Characterization of a Human Small Cell Lung Carcinoma Cell Line with Acquired Resistance to cis-Diamminedichloroplatinum(II) in Vitro


Division of Medical Oncology, Department of Internal Medicine [G. A. P. H., N. H. M., E. G. E. d. V.], Department of Clinical Immunology [L. d. L.], Department of Human Genetics [B. d. J.], and Pharmaceutical Department [D. R. A. U.], University Hospital, Groningen, The Netherlands; Medical Biological Laboratory T.N.O., Rijswijk, The Netherlands [A. M. J. F-S]; and Department of Pathology, Free University Amsterdam, The Netherlands [R. J. S.]

ABSTRACT

A 6.4-fold cis-diamminedichloroplatinum(II) (CDDP) resistant human small cell lung carcinoma cell line (GLC4-CDDP) was developed to study acquired CDDP resistance in vitro. Compared to the sensitive cell line (GLC4), the GLC4-CDDP showed an increase in doubling time and a decrease in cloning efficiency, cellular size, double minutes per cell, cellular protein, and nuclear protein content.

While a complete cross-resistance for tetraplatin and a partial cross-resistance for doxorubicin, melphanalin, cadmium chloride, carboplatin, and cis-dichloro-trans-dihydroxy-cis-bis(isoprolylamine)platinum(IV) (resistance factor, respectively, 4.0, 5.8, 2.1, 1.5, 2.9) was found, no cross-resistance for vincristine was found. In the GLC4-CDDP line in comparison to the GLC4 line, glutathione and total amount of sulfhydryl compounds was significantly increased, while glutathione S-transferase and glutathione reductase was the same.

The platinum content in cells and nuclei was lower in the resistant line, but after correction for cellular protein or volume no difference was found. The amount of platinum bound to DNA was significantly lower in the GLC4-CDDP line.

After a 1-h incubation with CDDP, the amount of Pt-GG adducts was the same and the amount of interstrand cross-links was reduced in the GLC4-CDDP line as compared to GLC4.

In conclusion, in the GLC4-CDDP line the phenotype and genotype are changed and various mechanisms, such as decreased Pt-DNA binding, elevated glutathione, and reduced interstrand cross-links, play a role in the development of the CDDP resistance.

INTRODUCTION

CDDP3 has become one of the most important cytotoxic agents now in use in clinical practice. The spectrum of applicability of platinum based drugs may even increase due to the development of new agents that either have substantially fewer side effects, such as carboplatin, or are active in situations where CDDP fails. One of the reasons for failure of any chemotherapeutic drug is the development of resistance. Although CDDP is an active drug for the treatment of SCLC, its chemotherapeutic drug is the development of resistance. Although CDDP is an active drug for the treatment of SCLC, its chemotherapeutic drug is the development of resistance. Although CDDP is an active drug for the treatment of SCLC, its chemotherapeutic drug is the development of resistance. Although CDDP is an active drug for the treatment of SCLC, its chemotherapeutic drug is the development of resistance.

On a more basic level, considerable discussion still exists on the precise mode of action of CDDP. Although the interference of the drug with tumor cell DNA is usually considered to be of major importance, a role for membrane and cytosolic effects still remains possible.

The availability of cell lines with acquired resistance to CDDP could be of importance in the development of measures to circumvent resistance in the clinic, in the screening of platinum based drugs for increased activity, and in achieving a better understanding of the critical modes of action of the drug.

We therefore developed a human SCLC cell line resistant to CDDP.

MATERIALS AND METHODS

Chemicals. CDDP, carboplatin, and cis-dichloro-trans-dihydroxy-cis-bis(isoprolylamine)platinum(IV) were provided by Bristol Myers S.A.E., Madrid, Spain. Doxorubicin was obtained from Farmitalia Carlo Erba, Milano, Italy; vincristine sulfate was from Eli Lilly, St. Cloud, France; melphanalin was from Wellcome Foundation, Ltd., London, England. Tetraplatin was kindly provided by E. Boven, Free University Amsterdam, The Netherlands. RPMI 1640 was obtained from Gibco, Paisley, Scotland. FCS was from Flow Laboratories, Irvine, Scotland.

