Single Step Selection of cis-Diaminedichloroplatinum(II) Resistant Mutants from a Human Ovarian Carcinoma Cell Line

Karen McLaughlin, Imogen Stephens, Nancy McMahon, and Robert Brown

Department of Medical Oncology, Cancer Research Campaign, Alexander Stone Building, CRC Beatson Laboratories, Garscube Estate, Bearsden, Glasgow G61 1BD, United Kingdom

Abstract

We have shown that cis-diaminedichloroplatinum(II) (DDP) resistant mutants can be isolated from the human ovarian carcinoma cell line A2780 using a single-step selection protocol with DDP. DDP resistant colonies were calculated to be present at a frequency of $1.7 \times 10^{-4}$/viable cell using a fluctuation analysis. The mutational origin of these surviving colonies is inferred by the fact that their frequency is increased by treatment of the A2780 cells with the chemical mutagen ethyl methanesulfonate, with a maximum frequency observed after a 3-day expression time. Independently isolated clones maintain, in the absence of selection, a DDP resistant phenotype up to 7-fold more resistant than the parental A2780 cells. The resistance modifiers aphidicolin and buthionine sulfoximine have no effect on the frequency of DDP resistant mutants. Therefore neither of these drugs appears to have an effect on increasing the sensitivity of DDP resistant mutants existing in a cell population prior to DDP exposure.

Introduction

DDP is an effective chemotherapeutic agent against a variety of tumor types (1). However, in many cases initial response is followed by relapse and failure of tumors subsequently to respond to chemotherapy. This has led to the suggestion that cells within the tumor have acquired specific cellular resistance mechanisms, causing these cells to have a selective advantage during treatment and thus to eventually predominate in the tumor (2). A variety of mechanisms of resistance to DDP have been suggested in cell lines selected in vitro for resistance (3-5). Most of these lines have been isolated after prolonged exposure to DDP, leading to the possibility of multiple mechanisms occurring together. It is not clear if these resistant cell lines will have mechanisms relevant to clinical resistance acquired during chemotherapy. Goldie and Coldman (6) have described a mathematical model for progression to resistance based on theoretical considerations of mutation frequencies and the assumption that drug resistant mutants exist or are induced in the tumor cell population. Using multiple exposure selection protocols it is not possible to measure the frequency of resistant variants in a cell population prior to DDP exposure or factors affecting this frequency.

A variety of drug selection protocols have been used to examine drug resistance mutations in mammalian cells (7, 8). The most reliable and quantitative selection procedures have used single step selection protocols that allow the identification of those rare cells that are drug resistant (8, 9). One important question concerning such drug resistant variants is whether or not they represent gene mutations in specific genes. In cases where the gene or enzyme conferring drug resistance is unknown, the mutational origin of drug resistance has been inferred by mutagens inducing an increase in the frequency of resistant cells and by the stable retention of the resistant phenotype in the absence of selection (8, 9).

DDP is used widely in the treatment of ovarian cancers (10). DDP resistant cells have previously been isolated from the human ovarian carcinoma cell line A2780 using repeated selection protocols (11). We have used this line to assess whether DDP resistant lines can be isolated using a single-step selection protocol. In order to provide evidence for a mutational origin, the effect of the chemical mutagen EMS on DDP resistant variant frequency has been examined. Isolated lines surviving DDP have subsequently been assayed for DDP resistance after prolonged periods of growth in nonselective media. Drugs which inhibit specific drug resistance mechanisms have been suggested as a means of increasing response of resistant tumors to chemotherapy or of increasing the efficacy of chemotherapy at first treatment (12). Aphidicolin (13) and BSO (14) have been suggested to increase the DDP cytotoxicity in resistant lines by inhibiting polymerase $\alpha$ mediated DNA repair and depleting glutathione levels, respectively. The DDP resistant lines used in these studies were isolated after multiple selections with DDP; therefore it is unclear how representative these lines are of all possible resistant mutants that survive DDP treatment. If resistance modifiers are to be used clinically at the time of first treatment in order to eliminate any resistant subpopulations, then it is important to know how generally effective they are against resistant mutants in the tumor population. In order to assess this, we have examined the effect of aphidicolin and BSO on the frequency of cells surviving the single-step selection with DDP in A2780 cells not previously exposed to DDP.

