Increased Platinum-DNA Damage Tolerance Is Associated with Cisplatin Resistance and Cross-Resistance to Various Chemotherapeutic Agents in Unrelated Human Ovarian Cancer Cell Lines

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ABSTRACT

We have examined a panel of 12 unrelated human ovarian cancer cell lines derived from patients who were either untreated or treated with platinum-based chemotherapy to determine whether a relationship is present between cisplatin sensitivity and: (a) cellular platinum accumulation; (b) glutathione levels; (c) platinum-DNA adduct formation; (d) platinum-DNA adduct repair; and (e) platinum-DNA damage tolerance. Multiple regression and correlation analysis revealed that of these resistance mechanisms, platinum-DNA damage tolerance correlates strongly with cisplatin sensitivity ($r = 0.84$, $P = 0.001$), whereas platinum accumulation ($r = -0.11$), cellular glutathione levels ($r = 0.13$), and platinum-DNA adduct removal ($r = 0.44$) correlate insignificantly. The correlation of platinum-DNA damage tolerance to cisplatin sensitivity ($IC_{50}$) is derived from the clustering of platinum-DNA adduct formation into three distinct groups spanning a 3-fold range, which is narrow relative to the corresponding 43-fold range in sensitivity. Adduct formation itself is not associated with cisplatin sensitivity ($r = -0.38$). Strong correlations were also observed between platinum-DNA damage tolerance and sensitivity to Adriamycin ($r = 0.80$, $P = 0.002$), paclitaxel ($r = 0.87$, $P = 0.0002$), etoposide ($r = 0.78$, $P = 0.003$), and mitomycin C ($r = 0.73$, $P = 0.007$). These results suggest that the failure of pathways that are involved in recognizing and processing platinum-DNA damage and other types of drug-induced damage that culminate in cell death may result in a broad resistance phenotype.

INTRODUCTION

Drug resistance is a major obstacle in the successful treatment of ovarian cancer with cisplatin or carboplatin-based combination chemotherapy. Although high response rates (60–80%) are initially observed in previously untreated ovarian cancer patients, relapse frequently occurs, and subsequent salvage therapy with a variety of drugs, including paclitaxel, is largely ineffective (1). Thus, an understanding of the molecular basis for platinum-based chemotherapeutic drug resistance may enable the design of more effective treatment regimens.

Most evidence indicates that DNA is the cytotoxic target of cisplatin and its analogues (2). Therefore, cells that are resistant to these drugs either have the capacity to limit the formation of platinum-DNA adducts or, alternatively, they must be able to repair or tolerate these lesions once they are formed. We have previously examined the mechanisms that may contribute to cisplatin resistance in a panel of human ovarian cancer cell lines with in vitro-induced cisplatin resistance. These cell lines (A2780/C-series) exhibit a wide range of primary cisplatin resistance (~1000-fold) relative to the parental cell line (A2780) from which they were derived. The resistant cell lines that comprise this model system exhibit multiple mechanisms that account for their cisplatin resistance including: (a) decreased platinum accumulation; (b) elevated GSH levels; (c) enhanced platinum-DNA adduct repair capacity; (d) alteration in the types of platinum-DNA lesions formed; and (e) an increased ability to tolerate platinum-DNA damage (3–6).

Despite the findings in in vitro-derived cisplatin resistance models, it remains to be determined which resistance mechanism(s) occurs in tumor cells from clinically drug-refractory patients. In the present study, we have examined a panel of cisplatin-sensitive and -resistant human ovarian cancer cell lines from tumors of untreated or platinum-based chemotherapy-treated patients with regard to the mechanisms that may contribute to cisplatin sensitivity/resistance. The relationship between these various mechanisms and sensitivity to cisplatin and other drugs is presented.