GSH, ethylene glycol-bis(β-aminethylether)-N,N′,N,N′-tetraacetic acid, dithionitrotetrazolium, Triton X-100, 5,5-dithio-bis(2-nitrobenzoic acid), cadmium chloride, 1-chloro-2,4-dinitrobenzene, RNase A, Colcemid, were obtained from Sigma Chemical Co., St. Louis, MO, NADPH and proteinase K were obtained from Merck, Darmstadt, West Germany, and ethidium bromide was from Serva, Heidelberg, West Germany.

The monoclonal antibodies mNF, RGE53, and Vim were supplied by Eurodiagnostics, Apeldoorn, The Netherlands. The a-HLA-ABC were supplied by Becton Dickinson, Sunnyvale, CA. RNase and the RNase-T1 were from Boehringer, Mannheim, West Germany.

Cell Lines and Production of Resistance. The GLC4 cell line is derived in the laboratory in Groningen from the pleural effusion of a patient with SCLC (2). The patient was not treated with a platinum containing regimen before establishing the cell line. These cells are growing partly in suspension and partly attached in RPMI 1640, 10% fetal calf serum in a humidified atmosphere with 5% CO2 at 37°C.

For production of a CDDP resistant cell line (GLC4-CDDP), the cells were first continuously exposed to CDDP. The drug was not washed out. When the cultures reached a density equal to or greater than initially determined with microscopic observation, new CDDP was added. Over the first year, CDDP could be added nine times. After 12 months of continuous incubation with CDDP and a resistance factor of 4 the cell line was exposed at least once a month to 1-h incubations with CDDP followed by washing out of the drug. The experiments described in the rest of this article were performed with CDDP resistant cells with a resistance factor of 6.4. In order to ensure stable resistance with GLC4-CDDP cells, all experiments were performed 6 weeks after the end of the last 1-h incubation with CDDP.

Cloning and Cytotoxicity. The clonogenic assay was performed as described earlier (3). For GLC4 and GLC4-CDDP, 3000 and 5000 cells, respectively, were plated per dish. After a 1-h incubation at 37°C with the drug, the cells were washed and resuspended in fresh medium. Cross-resistance was determined for doxorubicin, vincristine, melphanalin, diamiine(1-cyclobutane-carboxylato)platinum(II), cis-dichloro-trans-dihydroxy-cis-bis(isoprolylamine)platinum(IV), tetra-chloro(d,trans)-1.2 dianminocyclohexane platinum(IV), and cadmium chloride. The drug concentrations inhibiting colony formation by 50% were calculated and expressed as the mean of three determinations.

Morphology. For morphological analysis, cell preparations were
stained with hematoxylin and eosin and May-Grünwald-Giemsa, according to routine procedures. Cell Size. For relative cell size determination, cell preparations made by cytocentrifugation were stained and cell size was determined with the texture analyzing system. As described before (4), the microscope connected to the texture analyzing system was provided with a ×40 oil immersion objective. Cells to be measured were centered in the image of the television camera and enclosed in a circular mask. This area was calculated. A total of 40 cells per cell line and per experiment were measured in two different experiments.

DNA Content. The DNA content in nuclei was measured in a FACStar flow cytometer (Becton Dickinson), using the procedure of Vindelov and Nissen (5). Chicken RBC were used as internal standard (DNA index = 0.35).

Chromosome Analysis. The tumor cells were harvested by brief trypsinization for chromosome preparation. Two h after harvesting, 0.05 μg colcemid/ml culture medium was added. Following harvesting, the cells were centrifuged for 5 min at 240 × g. The pellets were resuspended in 0.06 M KCl, incubated for 15 min at 37°C, centrifuged, resuspended in a mixture of ethanol/glacial acetic acid (3:1), centrifuged, resuspended, and left in the tubes for 20 min. After a final centrifugation, the cell suspension was pipetted onto slides and air dried. For the GLC4 line 10 metaphases were analyzed, and for the GLC4-CDDP line 4 metaphases were analyzed and 25 were chromosomally counted. Chromosomes were banded by acetic acid or trypsin with Giemsa. For counting the amount of DM the slides were stained with Giemsa for 60 min.

