cis-Diaminedichloroplatinum(II) Resistance in Vitro and in Vivo in Human Embryonal Carcinoma Cells


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ABSTRACT

In the embryonal carcinoma cell line Tera and its 3.7-fold cis-diaminedichloroplatinum(II) (CDDP)-resistant subline, Tera-CP, parameters were studied that might have changed in relation to induction of CDDP resistance. Phenotypes of both lines were embryonal carcinoma. Karyotypes were related with a decreased mean number of chromosomes and fewer copies of the short arm of chromosome 12 in Tera-CP. Tera-CP showed cross-resistance for melphan and 4-hydroperoxycyclophosphamide and had an 1.4-fold increased glutathione (GSH) level, a 1.5-fold increased GST~r expression compared to Tera. Tera-CP was cross-resistant to 5-fluorouracil, but thymidylate synthase activity was not increased. Tera-CP tumors were 2.8-fold resistant to CDDP compared to Tera tumors. In new cell lines derived from xenografts of Tera and Tera-CP CDDP sensitivity, GST activity, and GSH level corresponded with their sensitivity and resistant origin. Tera-CP is a model of in vitro and in vivo CDDP resistance with the GSH/GST detoxifying system as an important mechanism. CDDP resistance could be induced without a concomitant increase in differentiation.

INTRODUCTION

Teratocarcinoma, a variant of germ cell tumor of the testis, is the most common form of cancer in young adult men (1). It consists of two histological components, namely EC3 and teratoma (1). EC cells are the stem cells from which the teratoma component is derived (2). Clinically, EC is highly malignant with a tendency to early metastatic spread but is also a good responder to CDDP-containing chemotherapy (3). In vitro, EC cell lines are known to be more sensitive to CDDP than their more differentiated counterparts (4). Patients with teratocarcinomas treated with CDDP-containing chemotherapy regimens have cure rates of 70–80% (5). However, in the remaining patients, the tumor becomes untreatable due to development of drug resistance.

Resistance to CDDP can be due to a variety of mechanisms. These include reduced drug accumulation and increased detoxification of CDDP in the cellular cytoplasm. In the cell nucleus, decreased DNA accessibility and increased DNA repair may play a role (6, 7). The latter is accompanied by increased amounts of repair enzymes (7, 8). Also, changes in the TTP synthesis might be an indication for increased DNA repair as this process requires a source of deoxyribonucleotides (8). The net effect of all these systems is reduced Pt-DNA and thus decreased cytotoxicity since the Pt-DNA interactions are considered to be the main cytotoxic lesions induced by CDDP (9). However, mechanisms of CDDP resistance are not uniform and vary among different cell lines.

In this study we describe a CDDP-resistant subline (Tera-CP) of NT2/D1 (Tera; Ref. 10), an EC cell line with the capacity to differentiate in vitro. This model enabled us to study not only mechanisms underlying CDDP resistance in extremely sensitive cells but also the effect of serial CDDP incubations on the EC phenotype of the cells. In addition, Tera cells in a xenograft in nude mice show marked differentiation (10), while in contrast, Tera in vitro is mainly composed of EC cells. To investigate the effects of this differentiation on CDDP resistance, Tera and Tera-CP were xenografted into nude mice, and tumor sensitivity for CDDP was determined. In new cell lines derived from the CDDP-treated sensitive and resistant xenografts, parameters thought to be relevant for resistance in the primary cell lines were measured in order to confirm their role in CDDP resistance.