MATERIALS AND METHODS

Chemicals and Reagents. Cisplatin, etoposide, and mitomycin C were obtained from Bristol Myers Squibb (Syracuse, NY). Adriamycin was obtained from Cetus Corp. (Emeryville, CA). Paclitaxel for clinical use was provided by the Division of Cancer Treatment, National Cancer Institute, and was suspended at a concentration of 6 mg/ml in 50% polyoxyethylated castor oil (Cremophor EL) and 50% dehydrated alcohol. Cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Cell Culture. The human ovarian cancer cell lines used in this study were isolated from patients who were either untreated or treated with platinum-based chemotherapy (Table 1). Cells were maintained at 37°C in a humidified incubator containing 5% CO2 in RPMI 1640 supplemented with 10% (v/v) FCS, 100 μg/ml streptomycin, 100 units/ml penicillin, 0.3 mg/ml glutamine, and 0.25 units/ml insulin (porcine).

Cytotoxicity. The tetrazolium-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine the relative sensitivities of the ovarian cancer cell lines to cisplatin. Adriamycin, paclitaxel, etoposide, and mitomycin C (7). Cells were plated in 150 μl of medium per well in 96-well plates (Corning Co., Corning, NY). Following overnight incubation, cells were exposed to various concentrations of drug, which were added in 10-μl volumes. Following a 72-h incubation, 40 μl of 5% (v/v) FCS, 100 μg/ml streptomycin, 100 units/ml penicillin, 0.3 mg/ml glutamine, and 0.25 units/ml insulin (porcine). was incubated for 48 h. While still subconfluent, cells were removed from the dish with trypsin, washed three times with Dulbecco's PBS (pH 7.1), sonicated in PBS, and centrifuged at 10,000 × g for 5 min. Protein was measured using the bicinchoninic acid protein determination kit (Sigma Chemical Co.). Protein was precipitated from the supernatant by adding 5-sulfosalicylic acid to a final concentration of 3% and incubating for 2 h at 4°C. Following centrifugation at 10,000 × g, the supernatant was removed, and the absorbance at 570 nm was measured for each well using a Bio-Rad model 3550 microplate reader. The reported IC50 for each cell line, which is the concentration of drug that resulted in a 50% reduction in absorbance relative to untreated cells, is the result of triplicate measurements made on at least two separate occasions.

Glutathione Measurement. To measure glutathione concentrations, cells (4 × 10⁶) were plated in triplicate or quadruplicate into 145-cm² dishes and incubated for 48 h. While still subconfluent, cells were removed from the dish with trypsin, washed three times with Dulbecco's PBS (pH 7.1), sonicated in PBS, and centrifuged at 10,000 × g for 5 min. Protein was measured using the bicinchoninic acid protein determination kit (Sigma Chemical Co.). Protein was precipitated from the supernatant by adding 5-sulfosalicylic acid to a final concentration of 3% and incubating for 2 h at 4°C. Following centrifugation at 10,000 × g, the supernatant was removed, and the absorbance at 570 nm was measured for each well using a Bio-Rad model 3550 microplate reader. The reported IC50 for each cell line, which is the concentration of drug that resulted in a 50% reduction in absorbance relative to untreated cells, is the result of triplicate measurements made on at least two separate occasions.

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The abbreviations used are: GSH, glutathione; AAS, atomic absorption spectrometry.
The value indicated is the point at which the curve crossed the 50% survival line.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Crated for each cell line, and the values reported are the result of duplicate
proteinase K. High molecular weight DNA was isolated by the phenol /
redissolved in 1 ml of 0.5 M Na2HPO4/NaH2PO4 (pH 8.5) containing 1% (w/v)
SDS, 1 mm EDTA, and 4 μm urea. Protein concentrations were measured for
these samples using the bicinchoninic acid protein determination kit. Relative
drug accumulation differences were determined by comparing the slopes of the
lines generated for each cell line.

DNA Platination. To measure platinum-DNA adduct formation, cells were
incubated for 4 h with 0—100 μM cisplatin, washed three times with PBS, and
incubated at 95°C in 10% HCl, 10 mm EDTA, 0.1% (w/v) SDS, and 200 μg/ml
proteinase K. High molecular weight DNA was isolated by the phenol /
chloroform method and restriction-digested with HindIII (6). For total platini-
num-DNA adduct determination, samples were incubated at 95°C in 10% HCl,
and platinum was measured by AAS as described (5). Relative differences in
DNA platination were determined by comparing the slopes of the lines gen-
erated for each cell line, and the relative values reported are the result of duplicate
measurements on DNA isolated from flasks treated on two separate occasions.

