Characterization of a Cisplatin-resistant Human Ovarian Carcinoma Cell Line Expressing Cross-Resistance to 5-Fluorouracil but Collateral Sensitivity to Methotrexate


In vitro exposure of the TR170 ovarian carcinoma cell line to six intermittent 24-h treatments with a 90% inhibitory concentration of cisplatin (CDDP) (0.15 μg/ml; 0.5 μM) resulted in a 2-fold stably resistant subline designated TR170/CP+ (B. T. Hill et al., Int. J. Cancer, 39: 219-225, 1987). Resistance to CDDP in these CP+ cells has now been associated with reduced uptake of 10 μM CDDP (2-fold; P < 0.01) and decreased removal of specific Pt-DNA adducts, quantitated immunochromatically, indicative of an apparent increased tolerance of CDDP-induced DNA damage. Specifically these resistant cells appeared deficient in removal of the major cis-Pt(NH3)2d(pGpG) adduct and the difunctional cis-Pt(NH3)2d(GMP)p lesion, showed less efficiency in removing cis-Pt(NH3)2d(pApG) adducts, but proved as proficient as the parental cell line in removing DNA-DNA interstrand cross-links. Activities of DNA polymerase-α and -β were comparable in both lines, and no significant alterations in glutathione metabolism were identified. Response to acute X-irradiation was not modified in these TR170/CP+ cells, but they showed marked (10-fold) cross-resistance to 5-fluorouracil and, unusually, proved collaboratively sensitive (12-fold) to methotrexate. Resistance to 5-fluorouracil was associated with significantly increased thymidylate synthase activity (P < 0.01), but this was not reflected in altered gene expression, while increased sensitivity to methotrexate was accompanied by increased drug uptake but by unaltered activity and expression of dihydrofolate reductase. These results indicate that exposure to CDDP can result in numerous alterations, both intracellularly and at the cellular membrane, reflected in significant changes in the tumor cells' responses to the cytotoxic effects of a range of antitumor drugs. The clinical relevance of these observations remains to be established.

INTRODUCTION

We reported earlier (1) that in vitro pulsed 24-h exposures of a human ovarian tumor cell line (TR170) to IC50 concentrations of CDDP resulted in the expression of a 2-fold order of resistance after six treatments. The resultant subline proved stably resistant after ≥6-mo growth in drug-free medium. We now describe the characterization of this subline in terms of its fundamental growth characteristics, CDDP uptake, CDDP-DNA binding, rates of formation and removal of platinated adducts, activities of DNA polymerase-α and -β, and levels of GSH and certain associated and antioxidant enzyme activities. In addition, while establishing responses to a range of other antitumor drugs, we found that this TR170/CP+ subline expressed significant collateral sensitivity to MTX, yet proved cross-resistant to 5-FU, so we have examined MTX uptake, cellular activities of DHFR and TS, and associated gene expression.

MATERIALS AND METHODS

Cell Lines and Culture Techniques. Details of the initiation and maintenance of the parental cell line, TR170, have been published previously, together with details of the establishment of the TR170/CP+ subline (1). Briefly, line TR170/CP+ was derived by six pulsed 24-h exposures to 0.15 μg/ml of CDDP (approximate IC50 concentration), permitting the cells to return to logarithmic growth in fresh complete medium between each drug exposure. Both lines were maintained in Ham's F-12 medium (Gibco, Paisley, Scotland) plus 10% FCS (Gibco) in an atmosphere of 5% CO2 in air at 37°C, except when cells were exposed to MTX or 5-FU, and then RPMI 1640 medium (Gibco) was substituted for 24 h prior to and throughout the period of drug exposure. MCF-7/PCx and MCF-7/ADRc breast carcinoma cell lines were kindly provided by Dr. K. Cowan (National Cancer Institute, Bethesda, MD) and were maintained in Eagle's minimal essential medium plus 10% FCS and 10 μg/ml of insulin in 10% CO2 in air at 37°C (2).

In Vitro Growth Characteristics of Logarithmically Growing Cultures. Population doubling times were calculated from cell counts of duplicate 5-cm dishes (Nunc, Roskilde, Denmark) containing 104 to 105 cells, at daily intervals, using a Model ZBI Coulter Counter. Cell volumes were determined using the Coulter Counter, cellular protein content was estimated by the method of Lowry et al. (3) or Bradford (4), and DNA content was determined as described by Burton (5).