MATERIALS AND METHODS

Chemicals

CDDP and teniposide were obtained from Bristol Myers (Weesp, the Netherlands); doxorubicin was from Farmitalia Carlo Erba (Milan, Italy); vincristine was from Eli Lilly (St. Cloud, France); melphan was from Wellcome (London, United Kingdom); bleomycin from Landbeck (Amsterdam, the Netherlands); and 5-fluorouracil (5-FUra) from Hoffman La Roche (Mijdrecht, the Netherlands). 4-HPC was a gift from ASTA Medica (Frankfurt, Germany). RPMI 1640 and fetal calf serum were obtained from Life Technologies (London, United Kingdom); cadmium chloride and 3,4-dimethylthiazol-2-yl)-2,5-diphenytreazolium bromide were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies directed against CK 4 (6B10), CK 7 (RCK105), CK 10 (RKSE60), CK 13 (1C7), CK 18 (RGE 53), neurofilaments, and vimentin were obtained from Eurodiagnostics (Apeldoorn, the Netherlands); the antibodies AE1/AE3 against a broad spectrum of CKs from Boehringer Mannheim (Mannheim, Germany); and those against desmin and GFAP from DAKO (Glostrup, Denmark). The antibodies against CKs 5 and 8 (M102) and CKs 8, 18, and 19 (M53D) were a gift from F. Raemakers (University of Maastricht, the Netherlands). The c-myc antibody OM-11-908 was purchased from Cambridge Research Biochemicals (Northwich, United Kingdom). The Bio-Rad Immuno-blot assay kit was obtained from Bio-Rad (Richmond, CA), and Tissue-Tek II O.C.T. compound was obtained from Miles Laboratories (Naperville, IL).

Cell Lines and Production of Resistance

Tera, kindly provided by P. W. Andrews, is an EC clone of a teratocarcinoma cell line with the capacity to differentiate in vitro as well as in vivo (10). In vitro it consists of nearly homogeneous EC populations when kept at high cell density (a minimum of 1.5 × 10^6 cells in a 25-cm² flask) (10). Cells were grown as a monolayer in RPMI 1640 supplemented with 10% fetal calf serum in a humidified atmosphere with 5% CO2 at 37°C. For production of the CDDP-resistant subline Tera-CP, cells were incubated with CDDP for 1 h and subsequently resuspended in fresh medium. This was repeated after cell re-
covery (determined by light microscopic observation) with stepwise increasing doses of CDDP. Every CDDP dose was added three times. Each cell recovery took about 3-4 weeks. The first dose of CDDP was 12.5 μg/ml, the CDDP concentration inducing 90% kill in the Tera line after a 1-h incubation. The final dose (after 11 months) was 100 μg CDDP.

Cell cycle distribution was determined by screening bromodeoxyuridine incorporation and propidium iodide intensity as described by Preisler et al. (11) with a FACStar flow cytometer (Becton Dickinson, Sunnyvale, CA).

Karyotype. To cells of Tera and Tera-CP in logarithmic phase of growth, 0.05 μg Colcemid/ml culture medium was added 2 h before harvesting. Chromosomes were GTG banded. From Tera and Tera-CP, five and seven metaphases were karyotyped, respectively.

The differentiation state of Tera and Tera-CP was determined by cellular antigen expression using immunocytochemistry. For these determinations, the antibodies anti-SSEA-1 (12), anti-SSEA-3 (13), TRA-1-60 and TRA-1-81 (14), anti-vimentin, anti-desmin, anti-neurofilaments, anti-GFAP, as well as antibodies against CKs (obtained as described in “Materials and Methods”), were used. Experiments were repeated at least three times.

For cytotoxicity measurements, the microculture tetrazolium assay was used (15). For Tera and Tera-CP, 103 cells/well were incubated continuously with CDDP, doxorubicin, vincristine, melphalan, bleomycin and for the drugs mentioned above was indicated by a RE The mean ID50 ± SD was determined in three independent experiments each performed in quadruplicate.

The conditions and measurements for GSH, TSH, and GST in the cell lines were as described before (16). For determination of the amount of Pt-DNA, 5 x 107 cells were incubated with CDDP concentrations ranging from 16.7 to 67 μg/ml for 4 h, and cells were washed with phosphate-buffered saline three times at 0°C. Dry pellets were dissolved in concentrated nitric acid and platinum was measured with AAS as described before (16). For determination of the amount of Pt-DNA, 5 x 107 cells were incubated with CDDP concentrations ranging from 16.7 to 67 μg/ml for 4 h, and cells were washed with phosphate-buffered saline three times at 0°C. DNA was isolated and dissolved in 200 μl of 1 m HCl; the amount of platinum was measured by AAS (detection threshold 2.5 pmol platinum), and the amount of DNA was measured by extinction at 260 nm as described before (16). The amount of DNA per sample analyzed was 10–20 μg. The reported values are the mean of three independent cell protein extracts.