DNA Repair Assay. Triplicate flasks (175 cm2) containing each cell line
were incubated for 4 h at 37°C with the concentration of cisplatin necessary to
obtain similar total platinum-DNA adduct levels (approximately 50 pg platini-
um/μg DNA). Cells were either harvested immediately or incubated in fresh
medium for 8 h. DNA was isolated and analyzed for platinum content as
indicated for the other cell lines are expressed relative to the IC50 for
A2780 (Table 2; Fig. 1). The cisplatin IC50 for the 12 cell lines ranged 43-fold (from 0.18 to 7.7 μM). For each of the five drugs examined, median IC50 for the cell lines derived from patients
without prior exposure to cisplatin (e.g., A2780, A1847, OVCAR5, and OVCAR7) were not significantly different from those of the other
cell lines. Decreased sensitivity to cisplatin was associated with decreased sensitivity (cross-resistance) to most, if not all, of the other four chemotherapeutic agents examined (Adriamycin (r = 0.70, P = 0.011), paclitaxel (r = 0.69, P = 0.014), etoposide (r = 0.58, P = 0.048), and mitomycin C (r = 0.54, P = 0.067)). OVCAR2, OVCAR4, and OVCAR7 showed particularly high levels of resistance to Adriamycin, etoposide, and mitomycin C. OVCAR2 and OVCAR7 also exhibited marked insensitivity to paclitaxel. An estimate for the paclitaxel IC50 for these two cell lines was made because their dose-response curves declined rapidly between 1.0 and 100 nm paclit-
taxel to 50—60% survival and plateaued at approximately 40—50% survival from 0.1 to 10 μM paclitaxel. This effect has been observed
in other cell lines treated with paclitaxel and is characteristic of a cell
cycle phase-specific drug (10). Steady-state glutathione levels were
also determined for each cell line and are listed in Table 3. GSH levels
varied 8-fold in the panel; however, there was a poor correlation
between GSH levels and cisplatin IC50 (r = 0.13, P = 0.697).

RESULTS

The sensitivities (IC50) of the 12 human ovarian cancer cell lines (Table 1) to cisplatin, Adriamycin, paclitaxel, etoposide, and mito-
mycin C are listed in Table 2. Of these cell lines, A2780 exhibited the lowest IC50 for all five drugs; therefore, the relative resistance levels
indicated for the other cell lines are expressed relative to the IC50 for
A2780 (Table 2; Fig. 1). The cisplatin IC50 for the 12 cell lines ranged 43-fold (from 0.18 to 7.7 μM). For each of the five drugs examined, median IC50 for the cell lines derived from patients
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between GSH levels and cisplatin IC50 (r = 0.13, P = 0.697).

Table 1 Human ovarian tumor cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>Untreated ovarian tumor</td>
</tr>
<tr>
<td>A1847</td>
<td>Untreated ovarian tumor</td>
</tr>
<tr>
<td>SKOV3</td>
<td>Ovarian tumor, cisplatin-sensitive</td>
</tr>
<tr>
<td>PEO1</td>
<td>Ovarian tumor, cisplatin-sensitive</td>
</tr>
<tr>
<td>PEO4</td>
<td>Ovarian tumor (PEO1) after patient became refractory</td>
</tr>
<tr>
<td>OVCAR2</td>
<td>Ovarian tumor cisplatin-refractory patient</td>
</tr>
<tr>
<td>OVCAR4</td>
<td>Ovarian tumor from a cisplatin-refractory patient</td>
</tr>
<tr>
<td>OVCAR5</td>
<td>Untreated ovarian tumor</td>
</tr>
<tr>
<td>OVCAR7</td>
<td>Untreated ovarian tumor</td>
</tr>
<tr>
<td>OVCAR8</td>
<td>Ovarian tumor from a high dose carboplatin-refractory patient</td>
</tr>
<tr>
<td>OVCAR10</td>
<td>Ovarian tumor from a high dose cisplatin and carboplatin-refractory patient</td>
</tr>
</tbody>
</table>