Drug and X-Ray Sensitivity Testing. CDDP, 5-FU, BSO, and cadmium chloride were purchased from Sigma Chemicals (Poole, Dorset, United Kingdom). The following drugs were kindly donated: carboplatin and VP-16 (etoposide) by Bristol-Myers Company, Inc. (Evansville, IN); Adriamycin by Farmitalia (Milan, Italy); vincristine and MTX by Lederle (Gosport, Hampshire, United Kingdom); and mitomycin C by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). For soft agar clonogenic assays logarithmically growing monolayers were exposed to drugs for 1 or 24 h. The colony-forming ability of surviving cells was measured by the assay of Courtenay et al. (6). All assays were performed in triplicate on a minimum of two independent occasions. IC50 concentrations were estimated from full dose-response curves.

For growth curve assays logographically growing cells were exposed to a range of drug concentrations for 24 h. After washing, cells were permitted to grow in fresh medium plus serum for a further 48 h, during which time control, non-drug-treated cells remained in logarithmic growth. Monolayers were then trypsinized, and the total cell numbers were counted using a Model ZBI Coulter Counter. GI50 concentrations were estimated from full dose-response curves.

Survival curves after X-irradiation were fitted using a linear least-

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3 The abbreviations used are: IC50, drug concentration reducing survival by 90% of control values as judged by clonogenic assay; IC50, drug concentration reducing survival by 50% of control values, as judged by clonogenic assay; BSO, buthionine sulfoximine; CDDP, cisplatin; CDNA, complementary DNA; CDNB, 1-chloro-2,4-dinitrobenzene; DHFR, dihydrofolate reductase; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; 5-FU, 5-fluorouracil; GI50, drug concentration resulting in 50% growth inhibition, as judged by counting cell numbers; GP, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione S-transferase; ISC, DNA-DNA interstrand cross-links; MTX, methotrexate; PAGE, polyacrylamide gel electrophoresis; PAG, phosphoglycerate kinase; Pt-GMP, Pt-(NH3)2d(GMP); Pt-AG, cis-Pt(NH3)2d(PGpG); Pt-GG, cis-Pt(NH3)2d(pGpG); Pt-(GMP); cis-Pt(NH3)2d(GMP)p; 2S, sodium deoxycyl sulfate; SOD, superoxide dismutase; TS, thymidylate synthase.
squares computer program, and X-ray doses reducing the survival fraction of cells to 0.37 on the exponential region of the survival curve (D0) and the extrapolation number (n) were derived.

Total GSH Levels and Total GST, GR, GP, SOD, Catalase, and DT-Diaphorase Activities. These measurements were made from each line 3 days after initial cell plating. The assays used were as described previously (7): GSH content by the GR-recycling method of Griffith (8); GST according to the procedure of Habig and Jackoby (9), using CDNB (Sigma Chemicals); GR by the method of Horn (10); and GP using H2O2 or cumene hydroperoxide according to the modified method described by Paglia and Valentine (11). For estimations of activities of catalase, DT-diaphorase and SOD cytosol preparations were prepared, as described by Akman et al. (12), and these enzymes were assayed by the methods of Beutler (13) and Ernest (14), as modified by Benson et al. (15) and Heikila and Cabbat (16), respectively.

Western Immunoblotting for GSTs. Cells growing logarithmically were harvested and lysed by sonication in 50 mM Tris-HCl (pH 7.5) and 2 mM EDTA. Cytosolic protein (150 µg/lane) was resolved by 14% SDS-PAGE followed by electrophoretic transfer to nitrocellulose according to the manufacturer's directions (Novoblot: LKB Instruments, Inc., Gaithersburg, MD). Triplicate blots were blocked in 5% nonfat milk for 30 min and then probed with rabbit antiserum directed against one of the three classes of human GST (17).

Uptake and Binding to DNA of [106P]CDDP. Drug uptake and DNA binding were measured immediately following a 1-h exposure to 2, 5, 10, or 20 µg/ml of [106P]CDDP (kindly supplied by Dr. H. L. Sharma, Department of Medical Biophysics, University of Manchester, Manchester, United Kingdom), as previously described (18).