Panel of Monoclonal Antibodies. A number of monoclonal antibodies reactive with different SCLC associated antigens were tested (6). These included antibodies reactive with antigens also expressed in normal and malignant tissues with a neuroendocrine differentiation state (MOC-1, MOC-21, MOC-32, MOC-51, MOC-52), and an antibody reactive with an antigen also expressed in subsets of normal and malignant tissues with an epithelial differentiation state (MOC-31). In addition, monoclonal antibodies detecting intermediate filaments were included. We used RGE 53 for detecting cytokeratin 18 (nomenclature according to Moll et al. (7)) and mNF for the M, 210,000 and 68,000 components of neurofilaments (8). Antibodies against other cellular antigens, Leu 7 (α-HNK, antigen) and α-HLA-ABC were also used. The monoclonal antibody JSB-1 against the MDR associated M, 170,000 glycoprotein was also tested (9).

P-Glycoprotein Gene Amplification and Expression. The P-glycoprotein gene amplification and expression were verified on DNA and RNA blots by hybridization with the cP-28 probe of Van der Bliek et al. (10).

Platinum Concentrations. The amount of platinum was determined with a Model 1275 atomic absorption spectrophotometer equipped with a Model GTA-95 graphite tube atomizer and an autosampler (Varian Techtron Pty., Ltd., Mulgrave, Victoria, Australia). For calibration, a solution of platinum chloride in 65% HNO3 was used. The platinum detection limit is 250 ng.

Cellular platinum content was measured after treating 2 × 10⁶ cells for 1 h with CDDP. After drug incubation, cells were washed with PBS and dried in an oven at 60°C. Thereafter the pellet was resuspended in 65% HNO3.

Nuclear platinum content was measured after washing 2 × 10⁶ cells with PBS after a 1-h incubation at 37°C with CDDP and resuspended in “nuclei buffer” (NaCl, 150 mM, KH2PO4, 1 mM; MgCl2, 5 mM; ethyleneglycol-bis(α-aminoethyl ether)-N,N′,N′′,N′-tetraacetic acid, 1 mM, dithiothreitol, 0.1 mM; pH 6.4) at 0°C. Then, the cells were centrifuged and resuspended in 1 ml nuclei buffer (0°C) and 9 ml nuclei buffer (0°C) containing 0.3% Triton X-100 and incubated for 10 min at 0°C. The nuclei were pelleted (250 × g, 5 min, 0°C), dried in an oven (60°C), and resuspended in 65% HNO3.

The amount of platinum bound to DNA was measured after treating 10⁶ cells with CDDP for 1 h. The cells were washed with PBS and the DNA was isolated according to the technique used by Fichtinger-Scheppman et al. (11), except that we used 20 μg/ml RNase instead of RNase A and RNase T1. The fibrous precipitate of DNA was washed in 80% ethanol hydrolyzed in 1 N HCl (250 μl) at 70°C for 2 h. The DNA content was estimated by absorption at 260 nm; the amount of platinum in the sample by atomic absorption spectrophotometer, as described in Roberts and Fraval (12).

Sulfhydryl Compounds, Glutathione S-Transferase, Glutathione Reductase, Protein, and Amino Acid Content. GLC4 and GLC4-CDDP cells in the logarithmic phase of growth were harvested 4 days after passage. The cells were washed with PBS and resuspended in a relevant volume of PBS or another solution mentioned in the assay procedure. The cells were then vortexed and sonicated. The cell suspension was centrifuged at 4°C at 100,000 × g for 1 h. The supernatant was then assayed. All measurements were performed under Vmax conditions and at least 4 separate determinations were performed. For GSH determination, cells were resuspended in cold 5% trichloroacetic acid, vortexed, and centrifuged at 4°C at 10,000 × g for 15 min. The supernatant was assayed for total GSH by the enzyme recycling method under conditions similar to those described by Tietze (13). The determination limit was 25 ng. Total sulfhydryl compounds were determined by the method of Ellman (14). Ellman’s reagent, 5,5-dithio-bis(2-nitrobenzoic acid), was used as substrate and absorbance was measured at 412 nm. The GST activity was measured according to the method of Habig et al. (15). 1-chloro-2,4-dinitrobenzene was used as substrate and the enzyme reaction was measured by the absorbance change at 340 nm. Glutathione reductase activity was measured by modification of the technique used by Carlberg and Mannervik (16). The consumption of NADPH was followed by an analysis of the absorbance change at 340 nm. For protein determination the method of Lowry et al. was used (17). For protein determination in nuclei the same isolation of the nuclei as described in a previous section was used. All measurements were performed on a Zeiss PMQ spectro-photometer. The amino acid concentrations were measured in 6 × 10⁶ cells according to the method of Hare (18). The analyses were performed on a Kontron Liquimat III analyser after hydrolysis of the samples with constant boiling in 6 M HCl in evacuated sealed tubes at 110°C. The detection limit was 1 nmol.