In Tera and Tera-CP, the catalytic activity of thymidylate synthase and the binding of FDUMP to thymidylate synthase were determined as published previously (18, 19). Measurements were repeated three times in independent cell protein extracts.

Tera and Tera-CP were screened for c-myc amplification by Southern blot and for c-myc expression at the RNA level by Northern blot using a 1.2-kilobase SacI fragment from the second exon of human c-myc as a probe (20). For c-myc protein expression, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed (21), followed by Western blotting; after transfer of proteins by semidy blot, c-myc was detected with OM-11-908 and the Bio-Rad Immuno-blots assay kit for visualization.

The topoisomerase II catalytic activity in 0.35 m NaCl nuclear extracts of cells in logarithmic phase of growth was measured by the decatenation of kinetoplast DNA networks (22). Topoisomerase I activity in the same extracts was assayed by relaxation of supercoiled pBR322 DNA (22).

For cellular platinum determinations, 7.5 x 106 cells were incubated with CDDP concentrations ranging from 10 to 67 μg/ml for 2 h; cells were washed with phosphate-buffered saline three times at 0°C. Dry pellets were dissolved in concentrated nitric acid and platinum was measured with AAS as described before (16). For determination of the amount of Pt-DNA, 5 x 107 cells were incubated with CDDP concentrations ranging from 16.7 to 67 μg/ml for 4 h, and cells were washed with phosphate-buffered saline three times at 0°C. DNA was isolated and dissolved in 200 μl of 1 m HCl; the amount of platinum was measured by AAS (detection threshold 2.5 pmol platinum), and the amount of DNA was measured by extinction at 260 nm as described before (16). The amount of DNA per sample analyzed was 10–20 μg. The reported values are the mean of three independent experiments.

In Vivo Experiments. In order to xenograft Tera or Tera-CP 5 x 106 cells of either cell line were injected s.c. in the left shoulder of male, athymic (nude) mice. Relative tumor size was determined using three-dimensional measurements, length x width x height, in mm (23). After the tumors had reached a relative size of 1000 mm3, the tumors were excised. For determination of the amount of Pt-DNA, 5 x 107 cells were incubated with CDDP concentrations ranging from 16.7 to 67 μg/ml for 4 h, and cells were washed with phosphate-buffered saline three times at 0°C. DNA was isolated and dissolved in 200 μl of 1 m HCl; the amount of platinum was measured by AAS (detection threshold 2.5 pmol platinum), and the amount of DNA was measured by extinction at 260 nm as described before (16). The amount of DNA per sample analyzed was 10–20 μg. The reported values are the mean of three independent experiments.

RESULTS

The cell line Tera-CP was defined (with a RF of 3.7 for CDDP), after treatment of cells as described in “Materials and Methods,” with a final induction dose of 100 μg/ml. In order to ensure stable resistance, proved by microculture tetrazolium assay, all experiments were performed within 14 weeks after the last 1-h CDDP incubation of Tera-CP. Every 14 weeks, a fresh culture of Tera-CP was started from a liquid nitrogen frozen stock.

In Fig. 1, the survival curve of Tera and Tera-CP, after continuous incubation with CDDP, is shown. Tera-CP cells were grown as a
monolayer. Doubling time, cell cycle distribution, cellular and nuclear protein, as well as relative cell size of Tera-CP cells, were comparable with Tera cells (Table 1). Tera-CP had a lower DNA content than Tera (Table 1).

Chromosome analysis of Tera and Tera-CP revealed a strongly abnormal chromosomal pattern with a modal chromosome number of 61 (range, 60–62) in Tera and 57 (range, 56–61) for Tera-CP. Fig. 2 shows one of the karyotypes from Tera (For a description, see Fig. 2 legend). All abnormalities described were clonal; other clonal abnormalities are an add(1)(p13) and der(1)add(1)(q24)del(1)(p36). Fig. 3 shows one of the karyotypes of Tera-CP (For a description, see Fig. 3 legend). Of the described abnormalities, the add(14)(p10) and one -21 were not clonal, while der(9),add(9)(q31) add(9)(p11),der(1)t(X;1)- (p11;p13) and add(d)(q13) were other clonal abnormalities. Of 12p, between five and nine copies and between four and five copies per karyotype were found in Tera and Tera-CP, respectively.