Quantitation of Platinum-DNA Adducts. For quantitation of Pt-DNA adducts, cells were exposed for 1 h to 10 µg/ml of CDDP (33.3 µM) dissolved in 0.9% NaCl solution immediately prior to use. After drug exposure, the cells were washed and harvested either immediately or 18 h after incubation in drug-free medium. DNA isolation was carried out, as detailed earlier (19), except that the overnight incubation was at 50°C, prior to digestion to nucleotides and platinum-containing oligonucleotides as described previously (20), except that sodium azide was omitted from the incubation mixture. Separation of platinated digestion products was carried out using an anion exchange chromatography column (MonoQ; Pharmacia, Sweden). Details of the enzyme-linked immunosorbent assay used to quantitate the Pt-DNA adducts have been described in an earlier publication (18). Antiserum W101 at a final 1:17,500 dilution was used to detect the adduct-derived platinum compounds Pt-GG and Pt-(GMP). Antiserum 3/43 was used at a final 1:4 x 106 dilution to detect the product Pt-GMP. Antiserum 3/65 was used at a final 1:100 dilution to detect the product Pt-Ag. Since the occurrence of Pt-DNA adducts was quantitated per gram of DNA isolated, correction for dilution by DNA synthesis during the 18-h postincubation period was carried out using parallel cell cultures, following the exact procedure described earlier (19).

Measurement of ISC. DNA-DNA ISC were measured in both cell lines at 0, 14, and 24 h after a 1-h exposure to CDDP using the alkaline elution technique of Kohn et al. (21) with minor modifications (18). The mean values ± SE from 2 to 3 experiments are provided.

DNA Polymerase Assays. DNA polymerase assays were performed using the method detailed recently by Mivechi et al. (22). Using logarithmically growing cells, total polymerase activity was determined and, in parallel, DNA polymerase-β was estimated in the presence of 10 µM N-ethylmaleimide and 200 µM KCl to inhibit DNA polymerase-α and -β.

Uptake of MTX. Cells (5 to 8 x 106 cells/ flask) were cultured in 25-cm² flasks for 48 to 72 h in Ham's F-12 medium plus 10% FCS. Twenty-four h prior to each experiment the medium was changed to RPMI 1640 containing 10% FCS. Using the procedure described by Pizzorno et al. (23), uptake was initiated by replacing the medium with 3 ml of 11 µM [3H]MTX (600 cpm/pmol) in RPMI 1640 medium with 10% filtered FCS. At time zero and at 10-, 30-, and 60-min intervals, the medium was aspirated from individual flasks and the adherent cells were washed 3 times with ice-cold phosphate-buffered saline and incubated for 1 h in 2 ml of 1 N NaOH. The radioactivity was counted after adding Optiphase scintillation fluid (LKB Scientific Products, USA).

RESULTS

Characterization of the Cell Lines. The fundamental growth characteristics of the TR170/CP+ subline were not significantly different from those of the parental line TR170/P (see Table 1). Population doubling times ranged from 29 to 32 h, colony-forming efficiencies in soft agar were 1 to 6%, while DNA and protein content were similar. The TR170/CP+ cells had a significantly larger cell volume, i.e., 1.3-fold (P < 0.002).

Table 1 In vivo characteristics of the two ovarian tumor cell lines

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TR170/P cells</th>
<th>TR170/CP+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population doubling time (h)</td>
<td>32 ± 4a</td>
<td>29 ± 1b</td>
</tr>
<tr>
<td>Cell vol. (µm³)</td>
<td>1235 ± 16</td>
<td>1653 ± 72b</td>
</tr>
<tr>
<td>DNA (µg/10⁶ cells)</td>
<td>16.1 ± 1.2</td>
<td>17.9 ± 1.5</td>
</tr>
<tr>
<td>Protein (µg/10⁶ cells)</td>
<td>288 ± 6</td>
<td>273 ± 3</td>
</tr>
<tr>
<td>% of clonogenicity in soft agar (range)</td>
<td>2-5</td>
<td>1-6</td>
</tr>
<tr>
<td>Modal chromosome nos.</td>
<td>41 (36-71)c</td>
<td>42 (33-46)</td>
</tr>
</tbody>
</table>

a Mean ± SE.
b P < 0.002.
c Numbers in parentheses, range.
TR170/P and TR170/CP+ cells were near-diploid with several chromosome rearrangements. Detailed karyotypic analysis of TR170/P cells has been presented elsewhere (34), and no gross alterations were apparent in this resistant subline.