Immunochemical Quantitation of cis-Pt(NH3)2(hgG-GP)(Pt-GG). The Pt-GG adduct, the main platinum-containing product in digested CDDP-DNA, was measured by using a polyclonal antibody according to the method of Fichtinger-Scheppman et al. (11). For this purpose the DNA was isolated from 5 × 10⁶ cells which had been treated with CDDP for 1 h and then subsequently washed with PBS.

After digestion of the DNA, the products were separated on the MonoQ column (Pharmacia, Sweden) and the Pt-GG adduct contents in the eluate fractions were determined in a competitive enzyme linked immunoabsorbent assay (11). The data are reported as the mean ± SE of one experiment performed in triplicate.

Ethisium Bromide Fluorescence Cross-Link Assay. Renaturation of DNA after heat denaturation and rapid cooling is related to the extent of DNA cross-linking. Double stranded renatured DNA can be stained selectively by ethidium bromide and measured by fluorescence spectrophotometry (19). For the study of CDDP induced cross-links per sample, 10⁶ cells were incubated for 1 h with various concentrations of CDDP. To stop the incubation, cells were washed three times with ice-cold PBS. Measurements were performed as described before (19) and cells were also lysed overnight. The data shown are the mean ± SE of 4–6 determinations. For repair measurements, DNA damage was induced by incubation with CDDP for 2 h with 100 μM CDDP. After stopping the incubation, the cells were washed and resuspended in fresh medium and allowed to repair at 37°C in 5% CO2 for a period of 22 h. For these experiments, apart from a lysis time of 30 min during heparin treatment, cells were processed as described previously.

Statistical Analysis. Differences were tested by using the unpaired Student’s t test.

RESULTS

Characteristics of the Sensitive and Resistant Line. The GLC4-CDDP cell line has a stable CDDP resistance for at least 8 months. All measurements described in this paper were performed at a resistance factor of 6.4. The GLC4 line grew partly floating, partly attached. The GLC4-CDDP cell line grows primarily attached. The doubling time of the latter cell line is
longer and the cloning efficiency was lower in the soft agarose assay compared to the former (Table 1). There was no clear morphological difference appreciable with light microscopy between the two cell lines. The relative cell size, however, is significantly reduced in the resistant line.

Chromosomal analysis showed that the average number of chromosomes was 58 with a range of 54–60. One of the karyotypes of GLC4 had the following description: 58,X,Xp; +1, +37, +34q, 4q-, -5p, +6q, +der(6)(12);q(27);q(13), +7, +10p, -13, +14p, -15, +16, +16, +6 markers. The other karyotypes showed a very related pattern. In the GLC4-CDDP line the average number of chromosomes was 54 (range, 46–62). One of the karyotypes of GLC4-CDDP was: 54,Xp; -Y, +3q, 4q, 6q, +der(6)(12);q(27);q(13), +7, +10p, -11, -12, -13, +13q, +14q, -15, +16q, +16q, +17, +20, +22, +22, +22, +22, +22, +22. The other karyotypes showed very related patterns. In both cell lines DM could be found. However, the amount of DM was significantly reduced in the resistant line (Table 1). Also, the DNA content was lower in the GLC4-CDDP line. With respect to cellular antigen expression, the intermediate filament pattern was identical for both lines and was in accordance with the pattern of a variant type human SCLC (20). The expression of the other assessed cellular antigens is identical in both cell lines except that GLC4-CDDP appeared to have a decreased HLA-ABC and a decreased HNK1 antigen content. Both cell lines expressed the neuroendocrine marker defined by MOC-1 (21). The stain with the antibody against the M., 170,000 glycoprotein was negative and no amplification in the GLC4-CDDP line was detected. The polyadenylate pattern of a variant type human SCLC (20). The expression of the other assessed cellular antigens is identical in both cell lines except that GLC4-CDDP appeared to have a decreased HLA-ABC and a decreased HNK1 antigen content. Both cell lines expressed the neuroendocrine marker defined by MOC-1 (21). The stain with the antibody against the M., 170,000 glycoprotein was negative and no amplification in the GLC4-CDDP line was detected. The polyadenylate