The differentiation states of Tera and Tera-CP were comparable. Expression of TRA-1-60 and TRA-1-81 was found in all cells; both cell lines were SSEA-3 positive in >90% of the cells and SSEA-1 negative in 80–90% of the cells, indicating an EC phenotype. A weak AE 1/AE 3 reaction was found and with the more specific monoclonal antibodies M102, RGE53, and M5D3, CKs were characterized as CK 8 and 18. Neither desmin nor GFAP-positive cells were detected in the lines. In Tera as well as Tera-CP, 50–100% of the cells contained vimentin. Neurofilaments were occasionally found in a few cells.

Cross-resistance was clearly demonstrated for 4-HPC, melphalan, and 5-FUra with RFs of 2.0, 1.9, and 2.3 respectively (Table 2). For the other drugs mentioned in Table 2, minimal or no cross-resistance was found.

Cellular platinum accumulation after 2-h CDDP incubation was equal in both lines (Fig. 4A). Pt-DNA (Fig. 4B) after 4-h CDDP incubation was decreased in Tera-CP compared to Tera (33 and 67 μM; P < 0.025).

In vivo CDDP sensitivity parameters, tumor measurements, and time to the start of regrowth, are shown in Fig. 5. After a single dose of CDDP, the mean percentage tumor reduction for TeraX was 73 ± 13% (n = 3) and 26 ± 15% for Tera-CPX (n = 4; TeraX versus Tera-CPX, P < 0.005). The time passing until tumor regrowth was 10.7 ± 3.1 days for TeraX (n = 3) and 5.5 ± 0.8 for Tera-CPX (n = 4; TeraX versus Tera-CPX, P < 0.025). From three mice with TeraX cell lines Teram2, Teram3, and Teram4 and from four mice with Tera-CPX cell lines, Tera-CPm1, Tera-CPm2, Tera-CPm3, and Tera-CPm4 were set up. CDDP sensitivity of these cell lines reflected the sensitivity of Tera and Tera-CP, respectively (Fig. 6). Cellular GSH levels of Teram(2–4) and Tera-CPm(1–4), as well as their respective GST activities, are shown in Fig. 7. The mean amounts of GSH were 1.6 ± 0.6 (n = 10) and 2.1 ± 0.5 (n = 14) μg/mg protein in the Teram group and the Tera-CPm group, respectively (Tera versus Tera-CPm, 1993 American Association for Cancer Research.)
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Fig. 3. Representative karyotype of Tera-CP: 56,add(X)(q23), +Y,+der(X)(p11;22),add(1)(p11);del(1)(p36), +der(1)(X)(p11; p13)add(X)(p22), +2,der(3)add(3)(p11)-del(3)(q23)1), +der(3)3qter->3p11:...->3p24:...-4,del(5)(q33);del(7)(q11);del(7)(q21)(1;7) (q25;q11.2), +der(7)(q17);7q22;add(9)(q22);der(9)(q:14)p11;q11), add(10)(p12);add(11)(q14);add(12)(p11), +del(12)(q14:21), +i(12)(p10);add(13)(q22);14;add(14)(p10);der(16)(q12;13.3), +del(18)(q22);21, -21, add(22)(p13), +6 mar.

Table 2: Sensitivity measured by microculture tetrazolium assay after continuous drug incubation of Tera and Tera-CP for various drugs

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IDSO Tera</th>
<th>IDSO Tera-CP</th>
<th>RF</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDDP (μM)</td>
<td>0.69 ± 0.10</td>
<td>2.57 ± 0.74</td>
<td>3.7 &lt;0.005</td>
<td></td>
</tr>
<tr>
<td>Cisplatin (μM)</td>
<td>157 ± 10</td>
<td>151 ± 19</td>
<td>1.0 NS</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin (ng)</td>
<td>12.8 ± 3.2</td>
<td>16.1 ± 4.5</td>
<td>1.3 NS</td>
<td></td>
</tr>
<tr>
<td>Vinblastine (μM)</td>
<td>0.97 ± 0.16</td>
<td>1.22 ± 0.07</td>
<td>1.3 &lt;0.05</td>
<td></td>
</tr>
<tr>
<td>4-HP (μM)</td>
<td>2.56 ± 0.70</td>
<td>5.16 ± 1.80</td>
<td>2.0 &lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Melphalan (μM)</td>
<td>0.98 ± 0.30</td>
<td>1.62 ± 0.42</td>
<td>1.9 &lt;0.025</td>
<td></td>
</tr>
<tr>
<td>Bleomycin (μg/mL)</td>
<td>0.16 ± 0.03</td>
<td>0.23 ± 0.13</td>
<td>1.3 NS</td>
<td></td>
</tr>
<tr>
<td>Teniposide (μM)</td>
<td>11.9 ± 0.6</td>
<td>15.9 ± 2.3</td>
<td>1.3 &lt;0.025</td>
<td></td>
</tr>
<tr>
<td>5-FU (μM)</td>
<td>3.8 ± 0.7</td>
<td>8.7 ± 1.1</td>
<td>2.3 &lt;0.0025</td>
<td></td>
</tr>
</tbody>
</table>