In Vitro Drug and X-Ray Sensitivity Drug Evaluations. Fig. 1 depicts the dose-response curves following a 24-h exposure of logarithmically growing cultures of the two cell lines to CDDP. From four individual repeat experiments, the mean IC50 values were calculated. These are listed in Table 2 and indicate that the cells express an approximate 2-fold order of resistance, which is statistically significant (P > 0.02), as judged by Student's t test. This level of resistance has proved stable in cells maintained in culture for ≥6 mo. Results in Table 2 also illustrate that a similar order of resistance could be identified by monitoring growth inhibition. Using this type of assay cross-resistance to carboplatin was shown in these TR170/CP+ cells, although increased sensitivity to cadmium chloride was retained. Resistance to 5-FU but marked collateral sensitivity to MTX (see Table 2) were comparable in the two cell lines.

Cellular Content of GSH, GR, GST, GP, SOD, DT-Diaphorase, and Catalase. Levels of GSH, GR, GST, and GP, with the latter activity compared using either H2O2 or cumene hydroperoxide, were not significantly different between TR170/P and TR170/CP+ cells (Table 3), as reported, in part, earlier (35). Western blot analyses indicated that the predominant GST isozyme expressed by both cell lines is GST-π (see Fig. 3). Depletion of total GSH content by 75 to 85% was achieved by incubation of both cell lines with 20 or 30 μM BSO for 25 h. When CDDP was added during the last hour of this incubation period, the GI50 values were not significantly modified in either cell line: GI50 values for TR170/P cells, 11.5 ± 0.4 and 10.3 ± 0.3 μg/ml with and without 30 μM BSO; and corresponding GI50 values for TR170/CP+ cells, 14.2 ± 0.3 and 12.8 ± 0.4 μg/ml with and without the addition of 30 μM BSO, respectively.

Activities of SOD and catalase were not significantly different in these two cell lines; however, a marked decrease (>4-fold, P < 0.01) in DT-diaphorase activity was noted in the TR170/CP+ cell line compared with the TR170/P line.

Uptake and Binding of [195mPt]CDDP. The dose dependency of [195mPt]CDDP uptake by the two cell lines is shown in Fig. 4, which illustrates an approximate 2-fold difference with the more resistant subline taking up significantly less [195mPt]CDDP (P < 0.01). Uptake normalized for extracellular drug concentration and cellular volume into the TR170/P and TR170/CP+ cells was 4.4 ± 0.4 and 2.0 ± 0.1 pmol/106 cells/μg of CDDP/unit of cell volume, respectively (P < 0.01). The extent of DNA binding determined immediately after exposure via radioactivity counting, normalized for extracellular drug concentration, was similar in the two lines, being 5.4 ± 0.4 and 4.4 ± 1.1 pmol/g of DNA/μg of CDDP (P < 0.2) in the TR170/P and TR170/CP+ cells, respectively.

Induction and Repair of Platinum-DNA Adducts. The specific activity of radiolabeled DNA decreased in both cell lines during the 18-h posttreatment incubation period to approximately 80 to 90% of the values immediately following treatment. Calculated dilution factors were the following: for TR170/P cells, 0.84 ± 0.06; for TR170/CP+ cells, 0.84 ± 0.03.

By correction for DNA synthesis and DNA content, the total amount of each adduct was calculated and the results are summarized in Table 4. From these data the total platination can be calculated, and these figures reveal slightly lower, but not statistically significant, levels associated with TR170/CP+ cells, as opposed to the parental TR170/P cells, with values of 28.6 ± 2.3 versus 35.6 ± 3.2 nmol of Pt/g of DNA, respectively. There were no significant differences in the distribution of these four adducts between the two cell lines. The major adduct formed in both lines was Pt-GG, comprising approximately 50% of the total platination immediately following drug exposure, with Pt-AG proving the next most abundant adduct, i.e., 21% (see Table 4). After an 18-h posttreatment incubation period, the extent of residual DNA platination was significantly higher (P < 0.05) in the resistant subline (18.6 ± 1.2 versus 13.6 ± 1.5 nmol of Pt/g of DNA). This suggests that the TR170/CP+ cells were less efficient at removing DNA platination.
CISPLATIN RESISTANCE IN OVARIAN CARCINOMA CELLS

Fig. 2. Survival curves for TR170/P (○) and TR170/CP+ (●) cells exposed to a range of concentrations of either 5-FU or MTX for 24 h. Points, mean of 2 experiments in which replicate cultures were treated and duplicate samples from each were analyzed; bars, SE.