<table>
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<th>Parameter</th>
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<th>GLC4</th>
<th>GLC4-CDDP</th>
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<tbody>
<tr>
<td>Doubling time (b)</td>
<td>3</td>
<td>24 ± 1.4</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>Cloning efficiency (%)</td>
<td>10</td>
<td>40.4 ± 2.3</td>
<td>23.4 ± 1.4</td>
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<tr>
<td>Relative cell size</td>
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</tr>
<tr>
<td>Double minutes/cell</td>
<td>2</td>
<td>49.3 ± 7.6</td>
<td>21.9 ± 3.5</td>
</tr>
<tr>
<td>DNA content (index)/cell</td>
<td>3</td>
<td>1.8 ± 0.05</td>
<td>1.5 ± 0.05</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* P < 0.0005.
* P < 0.025.

Resistance and Cross-Resistance Studies. Table 2 shows the drug concentration inhibiting colony formation by 50% and the degree of resistance for various chemotherapeutic drugs, including CDDP and three CDDP analogues. There is a significant cross-resistance with doxorubicin and melphalan.

The resistance factor of 2.1 for cadmium chloride is compatible with the raised sulfhydryl content, although the rise in the resistant line is most probably not due to a rise in metallothioneins but rather to elevated GSH content. Cross-resistance for the platinum analogues is highest for tetraplatin (8.1) and only 1.5 for carboplatin.

Sulfhydryl Compounds, Glutathione S-Transferase, Glutathione Reductase, and Amino Acid Content. Table 3 shows the levels of sulfhydryl compounds and the results of the enzyme assays and protein measurements in the two cell lines. GSH level was significantly raised in the CDDP resistant cell line. The significant rise in total sulfhydryl compounds is most probably due solely to the rise in GSH. There are no elevated levels of GST and glutathione reductase in the resistant cell line. Cellular and nuclear protein were significantly reduced in the resistant line, probably due to the reduction in cellular size. Amino acid analysis of the cell content was performed in order to evaluate whether this reduction was due to the fact that in the Lowry assay tryptophan and tyrosine are measured. A significant reduction of amino acid content could be found in the resistant cell line for all amino acids except glutamic acid and cysteine. Interestingly, glutamic acid and cysteine are both constituents of GSH.

Platinum Content. Fig. 1A shows the cellular and nuclear platinum content after a 1-h CDDP treatment. The GLC4-CDDP cells contain significantly less, namely 78.3 ± 1.5%, of the platinum content of the GLC4 cells at all concentrations.

When the platinum content was corrected for cellular protein or cellular volume, no significant difference was found at all CDDP concentrations tested. Further, no difference was found for the platinum content in the nuclei of both cell lines. In the GLC4 cell line, the nuclei contain 38.1 ± 3.1% of the platinum content of the GLC4 cells. For the GLC4-CDDP line this is significantly higher, namely 52.8 ± 3.8% (P < 0.025). The amount of Pt-DNA binding was significantly higher in the sensitive as compared to the resistant cell line (Fig. 1B). The CDDP incubation concentrations used for Pt-DNA binding were slightly higher than those used for the cellular and nuclear platinum measurements in order to obtain measurable platinum levels.

DNA Damage. For the intrastrand adducts Pt-GG, cells were incubated with 17, 33, or 67 μM CDDP for 1 h. The only significantly higher amount of intrastrand cross-links was detected at 17 μM (P < 0.025) in the sensitive cell line (Table 4).
The number of DNA interstrand cross-links after a 1-h CDDP treatment was reduced by 45% at high concentrations in the resistant cell line (at 333 μM, $P < 0.01$) (Table 4). For repair studies a 2-h incubation with 100 μM was chosen in order to obtain enough cross-links to study repair (Fig. 2). After 2 h of CDDP incubation, there was no significant difference in the amount of interstrand cross-links between both lines. The increase of cross-links after drug incubation in the sensitive line was higher and took place over a longer period of time than in the resistant line. There was a significant decrease in cross-links in the resistant line between 4 and 20 h ($P < 0.05$).