a IDSO Tera versus IDSO Tera-CP.

P < 0.05). GST activity was significantly increased (P < 0.0025) in the Tera-CP group compared to the Teram group; means were 192 ± 59 (n = 9) and 120 ± 27 (n = 13) nmol CDNB/min/mg protein, respectively.

DISCUSSION

The development of resistance to CDDP, a drug exquisitely effective in teratocarcinomas, has severe clinical consequences. Since the understanding of the mechanisms underlying this resistance may lead to determination of ways to prevent or to circumvent resistance induction, the development of models that permit such studies is relevant. In this report, we present an EC cell line with a level of CDDP resistance that is comparable with that found in other human cell lines with in vitro acquired resistance (7, 16, 24).

Cytogenetic analysis of Tera and Tera-CP revealed a strongly abnormal chromosomal pattern with a lower chromosome number in Tera-CP than in Tera. The 2 cell lines had 13 chromosomal abnormalities in common, pointing to their relationship. In addition, Tera has 5 and Tera-CP has 13 unique structural abnormalities. The chromosome number of both cell lines is in the range of primary testicular germ cell tumors; the number of abnormalities of the cell lines is higher. Both Tera and Tera-CP showed i(12p), found in about 80% of all testicular germ cell tumors (25). In addition, Tera-CP showed a del(12)(q14q21). Allelic deletions in this region are frequently observed; 12q13 and 12q22 might be the chromosomal location of a tumor suppressor gene (26). All karyotyped cells of Tera showed, besides 3 normal copies of chromosome 12, 1 (3x) and 2 and 3 copies of the i(12p) (between 5 and 9 copies of 12p). The 7 karyotyped cells of Tera-CP showed 1 or 2 normal chromosomes 12 and 1 copy of i(12p). In all 7 cells, the del(12)(q14q21) was found, and in 4 cells the add(12)(p11.2) was found. Therefore, the number of 12p copies in Tera-CP is either 3 or 4. In male germ cell tumors, BosI et al. (27) found the presence of 3 or more additional copies of 12p associated with a greater likelihood of treatment failure. Because both cell lines show 3 or more additional 12p copies, in this in vitro model no evidence for a positive correlation between CDDP resistance and number of copies of 12p is found.

a J. van Echten et al., manuscript in preparation.
Immunochemistry demonstrated that both cell lines mainly consisted of EC. The amount of SSEA-1-positive cells was higher than data reported in the literature (10). This is probably due to the long time during which cells were cultured in vitro in order to keep their passage number comparable with that of Tera-CP, combined with the tendency to differentiation shown by the cell line. The expression of neurofilaments in both lines suggests an early neuroepithelial differentiation in some cells. The relevance of the presence of vimentin in cultured cells as a parameter of differentiation in vitro is uncertain since it is described to vary with culture conditions (28). Differentiated germ cell tumor cells are found to be more resistant to CDDP than EC cells (4). However, in Tera-CP, resistance to CDDP in EC cells was induced without a concomitant increase in differentiation. In addition, the marked differentiation of TeraX and Tera-CPX (determined by morphometry of tumor slides; data not shown) shows that differentiation of the cells in the in vivo model does not influence their degree of resistance (in vivo RF = 2.8; based on differences in tumor reduction). This close correlation of in vitro and in vivo degree of resistance also excludes a role for in vivo active, tumor-associated factors that influence the pharmacokinetics of CDDP in this nude mouse model, a phenomenon described for a model with in vivo acquired resistance (29).

