chloroethyl)-1-nitrosourea. That the resistance was stable was demonstrated by three of the platinum compounds in the L1210PtR4 line was 3-fold cross-resistant to l-3-bis(2 chloroethyl)-l-nitrosourea. That the resistance was stable was demonstrated by three of the platinum compounds in the L1210PtR4 line was 3-fold cross-resistant to l-3-bis(2 chloroethyl)-l-nitrosourea. That the resistance was stable was demonstrated by these cell lines to three of the platinum compounds in the L1210PtR4 line was 3-fold cross-resistant to l-3-bis(2 chloroethyl)-l-nitrosourea. That the resistance was stable was demonstrated by these cell lines to three of the platinum compounds.

Intracellular glutathione was measured to determine whether elevated levels contributed to the resistant phenotype as has been investigated elsewhere (12, 25-27). The data in Table 2 show no significant differences in glutathione content between sensitive and resistant lines.

To determine whether the basis for the resistance demonstrated by these cell lines to three of the platinum compounds (Fig. 1) was related to alterations in drug accumulation, the rate of drug accumulation in RPMI 1640 or Fischer’s medium supplemented with 10% serum was measured in sensitive and resistant cell lines. Initial experiments were performed in Earle’s balanced salt solution, in a serum-free salts-glucose medium (28), or in serum-free RPMI 1640, but in each case the amount accumulated was less than in complete nutrient media supplemented with serum (data not shown). To allay concern about potential interactions of platinum-containing drugs with serum proteins leading to alterations in activity, an ID₅₀ concentration was determined in which drugs were incubated with serum-containing cell culture medium for 1 h prior to exposure of the cells to the drug-medium combination. The results of this experiment are shown in Table 3 in which none of the fifteen drug-cell line combinations showed an ID₅₀ value outside the range of variation normally present in this test.

Attempts to measure rates of intracellular drug accumulation at concentrations lower than 50–70 μM were not successful since the amounts found in cells at early times were below the detection limit. These concentrations were relatively nontoxic to the cells because the cells were only exposed to drug for up to 75 min. The doses of cis-DDP required to inhibit the cell growth over 72 h by 50% of control cell growth after only a 1-h exposure of the cells to drug were 21 μM for L1210S, 245 μM for L1210PtR4, 290 μM for L1210DDP5, 42 μM for L1210DACH, 5.0 μM for P388S, and 325 μM for P388PtR4 cell lines. Therefore, the ratio of ID₅₀ concentrations resulting from a 1-h treatment of cells with drug to the concentration of cis-DDP used for uptake ranged from 13-fold for P388S to 0.2-fold for P388PtR4.

An uptake study of CBDCA was attempted, but under conditions of this study, the amounts of platinum were close to undetectable (data not shown). The low potency of CBDCA in comparison with cis-DDP may result from the relative lack of intracellular accumulation in both sensitive and resistant lines.

Since large differences in sensitivity of these cell lines to the platinum-containing drugs existed (Table 1), the accumulation of platinum from these drugs was examined over a 75-min incubation at 37°C. The net uptake of cis-DDP by the two cis-DDP-resistant lines (L1210PtR4 and L1210DDP5) and the DACH-resistant line was reduced when compared to the sensitive L1210 line (Table 4). The uptake of DACH-platinum complexes in the cis-DDP-resistant lines was not appreciably altered over that of the sensitive L1210 line. A more striking result was the lower net accumulation of platinum from DACH compounds in the L1210DACH line (Fig. 2). The net uptake was approximately 25% of that in the sensitive cell line. The accumulation of platinum in the P388PtR4 line was approximately 50% of the sensitive line for the three platinum complexes characterized.