Table 2 Sensitivities of 12 human ovarian cancer cell lines to various drugs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin (μM)</th>
<th>Adriamycin (μM)</th>
<th>Taxol (nm)</th>
<th>VP-16 (μM)</th>
<th>Mitomycin C (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>0.18 ± 0.004</td>
<td>0.027 ± 0.001</td>
<td>1.4 ± 0.1</td>
<td>0.012 ± 0.000</td>
<td>0.01 ± 0.000</td>
</tr>
<tr>
<td>A1847</td>
<td>3.8 ± 0.566</td>
<td>0.053 ± 0.030</td>
<td>4.6 ± 2.8</td>
<td>0.75 ± 0.26</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td>SKOV3</td>
<td>1.08 ± 0.188</td>
<td>0.037 ± 0.000</td>
<td>3.0 ± 0.9</td>
<td>0.26 ± 0.000</td>
<td>0.10 ± 0.000</td>
</tr>
<tr>
<td>PEO1</td>
<td>0.45 ± 0.057</td>
<td>0.028 ± 0.012</td>
<td>1.9 ± 0.4</td>
<td>0.068 ± 0.006</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>PEO4</td>
<td>5.6 ± 0.493</td>
<td>0.172 ± 0.028</td>
<td>3.0 ± 0.8</td>
<td>1.19 ± 0.26</td>
<td>0.91 ± 0.77</td>
</tr>
<tr>
<td>OVCAR2</td>
<td>2.6 ± 0.671</td>
<td>0.353 ± 0.237</td>
<td>100.0⁰</td>
<td>5.2 ± 0.8</td>
<td>6.4 ± 3.6</td>
</tr>
<tr>
<td>OVCAR3</td>
<td>1.4 ± 0.120</td>
<td>0.036 ± 0.015</td>
<td>2.4 ± 0.7</td>
<td>0.164 ± 0.001</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>OVCAR4</td>
<td>5.0 ± 1.216</td>
<td>0.485 ± 0.276</td>
<td>7.2 ± 3.9</td>
<td>6.95 ± 1.63</td>
<td>2.9 ± 1.6</td>
</tr>
<tr>
<td>OVCAR5</td>
<td>2.1 ± 0.382</td>
<td>0.104 ± 0.023</td>
<td>4.3 ± 0.3</td>
<td>0.80 ± 0.03</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>OVCAR7</td>
<td>4.1 ± 0.707</td>
<td>1.205 ± 0.771</td>
<td>1000.0⁰</td>
<td>4.5 ± 0.3</td>
<td>3.5 ± 2.3</td>
</tr>
<tr>
<td>OVCAR8</td>
<td>1.9 ± 0.424</td>
<td>0.094 ± 0.003</td>
<td>5.0 ± 0.1</td>
<td>1.48 ± 0.46</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>OVCAR10</td>
<td>7.7 ± 0.849</td>
<td>0.028 ± 0.008</td>
<td>6.8 ± 0.9</td>
<td>0.22 ± 0.05</td>
<td>0.08 ± 0.03</td>
</tr>
</tbody>
</table>

⁶ Cytoxicity is expressed as IC50 for each cell line (± SD), which is the concentration of drug that caused a 50% reduction absorbance at 570 nm relative to untreated cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

⁷ Etoposide.

The survival curves for OVCAR2 and OVCAR7 plateaued at 40—60% survival at Taxol concentrations above 100 nm, which did not allow for a reliable assessment of cytoxicity.