Table 3 Glutathione levels and related enzyme and antioxidant enzyme activities

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>TR170/P cells</th>
<th>TR170/CP+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (nmol)</td>
<td>37.0 ± 0.1*</td>
<td>26.7 ± 5.8</td>
</tr>
<tr>
<td>GR (nmol of NADPH/min)</td>
<td>38.0 ± 1.0</td>
<td>41.7 ± 0.6</td>
</tr>
<tr>
<td>GST (nmol of CDNB/min)</td>
<td>181 ± 21</td>
<td>170 ± 13</td>
</tr>
<tr>
<td>GP (nmol of NADPH/min)</td>
<td>1.6 ± 0.1</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>H2O2</td>
<td>1.7 ± 0.5</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>SOD (μg)</td>
<td>3.6 ± 0.1</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>Catalase (μmol of H2O2/min)</td>
<td>434 ± 29</td>
<td>102 ± 5*</td>
</tr>
</tbody>
</table>

* All the values were normalized per mg of cellular protein content.
† Mean ± SD.
‡ P < 0.01.

Fig. 3. Western blot analysis of protein extracts from TR170/P and TR170/CP+ cells. Cytosolic protein (100 to 200 μg/lane) was resolved by 14% SDS-PAGE followed by electrophoretic transfer to nitrocellulose. The blot was probed with antisera directed against GST-α (16). The MCF-7/WTKc and ADRKc lines were used as negative and positive controls, respectively. MWM, molecular weight markers.

Fig. 4. Uptake of 153mCDDP by monolayer cultures of TR170/P (○) and TR170/CP+ (●) cells following a 1-h incubation at 37°C. Points, mean of 2 experiments utilizing duplicate samples at each point; bars, SE.

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Since it is not possible to distinguish between Pt-(GMP)2 derived from ISC or from long-distance intrastrand cross-links formed by binding of CDDP to two nonadjacent guanines in the sequence 𝒑G(𝒑X)₂𝒑G, alkaline elution was used to quantify ISC. An initial time-course study showed that peak ISC formation occurred in these cell lines 14 h after a 1-h CDDP exposure (data not shown). Using a CDDP concentration of 10 μg/ml, the number of ISC in rad equivalents formed at 14 h was 59.7 ± 6.4 and 52.6 ± 7.9 for the TR170/P and TR170/
Table 4. Pt-GMP, Pt-AG, Pt-GG, and Pt-(GMP)2 in the TRI 70 human ovarian tumor cell lines at 0-h and 18-h posttreatment incubation times

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Adduct</th>
<th>0 h</th>
<th>18 h</th>
<th>% of decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRI70/P</td>
<td>Pt-GMP</td>
<td>8.0 ± 1.3</td>
<td>1.4 ± 0.1</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Pt-AG</td>
<td>7.4 ± 0.8</td>
<td>0.9 ± 0.2</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Pt-GG</td>
<td>17.1 ± 1.1</td>
<td>9.8 ± 1.4</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Pt-(GMP)</td>
<td>3.1 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>52</td>
</tr>
<tr>
<td>TRI70/CP+</td>
<td>Pt-GMP</td>
<td>4.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Pt-AG</td>
<td>6.2 ± 0.2</td>
<td>2.7 ± 0.3</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Pt-GG</td>
<td>14.0 ± 1.8</td>
<td>11.7 ± 0.9</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Pt-(GMP)</td>
<td>3.5 ± 0.5</td>
<td>3.0 ± 0.6</td>
<td>14</td>
</tr>
</tbody>
</table>

* The number of adducts was determined immediately after a 1-h exposure to 10 μg/ml (33.3 μM) of CDDP and following an 18-h posttreatment incubation period. Mean ± SD, normalized for DNA content, of two different competitive ELISAs which were performed in four dilutions in triplicate wells. Values at 18 h were corrected for dilution by DNA synthesis as previously described (18).

Table 5. Activities of DNA polymerase-α and -β in logarithmically growing cultures of TRI 70/P and TRI 70/CP+ cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DNA polymerase activity (pmol/10^9 cells/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
</tr>
<tr>
<td>TRI70/P</td>
<td>131 ± 17*</td>
</tr>
<tr>
<td>TRI70/CP+</td>
<td>140 ± 19</td>
</tr>
</tbody>
</table>

* Mean ± SD of replicate samples from duplicate experiments.

Fig. 5. Uptake of [3H]MTX by monolayer cultures of TRI70/P (●) and TRI70/CP+ (◼) cells following a 10-, 30-, or 60-min incubation at 37°C. Columns, mean of 3 experiments utilizing duplicate samples at each point; bars, SE.