**DISCUSSION**

The aim of this study was to develop a model for CDDP resistance that could be relevant for inquiries into the mechanism of resistance and ultimately to aid in circumvention of resistance in the clinic.

Experimental evidence suggests that clinical resistance will be complete at an *in vitro* resistance level of no more than 5-fold (22). We therefore chose to study the GLC4-CDDP cell line at an intermediate level of resistance. At that level, no change in overall morphology of the cells was detected. This is in contrast to the observations obtained in resistant ovary cancer (23). The GLC4-CDDP cells are, however, smaller than the GLC4 cells, and the growth pattern *in vitro* did clearly change. GLC4 cells grow partly in suspension and partly attached and GLC4-CDDP cells grow largely attached. In accordance with findings of Frei et al. (24), slower growth kinetics emerged in GLC4-CDDP during development of resistance to CDDP.

The average number of chromosomes of the GLC4 and GLC4-CDDP lines is in the same range. The finding of a different DNA index in the two cell lines may mean that, although the number of chromosomes is comparable, the amount of chromosomal material is not. Comparison of the karyotypes of the GLC4 and GLC4-CDDP cell lines revealed that they have a lot of specific chromosomal abnormalities in common, except for abnormalities of chromosome 16 (16q+) and 17 (17p+). These abnormalities appeared only in the GLC4-CDDP line and a 15p+ chromosome was specific for the GLC4 line. The amount of DM was significantly lower in the resistant line. In contrast, during development of acquired resistance to doxorubicin *in vitro* (the GLC4-ADR line), the number of DM rose significantly (25). It is possible that these abnormalities are in some way involved in the resistance of the cell line. The expression of cellular antigens remained the same for both lines with the exception of Leu 7 and the HLA-ABC antigens. Although cross-resistance with the natural product doxorubicin is found in this cell line, no amplification of the MDR gene or expression of the M, 170,000 glycoprotein was found in the GLC4-CDDP cell line. Doxorubicin resistance in small cell lung cancer can be found *in vitro* without the expression of the MDR phenotype (26). The platinum analogues had varying effects on this resistant cell line (Table 2). Complete cross-resistance was found for tetraplatin but only partial cross-resistance was found for carboplatin. This cell line might prove to be a useful model to study differences in the modes of action of the various platinum analogues.

It can be concluded from the presented data that many changes occur during the process of development of resistance. It is possible to speculate on the relevance of a number of these changes. This discussion, however, is complicated by the relative insensitivity of the techniques used to detect changes in the binding of platinum by these cells.

We found no arguments for a decreased cellular or nuclear concentration of platinum (Fig. 1A). Some indications, however, were found for changes in the DNA platinum interaction. In the resistant cell line, high concentrations of CDDP caused less DNA binding (Fig. 1B). Identical amounts of Pt-GG adducts were found and less interstrand cross-links were found at a high CDDP concentration (Table 4). Although the occurrence of such a decrease in interstrand cross-linking was previously reported (27–29), it has not been demonstrated in all cases of resistance (30). It is possible that even if interstrand cross-links are only a small part of the mode of action of CDDP, their increased elimination or reduced formation could lead to a modest level of resistance. Although an increased GSH level...
was found in the CDDP resistant line, the role of GSH in CDDP resistance is controversial. In ovarian cancer cell lines, buthionine sulfoximate mediated glutathione depletion resulted in an increase in CDDP sensitivity (31). This is, however, not the case in all cell lines (32). In this study, the GST level is not different in both lines but there is a reduced sensitivity to cadmium chloride in the GLC-CDDP line. It is possible that the elevated GSH in the CDDP resistant line results in a different binding of CDDP at the cytoplasmic or DNA level.

The substantial difference in resistance to carboplatin and CDDP suggests that it is important that the formation of cross-links is changed in GLC-CDDP, especially since the rate of the production of cross-links was found to differ between the two agents (33). It is possible that this process of cross-link formation is influenced by GSH (34, 35). This compound appears to be present in excess in GLC-CDDP (Table 3).

In summary, in this human small cell lung carcinoma cell line with in vitro acquired CDDP resistance, the phenotype and genotype are changed. Various mechanisms may play a role in the development of CDDP resistance in this line, namely decreased Pt-DNA binding, elevated GSH, and reduced interstrand cross-link formation.

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