The value indicated is the point at which the curve crossed the 50% survival line.
Table 3 Steady-state GSH levels in human ovarian cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GSH concentration (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>17.1 ± 0.8</td>
</tr>
<tr>
<td>A1847</td>
<td>30.0 ± 2.6</td>
</tr>
<tr>
<td>SKOV3</td>
<td>64.8 ± 18.9</td>
</tr>
<tr>
<td>PEO1</td>
<td>15.7 ± 2.0</td>
</tr>
<tr>
<td>PEO4</td>
<td>27.2 ± 7.3</td>
</tr>
<tr>
<td>OVCAR2</td>
<td>8.3 ± 3.0</td>
</tr>
<tr>
<td>OVCAR3</td>
<td>43.9 ± 5.5</td>
</tr>
<tr>
<td>OVCAR4</td>
<td>45.6 ± 16.9</td>
</tr>
<tr>
<td>OVCAR5</td>
<td>29.0 ± 3.8</td>
</tr>
<tr>
<td>OVCAR7</td>
<td>29.3 ± 6.4</td>
</tr>
<tr>
<td>OVCAR8</td>
<td>32.2 ± 4.2</td>
</tr>
<tr>
<td>OVCAR10</td>
<td>31.5 ± 12.2</td>
</tr>
</tbody>
</table>

Further statistical analysis of the data indicated that the IC_{50}s for Adriamycin, paclitaxel, etoposide, and mitomycin C are nonnormally distributed, each skewed by the presence of the highly drug-resistant OVCAR2, OVCAR4, and OVCAR7 cell lines (Fig. 1). In contrast, cisplatin IC_{50}s, GSH levels, cellular platinum accumulation, platinum-DNA adduct formation and repair, and platinum-DNA damage tolerance all approximate a normal distribution. Because the conventional Pearson correlation coefficient assumes normality of distribution, all correlation coefficients reported here (with the exception of those resulting from multiple regression) are computed by the more robust Spearman rank order method (11).

Platinum accumulation in the 12 human ovarian tumor cell lines was linear following a 4-h incubation with a range of cisplatin concentrations (0–100 μM) as measured by AAS (Fig. 2). The relative levels of cell-associated platinum varied approximately 5-fold; however, decreased accumulation was not associated with cisplatin sensitivity among the panel of cell lines (r = -0.11, P = 0.729). When platinum accumulation was expressed as nanograms of platinum per 10^6 cells, the correlation with cisplatin sensitivity was also poor (r = 0.46, P = 0.131). Using the same cisplatin exposure conditions, total platinum-DNA adduct formation was measured by AAS. Platinum-DNA adduct formation increased linearly with cisplatin concentra-
platinum-DNA damage tolerance and sensitivity to Adriamycin (r = 0.80, P = 0.002), paclitaxel (r = 0.87, P = 0.0002), etoposide (r = 0.78, P = 0.003), and mitomycin C (r = 0.73, P = 0.007).

The removal of total platinum-DNA adducts was determined by incubating cells for 4 h with cisplatin concentrations required to obtain similar platinum-DNA adduct levels, followed by an 8-h incubation in drug-free medium to allow for repair (Table 5). The percentage of platinum removed from DNA over this time ranged from 0 to 39% among the cell lines; however, there was no strong correlation between total platinum-DNA adduct removal and cisplatin sensitivity (r = 0.44, P = 0.152).

Stepwise regression analysis of the data indicated that cisplatin sensitivity (IC50s) is best predicted by a linear combination of tolerance and adduct formation with Pearson R2 and adjusted R2 values of 0.82 and 0.78, respectively (Table 6). To enable direct comparison of the magnitudes and confidence intervals from multiple regression, the parameters in Table 6 were obtained using standardized data. That is, for each parameter, the data were transformed by subtracting their mean and then dividing by their standard deviation. That is, for each parameter, the data were transformed by subtracting their mean and then dividing by their standard deviation. The precise mechanism(s) of cisplatin uptake is unknown; however, the process is believed to occur through a combination of passive diffusion and carrier-mediated transport processes (22). Although a variation in platinum accumulation was observed in the present study, no significant correlation existed with cisplatin sensitivity in this panel of cell lines.