CP+ cells, respectively. By 24 h only 27.9 ± 5.6 and 23.4 ± 3.6 rad equivalents remained in the respective cell lines indicating substantial, but comparable, repair of ISC in both these cell lines, i.e., approximately 50% of lesions being removed.

Activities of DNA Polymerase-α and -β. The levels of these enzymes were comparable in both cell lines (see Table 5). The less efficient removal of Pt adducts by the TRI70/CP+ resistant subline was therefore not reflected in an altered level of DNA polymerase-β.

Uptake of MTX. The results illustrated in Fig. 5 indicate significantly increased uptake of [3H]MTX by the TRI70/CP+ cells following in vitro exposure to 1 μM drug at 37°C. This difference is particularly noticeable at the later time points, consistent with an apparent plateau in the time course for the TRI70/P cells between 30 and 60 min of incubation, while uptake continued throughout the hour into the TRI70/CP+ cells. Indeed, if a correction is made for the larger cellular volume of the TRI70/CP+ cells, then the difference in [3H]MTX uptake is only significant (P > 0.05) at the 60-min time point.

Activities of TS and DHFR. The results presented in Table 6 provide evidence of unaltered DHFR activity, but significantly enhanced levels of TS in the TRI70/CP+ cells, compared with parental cell values. This significant (P > 0.01) elevation of TS activity is consistent with the expression of cross-resistance to 5-FU by the TRI70/CP+ line.

Analyses of Levels of RNA for TS, DHFR, and DNA Polymerase-β. Northern blots of total RNA (see Fig. 6) probing for the expression of the genes encoding these TS cycle enzymes

Table 6. Activities of DHFR and TS in logarithmically growing cultures of TRI70/P and TRI70/CP+ cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DHFR (nmol of NADPH/min/mg)</th>
<th>TS (nmol/h/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRI70/P</td>
<td>2.37 ± 0.10*</td>
<td>11.47 ± 2.15</td>
</tr>
<tr>
<td>TRI70/CP+</td>
<td>2.56 ± 0.16</td>
<td>20.42 ± 2.26*</td>
</tr>
</tbody>
</table>

* Mean ± SD of duplicate samples from 2 to 3 experiments.

Fig. 6. Gene expression in TRI70/P and TRI70/CP+ cells. Total RNA was extracted and loaded (10 μg/lane) for analysis by Northern blotting for TS, DHFR, DNA polymerase-β (DNA Pol beta), and PGK expression.
and for DNA polymerase-β failed to reveal any significant differences between the two cell lines. Northern analysis of polyadenylate-enriched RNA provided similar results. Using probes for PGK and β-actin to control for equal loading and RNA transfer, the relative amounts, respectively, in the TR170/CP+ cells compared with the parental cells, as judged by densitometry, were the following: 0.79 and 1.04 for DHFR; 0.98 and 1.29 for TS; and 0.54 and 0.71 for DNA polymerase-β.

DISCUSSION

In these studies we have attempted to determine the mechanism(s) of resistance operating in an ovarian cancer cell line expressing a modest, yet clinically relevant, level of resistance to CDDP resulting from pulsed 24-h drug exposures. While there are now numerous reports describing the establishment and characterization of CDDP-resistant sublines in the literature, this TR170/CP+ line is unusual since the resistance was expressed as a result of intermittent 24-h exposures in vitro to IC₅₀ concentrations of CDDP, allowing the tumor cell population to return to logarithmic growth behavior between each treatment. This procedure was adopted in an attempt to mimic the manner of administration of CDDP to patients with ovarian cancer. A similar type of rationale formed the basis for the procedure adopted by Scanlon and coworkers (29, 36, 37) using A2780 human ovarian cancer cells and HCT8 human colon carcinoma cells to develop CDDP-resistant sublines. They exposed the cells to increasing concentrations of CDDP weekly for 1 h, a condition that closely relates to the clinical half-life of the drug, and obtained approximately 10- and 5-fold stably resistant sublines, respectively, from the two tumor types.

