Accumulation of cis-Diaminedichloroplatinum(II) and Platinum Analogues by Platinum-resistant Murine Leukemia Cells in Vivo

Alan J. Kraker and Charles W. Moore

ABSTRACT

Three murine leukemia lines resistant to cis-diaminedichloroplatinum(II) and one line resistant to diaminocyclobutane (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-diaminedichloroplatinum(II)-resistant lines (L1210PtR4, L1210DDP5, P388PtR4) and the DACH-resistant line (L1210DACH) were incubated in vitro with cis-diaminedichloroplatinum(II), [sp-4-2-(R,R)-1,2-cyclohexanedi-amine-N,N']dichloroplatinum(II), [sp-4-2-(R,R)-1,2-cyclohexanedi-amine-N,N'](ethanedioato(2-))O,O'platinum(II), or diaminocyclohexane (DACH) oxalate, [sp-4-2-(R,R)-l,2-cyclohexanediamine-A'A'[dichloroplatinum(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 diaminocyclobutanedicarboxylato(2-)platinum(II), or diaminocyclobutanedicarboxylatoplatinum(II) and the time-dependent cellular platinum levels determined by flameless atomic absorption spectrophotometry. Cells were removed from drug-containing media and passed for 1 wk before measurement. The L1210S and P388S lines. The L1210PtR4 and P388PtR4 lines reached confluence within 10 d at the same time point. The L1210DDP5 lines was maintained in 5 /¿g/ml cis-DDP. DACH C12 at 2.5 /¿g/ml was added to the L1210DDP5 lines at 37°C until diluted at time of use. DACH C12 was dissolved in N,N-dimethylformamide at 2 mg/ml and stored at ~20°C until dilution at 0.9% NaCl and stored at ~2°C until diluted at time of use. DACH CI1 was dissolved in N,N-dimethylformamide at 2 mg/ml and stored at ~2°C until dilution at 0.9% NaCl immediately prior to use. Solutions of the two carboxylate compounds were made immediately prior to use in 0.9% NaCl. N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid, reduced glutathione, type IV glutathione reductase, and 5,5'-dithiobis(2-nitrobenzoic acid) were from Sigma (St. Louis, MO). Doxorubicin and L-phenylalanine musturd (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-nitosourea was a gift from J. Shillies (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).

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 alkylation, 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 Cl1, 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 ~2°C until diluted at time of use. DACH Cl1 was dissolved in N,N-dimethylformamide at 2 mg/ml and stored at ~2°C until dilution at 0.9% NaCl immediately prior to use. Solutions of the two carboxylate compounds were made immediately prior to use in 0.9% NaCl. N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid, reduced glutathione, type 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-nitosourea was a gift from J. Shillies (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).
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-hydroxyethylpiperazine-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).

**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 melphanal 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 these cell lines to three of the platinum compounds. None of the cell lines showed appreciable differences in glutathione content between the two DACH-containing complexes while the DACH-resistant L1210 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 used for uptake ranging 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 1 Drug resistance in platinum-resistant murine lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cis-DDP</th>
<th>DACH Cl₂</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>0.40</td>
<td>0.30</td>
<td>0.31</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>7.84 (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.

- DACH Cl₂ 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.

- BCNU, 1-3-bis(2-chloroethyl)-1-nitrosourea.

- Numbers in parentheses, fold resistance defined as ID₅₀ of resistant line/ID₅₀ of sensitive line.
Reduced glutathione levels were determined by an enzymatic assay (22). The levels of reduced glutathione in the resistant lines were not significantly elevated from the corresponding sensitive lines (n = 3; P < 0.05 by the two-tailed t test).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Reduced glutathione (mmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210S</td>
<td>3.18 ± 0.16*</td>
</tr>
<tr>
<td>L1210PrR4</td>
<td>3.84 ± 0.24</td>
</tr>
<tr>
<td>L1210DACH</td>
<td>3.05 ± 0.18</td>
</tr>
<tr>
<td>P388S</td>
<td>2.56 ± 0.10</td>
</tr>
<tr>
<td>P388PrR4</td>
<td>3.05 ± 0.05</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Fig. 1. Structure of platinum compounds utilized for uptake and accumulation studies. The two DACH-containing compounds were comprised of the trans-1 form of the ligand. A, cis-DDP; B, DACH Cl₂; C, DACH oxalate.

Table 3 Effect of preincubating drug in serum-containing media on ID₅₀ values

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cis-DDP</th>
<th>DACH Cl₂</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>L1210PrR4</td>
<td>1.1</td>
<td>0.73</td>
<td>2.2</td>
</tr>
<tr>
<td>L1210DACH</td>
<td>0.85</td>
<td>1.11</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>P388PrR4</td>
<td>1.9</td>
<td>0.74</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* Dose modification factor determined by:

ID₅₀ after 1-h preincubation
ID₅₀ with no preincubation

ACCUMULATION AND RESISTANCE TO PLATINUM COMPOUNDS

Table 4 Platinum accumulation in sensitive and resistant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cis-DDP</th>
<th>DACH Cl₂</th>
<th>DACH oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210S</td>
<td>0.079</td>
<td>0.066</td>
<td>0.064</td>
</tr>
<tr>
<td>L1210PrR4</td>
<td>0.027</td>
<td>0.053</td>
<td>0.064</td>
</tr>
<tr>
<td>L1210DACH</td>
<td>0.020</td>
<td>0.053</td>
<td>0.056</td>
</tr>
<tr>
<td>P388S</td>
<td>0.031</td>
<td>0.027</td>
<td>0.017</td>
</tr>
<tr>
<td>P388PrR4</td>
<td>0.029</td>
<td>0.022</td>
<td>0.022</td>
</tr>
</tbody>
</table>

* Values are means of two determinations done over the course of 90 min.

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 ID₅₀ values of a 1-h preincubation of drug with serum-containing media before exposure to cells (Table 3). The magnitude of the dose modification factors was within the ordinary amount of variation seen with in vitro ID₅₀ determinations.

Thus, upon first appearance the 50–70 µM concentration of drugs used for the accumulation studies seems elevated, especially in relation to the ID₅₀ values shown in Table 1, further examination shows this not to be the case. The 72-h growth delay ID₅₀ values in Table 1 were determined in cells exposed to drug for the entire length of the test. An ID₅₀ 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 ID₅₀ value, thus bringing the concentration of drugs used for accumulation over the course of an hour within the range of the ID₅₀ 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 ID_{50} 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 ID_{50} for cis-DDP in the L1210DACH line was 2-fold greater than the ID_{50} for cis-DDP in the L1210S cells, unlike the case of the two other L1210 lines where the ID_{50} 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 ID_{50}. In the L1210PtR4 and L1210DDP5 lines, a 65 and 75% reduction in rate accompanied a 13- and 26-fold increase in the ID_{50} value. 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 ID_{50} than was found for L1210DACH.

Examination of accumulation of DACH platinum compounds also showed reduced intracellular content associated with an increased ID_{50} value. Resistance resulting from reduced uptake of drug has been widely reported (2-6). In every case of resistance or cross-resistance to platinum complexes exhibited with an increased ID_{50} value. Resistance resulting from reduced transport of the ID_{50} was evident in these platinum-resistant lines. Since the degree of platinum initially present in cells. Studies relating intracellular platinum content. P388PtR4 cells by glutathione depletion. Cancer Res., 45:6250-6253, 1985.

The differences in rates of accumulation reported here contrast with previously published work which showed a small reduction in intracellular platinum in a cis-DDP-resistant line. The same resistant line treated with melphalan 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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