In addition to reduced drug accumulation, a variety of intracellular reports, however, comprehensively examining the mechanisms which may cause resistance to cisplatin in unrelated tumor cell lines with a large difference in drug sensitivity. Therefore, we have performed such a study in a panel of human ovarian cancer cell lines derived from patients who were either untreated or treated with platinum-based chemotherapy. This enabled us to gain some insight into which mechanism(s) may be relevant to clinical cisplatin resistance. The cell lines we used exhibited a wide range of sensitivity to cisplatin (43-fold), and the cell lines that showed decreased cisplatin sensitivity also exhibited cross-resistance to several other clinically important anticancer drugs.

Decreased platinum accumulation is a common phenotype observed in cell lines selected for cisplatin resistance in vitro (5, 13–20), and we have shown that the accumulation difference between the sensitive and resistant counterpart may reach as high as 14-fold (21). The precise mechanism(s) of cisplatin uptake is unknown; however, the process is believed to occur through a combination of passive diffusion and carrier-mediated transport processes (22). Although a variation in platinum accumulation was observed in the present study, no significant correlation existed with cisplatin sensitivity in this panel of cell lines.

In addition to reduced drug accumulation, a variety of intracellular
factors may affect the activity of cisplatin and also preclude it from binding to DNA. Sulphhydryl-rich proteins and glutathione have been implicated in the inactivation of cisplatin and other alkylating agents by drug-resistant cells. Glutathione is a ubiquitous tripeptide that is involved in the detoxification of a variety of exogenous and endogenous molecules. Evidence for the formation of a GSH-platinum complex in cells exposed to cisplatin has been reported by two groups (23, 24), and several investigators have observed increased glutathione levels in cell lines selected for cisplatin resistance in vitro (3, 25, 26). GSH has also been found to be associated with cisplatin sensitivity in unrelated cell lines. For example, Mistry et al. (27) found a strong correlation between glutathione levels and the sensitivities of eight human ovarian cancer cell lines to cisplatin, carboplatin, and iproplatin. In another study, Hosking et al. (25) also reported a correlation between GSH levels and sensitivity to cisplatin in 20 tumor cell lines. In contrast, Pendyala et al. (28) reported recently no significant association of GSH levels with cisplatin cytotoxicity in a panel of 10 human tumor cell lines; however, a significant correlation was observed with iproplatin sensitivity. In the present study, we found no significant correlation (r = 0.13) between GSH levels and cisplatin sensitivity in the 12 unrelated human ovarian cancer cell lines examined.

Decreased platinum-DNA adduct formation and increased repair of platinum-DNA adducts have also been shown to be associated with cisplatin resistance in several in vitro model systems (6, 29–34). In the present study, the kinetics of formation and removal of platinum-DNA adducts varied in the 12 ovarian cancer cell lines, but neither parameter was related to cisplatin cytotoxicity. The observed clustering of the cell lines into three distinct groups based on platinum-DNA adduct formation (Fig. 3B) may result from the cellular pharmacodynamics of platinum accumulation, inactivation, and repair. It should be noted, however, that one limitation to our method of measuring repair capacity is that cisplatin-sensitive cells may respond differently to equivalent platinum-DNA lesion densities as compared to cisplatin-resistant cells. Because such a concentration is more cytotoxic to the sensitive cells, they may respond by not continuing to synthesize the proteins required for nucleotide excision repair, whereas the relatively resistant cells might continue or even increase synthesis of repair proteins. At equitoxic doses of drug, the values for platinum removal may be different, although it becomes much more difficult to measure platinum-DNA damage reliably under these conditions of low lesion density. Alternatively, using an in vitro assay for measuring DNA repair may enable such a comparison to be made (35).