Materials and Methods

Cell Lines and Routine Culture Conditions. A2780, an ovarian carcinoma cell line derived from an untreated patient, and A2780CP, a DDP resistant line produced by exposure of the A2780 line to multiple increasing concentrations of DDP (11), were the kind gifts of Drs. R. F. Ozols and T. C. Hamilton, Fox Chase Cancer Centre, Philadelphia, PA. Both cell lines were maintained as monolayers in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum and 0.005 unit/ml penicillin at 37°C in a 5% CO$_2$/95% air atmosphere. All lines were free of Mycoplasma contamination.

Drug Selection. A2780 cells were plated at $2 \times 10^6$ cells/75-cm$^2$ flask for $5-50 \mu$M DDP concentrations or at $10^3$ for $0-5 \mu$M DDP concentration. After 24 h the cells were treated with various doses of DDP for 4 h, washed twice with phosphate-buffered saline, and incubated in RPMI for 2 weeks; surviving colonies of more than 100 cells were then counted. The A2780 cells had a plating efficiency of about 20% under these conditions. The frequency of resistant colonies was calculated as:

Received 1/2/91; accepted 3/1/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Grant support by Cancer Research Campaign of Great Britain and Caledonian Research Foundation grant to K. M.

2 To whom requests for reprints should be addressed.

The abbreviations used are: DDP, cis-diaminedichloroplatinum(II); EMS, ethyl methanesulfonate; BSO, buthionine sulfoximine; ID$_{50}$, concentration giving 50% survival; GSH, glutathione.
surviving colony number per viable cell or using fluctuation analysis (15) based on the number of cultures containing no surviving colonies. This latter analysis avoids any uncertainties due to daughter colony formation.

Mutagen Exposure Prior to DDP Selection. Cells ($5 \times 10^6$) were treated with 2.5 mg/ml EMS (Sigma) for 2 h in serum free medium. This concentration of EMS gave 22% survival of the A2780 cells. At various times after the exposure to EMS the cells were selected at $2 \times 10^6/75$-cm$^2$ flask in 15 $\mu$M DDP as described above.

Aphidicolin and BSO Treatment. Aphidicolin (Sigma; 2.5 and 5 $\mu$g/ml) was added 1 h prior to DDP and maintained during the DDP selection. These are conditions previously shown to inhibit removal of DNA bound platinum in A2780 cells treated with DDP (13). For BSO treatment, BSO (Sigma) was added 24 h prior to DDP and maintained during the DDP selection. Concentrations of BSO used (25 and 50 $\mu$M) were previously shown to deplete glutathione levels in A2780 cells and increase the sensitivity of A2780CP cells to DDP in short term cell viability assays (14).

Results and Discussion

Selection of DDP Variants. The surviving fraction of clonogenic cells of the human ovarian cell line A2780 after exposure to different concentrations of DDP is shown in Fig. 1. The DDP ID$_{50}$ was 0.6 $\mu$M which is comparable with the ID$_{50}$ observed by others using clonogenic assays of A2780 cells (11, 17). At concentrations of DDP between 15 and 30 $\mu$M a surviving fraction in the range 2 to $5 \times 10^{-6}$ was observed. This appears as a tail in the survival curve suggesting that a subpopulation of cells are resistant to this concentration of DDP (Fig. 1). Selection of the cells with 15 $\mu$M DDP (Table 1) gives a frequency of $3.2 \times 10^{-6}$ surviving colonies/viable cell. Using a fluctuation analysis (15) a spontaneous frequency of colonies surviving 15 $\mu$M DDP selection of $1.7 \times 10^{-6}$/viable cells was observed. This is a frequency comparable with known mutation frequencies for drug resistant mutants in mammalian cells (18). This questions whether the cells surviving DDP are mutants or simply variants which will survive DDP in a stochastic manner but do not represent stable genetic changes.