There were no differences in cellular TSH level between Tera and Tera-CP and no cross-resistance for cadmium chloride was observed. This indirectly indicates that cellular methallothionein content does not play a role in this model, although a role for these proteins is described in another CDDP-resistant germ cell tumor cell line (24). Elevated GSH levels were found to be part of the CDDP-resistant phenotype in several studies but not all (30). Also, depletion of GSH in resistant cells did not always lead to sensitization to CDDP (30). A causative role of GSH in CDDP resistance therefore remains unclear. In Tera-CP, GSH levels were increased, which is compatible with the cross-resistance of Tera-CP to melphalan (31) and 4-HPC (32). It was demonstrated that Tera-CP could be sensitized to CDDP by depletion of cellular GSH with DL-buthionine-S,R-sulfoximine up to 50% reduction in RF (33). Because GSH seemed to be a possible mechanism of resistance in Tera-CP, it was measured in the cell lines Teram and Tera-CPm created after passage through mice. In Teram4, although CDDP sensitivity was comparable with the other Teram lines, the GSH level was comparable with that of the Tera-CPm lines. This increase in GSH without a concomitant decrease of sensitivity indicates that an increased amount of GSH alone does not necessarily predict drug resistance. Other mechanisms that influence CDDP sensitivity but that were not determined here might play a role. GST
activity was elevated in Tera-CP. It was also consistently high and low, respectively, in resistant Tera-CPm and sensitive Tera lines. This together with a report about the low activity of GST in testicular germ cell tumor samples compared with adjacent normal testicular tissue (34) indicates that GST activity could be an important marker of tumor cell CDDP sensitivity in germ cell tumors. The role of the GST isoenzyme GSTπ is controversial in CDDP resistance. Low levels in small cell lung cancer cells correlated with sensitivity to CDDP (35).

In CDDP-resistant sublines of Chinese hamster ovary cells, the observed increased mRNA and protein expression of GSTπ were normalized in a revertant subline (36). But transfection of the GSTπ gene into NIH3T3 and MCF7 cells did not decrease CDDP sensitivity of these cells (35, 37). In two CDDP-resistant small cell lung cancer cell lines, GSTπ mRNA overexpression was found in only one of the two cell lines (38). In our model, GST was especially present as the isoenzyme GSTπ, which is in accordance with the relatively high amount of GSTπ found by Strohmeyer et al. (34) in germ cell tumors compared to normal testicular tissue. Immunohistochemistry of other germ cell tumors also demonstrated that GSTπ was the predominant isoenzyme in EC cells but did not correlate with response to therapy (39).

In contrast to a CDDP-resistant germ cell tumor cell line described by Kelland et al. (24), accumulation defects did not seem to play a role in Tera-CP. The observed decreased binding of platinum to DNA might therefore be due to an increased efficacy of the detoxifying system, leading to increased scavenging of reactive CDDP metabolites in the cellular cytosol. On the other hand, an effect of GSH on Pt-DNA adduct formation and/or repair has been described (40, 41). From the many indicators of DNA repair and handling, topoisomerase I and II were studied because of their potential role in CDDP resistance. While in other CDDP-resistant cell lines increased topoisomerase I (42) and II (43) activities have been reported, we could not demonstrate an altered activity of the DNA topoisomerases in the resistant line. Although cross-resistance for 5-FUra could be an indication for an increased thymidine metabolism in the resistant cell line (44), the FdUMP-binding capacity and the activity of thymidylate synthase found in Tera-CP showed a tendency to be decreased. The cross-resistance to 5-FUra could be due to an alteration in cellular reduced folate levels in Tera-CP since the complexation of the 5-FUra metabolite FdUMP to thymidylate synthase is stabilized by such reduced folates.

Tera and Tera-CP are good models for in vitro and in vivo CDDP resistance. The total binding of platinum to DNA was reduced, probably due to an increased efficacy of the detoxifying system. Induction of CDDP resistance did not affect the EC phenotype of Tera-CP.
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model can be used for testing modulators of CDDP sensitivity and for studying the correlation between CDDP resistance and phenotype of germ cell tumors.

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