In an effort to determine how all five of the explanatory variables jointly affect the cisplatin IC50s of the 12 cell lines, multiple regression analysis was performed. Platinum accumulation, GSH levels, and platinum-DNA adduct removal were found to contribute insignificantly to the overall regression equation. Sensitivity to cisplatin is best described through a linear combination of platinum-DNA damage tolerance and adduct formation (Table 6). The inclusion of tolerance in the final regression equation is not surprising, given the strong correlation between it and cisplatin sensitivity (r = 0.84, P = 0.001). Biochemically, tolerance is defined as the density of platinum-DNA lesions present at equitoxic doses of cisplatin, in this case, at the IC50 value. Operationally, the determination of tolerance requires a priori knowledge of the cisplatin IC50 of a cell line because tolerance is calculated as the product of adduct formation and cisplatin IC50. Tolerance and cisplatin IC50 necessarily show some correlation because these are not independent quantities. For this reason, the degree of correlation is not directly comparable to the typical situation where the two quantities compared for correlation are believed to be independent of each other, for example, in correlating tolerance to the IC50 of one of the other drugs examined (Adriamycin, etoposide, paclitaxel, or mitomycin C). Nonetheless, the comparison of cisplatin sensitivity and tolerance remains an important one because the degree of correlation is also dependent on adduct formation, an independently determined quantity, and because tolerance also correlates strongly with the IC50 of the other four drugs studied. Finally, the purpose of multiple regression as used here is not predictive; rather, it is to identify those explanatory variables that do not influence cisplatin sensitivity. It is conceivable that correlation between platinum-DNA damage tolerance and cisplatin IC50 might be so strong as to obscure the contribution from the other four explanatory variables. Consequently, multiple regression was redone excluding tolerance. Neither platinum accumulation, GSH levels, platinum-DNA adduct formation, nor repair could be entered into the regression equation under the P = 0.05 inclusion and P = 0.10 exclusion criteria.

As discussed above, previous studies have linked cisplatin resistance to mechanisms distant from the locus of DNA, such as platinum uptake/exflux and detoxication. If resistance in the present cell lines were mediated largely or exclusively by these mechanisms, then tolerance could be expected to be unrelated to cisplatin sensitivity. Through the definition of tolerance, this hypothesis also implies that, as cisplatin IC50 increases, platinum-DNA adduct formation should decrease in a compensating manner. As Fig. 4A illustrates, data from the 12 cell lines contradict the hypothesis through the strong correlation (r = 0.84, P = 0.001) between tolerance and cisplatin IC50. This strong correlation originates in the clustering of platinum-DNA adduct formation into three groups (Fig. 4B) over a range (~3-fold) which is narrow compared to the 43-fold range of IC50. Platinum-DNA adduct formation in these three groups is statistically distinct (P = 0.012, Kruskal-Wallis test). If one considers only cell lines comprising an individual group, for example, cell lines A1847, PEO1, PEO4, OVCAR5, and OVCAR10, each having an adduct formation of approximately 1 pg platinum/μg DNA/μM cisplatin (Fig. 4B), the corresponding relationship between tolerance and cisplatin IC50 is stronger still (r = 0.998), with this subset of points forming a nearly straight line (Fig. 4A), the slope of which is an average of the adduct formation values for this group. Within each group, cisplatin IC50 varies widely (Fig. 4B), with only weak correlation between cisplatin IC50 and adduct formation (r = −0.38, P = 0.217).

From this data, we believe that the significant contribution of tolerance to cisplatin sensitivity originates in the similarity among cell lines with diverse cisplatin IC50s in their degree of platinum-DNA adduct formation. To explore the influence of the narrow range of adduct formation in determining the strong correlation between tolerance and cisplatin IC50, a computer simulation experiment was performed. In each of 1000 trials, measured platinum-DNA adduct
formation for the 12 cell lines was randomly assigned among the 12 cell lines, with platinum-DNA damage tolerance and the correlation coefficient subsequently recalculated. By randomizing the pairing of platinum-DNA adduct formation and cisplatin IC_{50}s, any remaining correlation between tolerance and cisplatin IC_{50} must reside in the IC_{50} and adduct formation distributions themselves, particularly in their relative breadths. Simulations produced a mean r of 0.89 ± 0.04, a value close to the measured value of 0.84. These results reinforce the importance of the narrow breadth of adduct formation.