The TR170/CP+ cell line expressed a 2-fold order of resistance, which has proved stable over 6 mo in culture without further exposure of the cells to CDDP. There were neither major differences in the fundamental growth characteristic nor any significant karyotypic alterations observed in this resistant subline compared with the parental line. This is in agreement with our earlier description of a CDDP-resistant bladder carcinoma cell line (6) and reports from various other groups (38–40). These TR170/CP+ cells expressed cross-resistance to carboplatin, a finding also reported for most other CDDP-resistant sublines (for example, Refs. 7 and 38–43), while retaining full sensitivity to Adriamycin, vincristine, and etoposide. Significant cross-resistance was noted to 5-FU in accord with other published reports (44–47). The unusual findings from these in vitro sensitivity evaluations, however, were the marked (12-fold) collateral sensitivity shown by these TR170/CP+ cells to MTX. Fram et al. (43) reported earlier that a 5-fold CDDP-resistant colon carcinoma line showed a 5-fold increased sensitivity to MTX, but this was associated with the general expression of collateral sensitivity to all the antimetabolites tested, including 5-FU and 1-β-d-arabinofuranosylcytosine. More generally, however, CDDP-resistant cells are cross-resistant to MTX (37, 39, 44, 47, 48).

Although Louie et al. (49) reported cross-resistance to X-rays in their ovarian tumor cell line with a 7-fold level of CDDP resistance, there was no evidence of cross-resistance to X-irradiation in this TR170/CP+ line. Indeed, in our experience working with a range of human cell lines expressing resistance to CDDP, derived from tumors of the bladder (7), testis (50), and ovary (51), we have not observed any expression of radiation resistance. Wallner and Li (52) similarly found no relation between CDDP and radiation resistance. However, Schwartz et al. (53) reported that radioresistance was strongly associated with CDDP resistance in 10 early passage lines derived from various human tumor biopsies.

The comparable levels of GSH and related enzyme activities in these two TR170 cell lines suggest that there is no differential inactivation of CDDP via these pathways in the resistant subline. Consistent with these findings, the depletion of GSH by BSO did not significantly enhance CDDP sensitivity in either cell line. Other groups have similarly shown unaltered GSH metabolism in CDDP-resistant cell lines (40, 45, 50, 54), and Richon et al. (55) showed that the increased levels of GSH in CDDP-resistant L1210 mouse leukemia cells were a recessive trait. The marked growth-inhibitory effects of cadmium chloride on the TR170/CP+ cells, as opposed to the TR170/P cells, argue against mediation of CDDP resistance by metallothioneins, implicated in other experimental tumor systems (39, 56–58). Our observation is consistent with earlier reports of increased sensitivity of CDDP-resistant cells to cadmium chloride (59, 60) and with the study by Fujiwara et al. (61) which identified reduced levels of metallothionein in a CDDP-resistant lung cancer cell line.

Activities of SOD and catalase were similar in both cell lines, consistent with their comparable sensitivities to Adriamycin and vincristine. The marked decrease in DT-diaphorase in the TR170/CP+ cells remains to be explained, but this is not reflected in any altered sensitivity to mitomycin C (62). While we have noted a similar decrease in another CDDP-resistant ovarian carcinoma subline, Mansouri et al. (63) found no changes in DT-diaphorase levels in a CDDP-resistant subline of murine RIF-1 cells. Resistance to CDDP in drug-selected sublines of both murine and human origin has been associated with significantly decreased CDDP accumulation (30, 45, 54, 64, 65, 66–68), although this is by no means a universal finding (7, 40, 42, 66–69). These TR170/CP+ cells accumulated significantly less [¹⁹⁵mPt]CDDP over a 1-h incubation period than did TR170/P cells. Whether this reflects diminished drug retention, as opposed to uptake, a feature highlighted in some recent detailed studies of CDDP accumulation (70), remains to be established. This reduced CDDP accumulation was not, however, reflected in a reduced level of drug bound to DNA, as judged in terms of [¹⁹⁵mPt] CDDP quantitation or of total adduct formation measured immunochromatically. Published reports indicate that reduced drug uptake may (54) or may not (71–73) be reflected in a reduced level of drug bound to DNA.

Resistance to CDDP in these TR170/CP+ cells was not associated with any apparent differential induction of the major Pt-DNA adducts. Similar adduct levels have also been reported in various other sensitive and resistant tumor cell lines (43, 65, 74). Although a number of studies have suggested a positive correlation between the extent of ISC formation and CDDP cytotoxicity, with decreased levels characteristic of a range of human tumor sublines (7, 17, 39, 43, 66, 67, 75, 76), this was not identified in these TR170/CP+ cells. Similar levels of formation and rates of removal of ISCs were quantitated in the two TR170 lines.