DISCUSSION A previous study (29) has indirectly characterized the transport of cis-DDP by using a colony-forming unit assay which resulted in the postulation of a passive diffusion mechanism for the membrane transport of platinum. An earlier study of cis-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cis-DDP</th>
<th>DACH Cl⁻</th>
<th>DACH oxalate</th>
<th>CBDCA</th>
<th>Melphalan</th>
<th>Adriamycin</th>
<th>BCNU*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210 S</td>
<td>0.80</td>
<td>0.20</td>
<td>0.41</td>
<td>10.9</td>
<td>2.32</td>
<td>0.29</td>
<td>9.71</td>
</tr>
<tr>
<td>L1210PtR4</td>
<td>10.8 (13.5)*</td>
<td>0.20 (1)</td>
<td>0.11 (0.3)</td>
<td>57.5 (5.3)</td>
<td>3.89 (1.7)</td>
<td>0.27 (0.9)</td>
<td>9.0 (0.9)</td>
</tr>
<tr>
<td>L1210DDP5</td>
<td>20.6 (25.8)</td>
<td>0.51 (2.6)</td>
<td>0.22 (0.5)</td>
<td>97.8 (9.0)</td>
<td>7.94 (3.4)</td>
<td>0.40 (1.4)</td>
<td>10.1 (1.0)</td>
</tr>
<tr>
<td>L1210DACH</td>
<td>1.87 (2.3)</td>
<td>11.2 (56.1)</td>
<td>21.9 (53.4)</td>
<td>18.7 (1.7)</td>
<td>1.47 (0.6)</td>
<td>0.18 (0.6)</td>
<td>8.26 (0.9)</td>
</tr>
<tr>
<td>P388S</td>
<td>0.67</td>
<td>0.40</td>
<td>0.97</td>
<td>7.45</td>
<td>0.26</td>
<td>0.24</td>
<td>0.61</td>
</tr>
<tr>
<td>P388PtR4</td>
<td>16.2 (24.1)</td>
<td>5.59 (14.0)</td>
<td>17.1 (17.6)</td>
<td>40.5 (5.4)</td>
<td>0.88 (3.4)</td>
<td>0.10 (0.5)</td>
<td>1.64 (2.7)</td>
</tr>
</tbody>
</table>

* Values are means of at least three experiments with the exception of BCNU which was done in duplicate.

1. DACH Cl⁻ was dissolved in N,N-dimethyl formamide at 2,000 μg/ml and diluted in 0.9% NaCl. The highest concentration of dimethyl formamide in the wells (0.005% v/v) had no effect on cell growth.
2. BCNU, 1-3-bis(2 chloroethyl)-1-nitrosourea.
3. Numbers in parentheses, fold resistance defined as ID₅₀ of resistant line/ID₅₀ of sensitive line.
with 10% heat-inactivated fetal bovine serum (for 1.1210 lines) or Fischer's levels of reduced glutathione in the resistant lines were not significantly elevated  

Pi platinum accumulation in resistant LI210 cells 70% that of  

were far greater than those reported here probably as a result  

form of the ligand. A, c/s-DDP; B, DACH C12; C, DACH oxalate.  

studies. The two DACH-containing compounds were comprised of the trans-1  

the addition of cells for II),,, determination.  

medium containing 10% equine serum (for P388 cells) for l h at 37°C prior to  

from the corresponding sensitive lines (n = 3; P < 0.05 by the two-tailed t test).  

the sensitive cells was found although the initial rates were  

of uptake done with a greater concentration of cells.  

 accumulation has reported a time-dependent uptake of cis-DDP  

dichlorobis(pyridine)platinum(II) uptake also concluded that  

dichlorobis(pyridine)platinum(II) uptake also suggested that this platinum complex was transported into the cell by passive diffusion (30). One other study dealing with platinum accumulation has reported a time-dependent uptake of cis-DDP into L1210 cells (31) but the intracellular levels of platinum were far greater than those reported here probably as a result of uptake done with a greater concentration of cells.

Two recently published studies have investigated transport of  

Table 2 Glutathione content of sensitive and resistant cells

| Reduced glutathione (nmol/10^6 cells) |
|-----------------|-----------------|-----------------|
| L1210S          | 3.18 ± 0.16*a   |                 |
| L1210PIR4       | 3.84 ± 0.24     |                 |
| L1210DACH5      | 3.05 ± 0.18     |                 |
| P388S           | 2.56 ± 0.10     |                 |
| P388PIR4        | 3.05 ± 0.05     |                 |

*a Mean ± SD.

Table 3 Effect of preincubating drug in serum-containing media on ID_{so} values

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cis-DDP*</th>
<th>DACH Cl(_2)*</th>
<th>DACH oxalate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210S</td>
<td>0.42</td>
<td>0.65</td>
<td>0.74</td>
</tr>
<tr>
<td>L1210PIR4</td>
<td>1.1</td>
<td>0.73</td>
<td>2.2</td>
</tr>
<tr>
<td>L1210DACH</td>
<td>0.85</td>
<td>1.1</td>
<td>0.82</td>
</tr>
<tr>
<td>P388S</td>
<td>1.0</td>
<td>0.95</td>
<td>0.84</td>
</tr>
<tr>
<td>P388PIR4</td>
<td>1.9</td>
<td>0.74</td>
<td>0.96</td>
</tr>
</tbody>
</table>