The kinetics of platinum accumulation, cellular distribution, and inactivation should determine the level of active drug that is available for DNA binding; however, to identify and determine the contribution of the individual components of these mechanisms is a complex task. As a result, the molecular basis for this narrow range in platinum-DNA adduct formation is unclear, and it is difficult to explain the pattern of adduct formation among the cell lines using only our platinum accumulation and GSH data. Another possibility that has not been examined is the capacity of different cells to influence the formation of different types of platinum lesion. Some of these lesions may be more cytotoxic than others, and there is evidence that this process occurs in related cell lines (5).

Based on the above results, we believe that platinum-DNA damage tolerance is the fundamental mechanism that causes decreased cisplatin sensitivity in this series of cell lines. Platinum-DNA damage tolerance has been identified in a few in vitro-derived cisplatin resistance models (6, 33, 36). Assuming that DNA is the cytotxic target of cisplatin, there are several ways a cell can exhibit increased platinum-DNA damage tolerance. Enhanced replicative bypass enables cellular DNA replication machinery to synthesize DNA past a lesion. Subsequent repair of the lesion could then be completed at the G2 checkpoint prior to mitosis (37). This process was demonstrated previously to occur in two cisplatin-resistant human ovarian cancer cell lines (38). It has also been shown that DNA polymerase β can efficiently bypass a (dGpG)Pt intrastrand adduct in a cell-free system (39). It is difficult to explain, however, how replicative bypass could result in the observed cross-resistance pattern of the human ovarian cancer cell lines to drugs that do not exert their cytotoxic effects through DNA binding (e.g., paclitaxel).

We hypothesize that a more likely explanation for the increased platinum-DNA damage tolerance observed in our panel of human ovarian tumor cell lines is that the more cisplatin-resistant cells require higher levels of DNA damage to activate programmed cell death (apoptosis) pathways. Although these pathways are incompletely characterized, it has been shown that treatment of cells with cisplatin and other DNA-damaging agents result in cell cycle arrest at the G2 checkpoint and the accumulation of hyperphosphorylated, inactive p34^{cdc2} protein (40–42). Demarcq et al. (42) demonstrated that following a protracted delay in G2 arrest in cisplatin-treated Chinese hamster ovary cells, dephosphorylation of p34^{cdc2} occurs, and cells undergo an aberrant mitosis that ultimately results in DNA degradation and the morphological features characteristic of apoptosis. Because apoptosis is a highly regulated process, it is conceivable that disruption of one or more components of the pathway could elicit the multidrug resistance phenotype observed in some of these clinically refractory tumor cells. Consistent with this idea, the failure of cisplatin-resistant murine leukemia cells to undergo apoptosis when exposed to normally toxic concentrations of cisplatin has been demonstrated recently (43). Alternatively, overexpression of a gene that suppresses apoptosis could also result in decreased drug sensitivity. For example, overexpression of members of the bcl-2 gene family, bcl-2 and bcl-x, have been shown to protect cells from apoptotic cell death when exposed to cisplatin and other chemotherapeutic agents (44, 45).

The association of cell cycle events with apoptosis requires that drug resistance be viewed as a dynamic process. Because regulation of the cell cycle occurs through signal transduction pathways, it is not surprising that modulating these pathways can impact on cellular drug sensitivity. A variety of agents that activate or inhibit receptors and protein kinases have been shown to influence cisplatin sensitivity. In addition, the expression of certain oncopgenes (e.g., Ha-Ras, Her-2/new, and abl) or inactivation of tumor suppressor genes (e.g., p53) can promote the survival of some cells in the presence of diverse drugs (46–50). It is possible that some of the genetic changes responsible for the transformed phenotype could also contribute to a multidrug-resistant profile in malignant cells. Consequently, it is tempting to speculate as to whether some of the tumor cell lines used in this study, such as A1847, OVCAR5, and OVCAR7 (derived from untreated patients), are examples of this phenomenon.

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Increased Platinum-DNA Damage Tolerance Is Associated with Cisplatin Resistance and Cross-Resistance to Various Chemotherapeutic Agents in Unrelated Human Ovarian Cancer Cell Lines

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