If the cells surviving 15 $\mu$M DDP are mutational in origin, then the frequency of these resistant cells should be increased with known mutagens. The requirement for an expression time of cells after mutagen treatment to allow phenotypic expression of resistance mechanisms has been well documented (9). The A2780 cells were treated with the chemical mutagen, EMS, and at varying times after exposure selected with 15 $\mu$M DDP. A maximum increase in DDP resistant colonies was observed 3 days after EMS exposure (Fig. 2). The increase in frequency of resistant colonies between days 1 and 3 supports a time dependent delay in the expression of a mutant phenotype and argues against the EMS cytotoxicity selecting for EMS resistant clones that are cross-resistant to DDP. The reduced frequency observed at later time points suggests that DDP resistant mutants may be selected against during nonselective growth conditions. Similar reductions in mutant frequency at longer expression times have been observed for other drug resistance selection systems (9). The resistant colony frequency 3 days after EMS treatment represents at least a 10-fold increase compared to the spontaneous frequency (Table 1). Thus EMS can induced DDP resistant clonogenic cells, supporting a mutational basis for the resistance.

If the cells maintain a drug resistant phenotype in the absence of DDP selection this also would support a stable genetic alteration leading to the drug resistant phenotype. After at least 40 generations of growth in nonselective media, the ID$_{50}$s to DDP of clones independently isolated after selection in 15 $\mu$M DDP were assessed using a short-term sensitivity assay, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction (Sigma) as a measure of cell viability as described previously (16).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viability$^a$</th>
<th>Mutant frequency$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
<td>$3.2 \pm 1.5 \times 10^{-4}$ (40)</td>
</tr>
<tr>
<td>EMS$^c$</td>
<td>0.22</td>
<td>$3.4 \pm 2.5 \times 10^{-4}$ (10)</td>
</tr>
</tbody>
</table>

$^a$ Viability is expressed as fraction of A2780 plating efficiency.
$^b$ The number of colonies surviving selection with 15 $\mu$M DDP per viable cell, with 95% confidence limits shown. Numbers in parentheses, number of experiments.
$^c$ EMS (2.5 mg/ml) for 2 h. Data shown are for 3-day expression time prior to DDP selection.

Fig. 2. Number of colonies surviving selection with 15 $\mu$M DDP per viable cell when assayed at the time shown after 2-h exposure to 2.5 mg/ml EMS. Bars, 95% confidence limits.
The low frequency of these resistant cells, their induction by treatment with the mutagen EMS, and the stable retention of the drug resistant phenotype support their mutational origin. Subpopulations of cells mutated at genes conferring resistance to chemotherapeutic drugs have been suggested to be the cause of eventual chemotherapy treatment failure for many types of tumors. A variety of possible resistance mechanisms have been suggested for cells resistant to DDP (2–5). Depletion of intracellular GSH levels have been shown in DDP resistant cell lines. Aphidicolin, an inhibitor of DNA polymerase α, has also been shown to increase the cytotoxicity of DDP in DDP resistant A2780CP cells (17). These studies have involved specific DDP resistant lines which have been isolated by multiple rounds of DDP selection. The lines to DDP compared to the parental A2780 cell line. A2780; A2780CP; D, DDP selected clones. Bars, 95% confidence limits.