*a Dose modification factor determined by:

\[ \text{ID}_{50} \text{ after 1-h preincubation} \div \text{ID}_{50} \text{ with no preincubation} \]

The accumulation studies reported here were done in culture media containing serum since transport performed in serum-free defined media took place at a lower rate (see above). Although some concern exists about potential interaction of drug with serum proteins, the kinetics of such interactions is slow enough that on the time scale of these uptake experiments the majority of drug remains unbound (34–37). Additional evidence for the lack of significant serum-drug interaction is given by the relative lack of effect on the ID_{50} values of a 1-h preincubation of drug with serum-containing media before exposure to cells (Table 3). The magnitude of the dose modification factor was within the ordinary amount of variation seen with in vitro ID_{50} determinations.

Although upon first appearance the 50–70 μM concentration of drugs used for the accumulation studies seems elevated, especially in relation to the ID_{50} values shown in Table 1, further examination shows this not to be the case. The 72-h growth delay ID_{50} values in Table 1 were determined in cells exposed to drug for the entire length of the test. An ID_{50} concentration determined by a 1-h exposure of cells to drug followed by a 72-h period of growth would be expected to be much higher, as is the case (see above). A 1-h exposure to drug, as opposed to a 72-h exposure, resulted in a 7- to 23-fold increase in the ID_{50} value, thus bringing the concentration of drugs used for accumulation over the course of an hour within the range of the ID_{50} concentrations. In addition, the concentrations of drugs used for the accumulation studies here were similar to those used in previously reported work (20, 21, 31).

Consideration of long-term accumulation (>10 min) of platinum in cells lends support to the notion that reduced accumulation of platinum compounds is involved in resistance. The difference in intracellular platinum levels is readily apparent in
the L1210S and L1210DDP5 cell lines. The accumulation in the resistant line was lower than in the sensitive line (Table 4), which was associated with an increased ID₅₀ over the sensitive line. This finding of reduced intracellular platinum in a cis-DDP resistant line is similar to the findings of Richon et al. (11), who reported a reduction in cellular platinum levels in their cis-DDP-resistant line.

The rate of platinum accumulation from cis-DDP in the L1210DACH line was reduced by 60% from that in the L1210S line. This reduction was comparable with the reduced rate seen in the two cis-DDP-resistant L1210 lines. The ID₅₀ for cis-DDP in the L1210DACH line was 2-fold greater than the ID₅₀ for cis-DDP in the L1210S cells, unlike the case of the two other L1210 lines where the ID₅₀ was increased 13- and 26-fold. A clear difference in the nature of resistance exists between the L1210DACH line and the two cis-DDP-resistant lines (L1210PtR4 and L1210DDP5) since a 60% reduction in the rate of accumulation of cis-DDP was associated with only a 2-fold increase in the ID₅₀. In the L1210PtR4 and L1210DDP5 lines, a 65 and 75% reduction in rate accompanied a 13- and 26-fold increase in the ID₅₀. Additional mechanisms of resistance besides reduction in transport apparently are present in the two cis-DDP-resistant L1210 lines since a similar reduction in accumulation was accompanied by a greater increase in the ID₅₀ than was found for L1210DACH.

Examination of accumulation of DACH platinum compounds also showed reduced intracellular content associated with an increased ID₅₀ value. Resistance resulting from reduced uptake of drug has been widely reported (2-6). In every case of platinum compounds also showed reduced intracellular content associated with an increased ID₅₀ value. Resistance resulting from reduced uptake of drug has been widely reported (2-6). In every case of platinum initially present in cells. Studies relating intracellular platinum levels to other mechanisms of resistance ought to be given consideration. Differential DNA cross-link formation resulting from treatment with cis-DDP in a cis-DDP-resistant line. The same resistant line treated with melphanol had numbers of DNA cross-links equal to those found in the sensitive line suggesting no difference in rates of DNA repair. If the concentration of platinum in the resistant line was reduced as is the case with the cell lines in this report, a reduction in DNA cross-links might be expected.

Differences in intracellular platinum levels in sensitive and resistant cells resulting from different rates of accumulation have implications on several subsequent mechanisms of resistance and therefore ought to be given consideration. Differential DNA cross-linking (15, 17), differential modification of DNA lesions (38), or differential repair of DNA damage in resistant versus sensitive lines (14, 39) may be dependent on the amount of platinum initially present in cells. Studies relating intracellular levels of platinum to other mechanisms of resistance ought to give a more thorough understanding of this multifaceted phenomenon.

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