Differential repair of CDDP-damaged DNA as a mechanism of CDDP resistance is currently the subject of intensive laboratory investigations. Increased repair capacity has been identified in a range of experimental model systems, utilizing various different methodologies including immunochromato-
titation of adducts (50, 72, 77, 78), monitoring of unscheduled DNA synthesis (69, 79), increased activity and gene expression of DNA polymerase-β (36, 80) transfection studies using CDDP-damaged plasmids (81, 82) and, most recently, evaluation of repair in specific genomic regions utilizing the *Escherichia coli* avidin-biotin complex exucinuclease (83). However, in this study, evidence is provided that TR170/CP+ cells were generally less efficient at removing platinumated DNA. This resulted from their deficiency in removing only certain adducts, specifically the major Pt-GG lesions and the difunctional Pt-(GMP)$_2$ adducts and their less efficient removal of Pt-AG adducts, since they proved as efficient as the TR170/P cells in removing ISCs. Significant alterations in either the activity or gene expression of DNA polymerase-β were not identified, although a small reduction (<2-fold) in polyadenylate-encrined RNA was recorded in the TR170/CP+, which would be consistent with their deficient "repair" capacity. The apparent deficiency of the more CDDP-resistant cell line in removing the major Pt-GG adduct was also identified in one of our earlier studies comparing two ovarian tumor cell lines with differing inherent sensitivities to CDDP (74). One explanation for this rather unexpected observation is that the more CDDP-resistant cells exhibit increased tolerance of these unrepaired lesions in the DNA and are able to replicate on a damaged template. Such increased tolerance of CDDP-induced DNA damage has been reported in a number of other experimental model systems (7, 50, 68, 84). The precise mechanisms associated with increased damage tolerance, which include alterations in DNA replication and postreplication repair (54, 85), remain to be defined. In addition, since the methods used only measure DNA damage and its repair in the overall genome, heterogeneity of drug-induced DNA damage and repair in specific genes, as recently reported in relation to CDDP (83), cannot be ruled out.

In view of the recent proposal by Scanlon et al. (36, 37) that the TS cycle may be involved in the development of CDDP resistance, we extended our study to consider this possibility. These authors reasoned that the TS cycle provides the sole source of de novo thymidylate required by repair enzymes in removing Pt-DNA complexes. This group has now provided evidence of elevated mRNA for TS and DHFR, with comparable increases in enzyme activities in CDDP-resistant sublines of human ovarian (A2780) and colon (HCT8) cells (29, 36, 37, 47). Monitoring these parameters in the TR170/P and TR170/CP+ cells has revealed a significant (2-fold; $P > 0.01$) elevation in TS activity, but without detectable alterations in gene expression and unchanged levels of DHFR. Clearly this modified TS activity does not appear to correlate with the TR170/CP+ cells' reduced "repair" capacity, but it remains to be established whether increased damage tolerance can also be linked to changes in the genes encoding the TS cycle. However, the increased TS activity is consistent with the expression of marked cross-resistance to 5-FU in these TR170/CP+ cells and, in this respect, the data are similar to those reported by Scanlon et al. (29, 36, 37) in their A2780DDP and HCTDTP sublines. However, while these two latter sublines also showed cross-resistance to MTX, associated with increased DHFR expression, in contrast, TR170/CP+ cells showed very significant collateral sensitivity to MTX with apparently unchanged DHFR activity. Initial studies indicate that MTX uptake may be modified in these cells, but further detailed studies are under way to identify the mechanisms associated with this unusual expression of MTX hypersensitivity, including investigating their ability to synthesize MTX polyglutamates. One objective of our ongoing research is to establish whether comparable effects can result from clinical treatment.

In summary therefore CDDP resistance in these TR170/CP+ cells has been shown to be associated with reduced drug uptake and decreased removal of specific Pt-DNA adducts, indicative perhaps of an apparent increased tolerance of drug-induced DNA damage. These cells expressed cross-resistance to 5-FU, with increased activity of TS, but showed an unusual collateral sensitivity to MTX involving altered drug uptake without significant changes in DHFR activity or gene expression. These data provide further evidence that exposure of tumor cells to CDDP results in a wide variety of alterations not only intracellularly, in terms of altered repair and tolerance of DNA damage, but also at the level of the membrane resulting in modified drug uptake. It would now appear also that significant changes in sensitivities to other antitumor drugs can also occur, resulting in the expression of significant cross-resistance or collateral sensitivity.

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Characterization of a Cisplatin-resistant Human Ovarian Carcinoma Cell Line Expressing Cross-Resistance to 5-Fluorouracil but Collateral Sensitivity to Methotrexate

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