Table 2: Effect of BSO and aphidicolin on DDP resistant mutant frequency

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Fraction of experiments with zero colonies</th>
<th>Mutant frequency*</th>
<th>Mutant frequency using fluctuation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
<td>5.2 × 10^-6</td>
<td>1.7 × 10^-6</td>
</tr>
<tr>
<td>2.5 μg/ml aphidicolin</td>
<td>0.38</td>
<td>4.5 × 10^-6</td>
<td>2.0 × 10^-6</td>
</tr>
<tr>
<td>5 μg/ml aphidicolin</td>
<td>0.17</td>
<td>1.3 × 10^-6</td>
<td>1.3 × 10^-6</td>
</tr>
<tr>
<td>25 μM BSO</td>
<td>0.41</td>
<td>2.6 × 10^-6</td>
<td>8.7 × 10^-7</td>
</tr>
<tr>
<td>50 μM BSO</td>
<td>0.20</td>
<td>8.3 × 10^-6</td>
<td>2.1 × 10^-6</td>
</tr>
</tbody>
</table>

* Viability is expressed as fraction of A2780 plating efficiency.  
* The number of colonies surviving selection with 15 μM DDP per viable cell. Numbers in parentheses. SEM.

cell line A2780 by a single exposure to DDP. The low frequency of these resistant cells, their induction by treatment with the mutagen EMS, and the stable retention of the drug resistant phenotype support their mutational origin. Subpopulations of cells mutated at genes conferring resistance to chemotherapeutic drugs have been suggested to be the cause of eventual chemotherapy treatment failure for many types of tumors. A variety of possible resistance mechanisms have been suggested for cells resistant to DDP (2–5). Depletion of intracellular GSH levels have been shown in DDP resistant cell lines. Aphidicolin, an inhibitor of DNA polymerase α, has also been shown to increase the cytotoxicity of DDP in DDP resistant A2780CP cells (17). These studies have involved specific DDP resistant lines which have been isolated by multiple rounds of DDP selection. The effect of these resistance modifiers on the frequency of DDP resistant cells in a population prior to DDP selection has thus far not been studied. Table 2 shows the effect of exposure of cells to aphidicolin or BSO prior to DDP selection. As shown BSO reduces the cell viability in clonogenic assays of the A2780 cells, as has previously been reported (19). Aphidicolin also markedly reduced the cell viability of A2780, in contrast to a previous report (17). Although both the BSO and aphidicolin treatments increase the number of experiments without DDP resistant colonies, when account is made of the reduction in viability there are no significant differences in the mutant frequencies. Therefore neither BSO nor aphidicolin has an effect on increasing the sensitivity of DDP resistant mutants existing in the cell population at time of exposure to DDP, although there is a combined cytotoxic effect.

Few studies have used single-step selections with chemotherapy drugs to examine the frequency of resistant mutants occurring in a cell population. Barranco et al. (20) have shown that a single exposure to melphalan (99% lethal dose) increased 10–50-fold resistance to the same agent 1 week later. The authors suggest that this is due to increased GSH levels in the cells and showed that BSO can partially reverse the resistance to melphalan. At present we are examining mechanisms of resistance to DDP in the single-step selection clones isolated, including alterations in drug accumulation, drug inactivation, and repair of induced lesions. However, since BSO has no effect on the frequency of DDP resistant mutants, this would suggest that GSH levels are not involved in resistance of these lines.

Both BSO and aphidicolin have previously been reported to increase the sensitivity of DDP resistant A2780 cells (13, 14, 17). These cells were selected after multiple exposure to DDP and may represent a different mutant type than that isolated by the single-step procedure used in the present study or may represent only a small subgroup of all possible mutant types. Which of the several possible mechanisms of DDP resistance have the highest probability of being selected for by DDP in human tumors is unclear. However, the type of single-step selection used in the present study will provide a means of examining the efficacy of modulators of DDP resistance to reduce the DDP resistant mutant frequency in cells not previously exposed to DDP.

Acknowledgments

We thank Dr. R. F. Ozols and T. C. Hamilton for kindly providing A2780 and A2780CP cells. We also thank F. Conway for secretarial assistance.

References

SINGLE STEP CISPLATIN SELECTION


15. Luria, S. E., and Delbruck, M. Mutations of bacteria from virus sensitivity to virus resistance. Genetics, 28: 491–495, 1943.


Single Step Selection of cis-Diamminedichloroplatinum(II) Resistant Mutants from a Human Ovarian Carcinoma Cell Line
