Characterization of a Human Squamous Carcinoma Cell Line Resistant to cis-Diaminedichloroplatinum(II)  

Beverly A. Teicher, Sylvia A. Holden, Michael J. Kelley, Thomas C. Shea, Carol A. Cucchi, Andre Rosowsky, W. David Henner, and Emil Frei III

Division of Cancer Pharmacology, Dana-Farber Cancer Institute, Boston, MA 02115

ABSTRACT

We have developed a human head and neck squamous cell carcinoma cell line (SCC-25/CP) which is relatively stably resistant to cis-diaminedichloroplatinum(II) (CDDP) after repeated exposure to escalating doses of the drug. The studies reported elucidate the mechanism(s) by which the SCC-25/CP cell line is resistant to CDDP. The SCC-25/CP cell line is approximately 30-fold resistant to CDDP, approximately 10-fold resistant to carboplatin, and about 9-fold resistant to ifosfamide.

Using [$^{195}$Pt]CDDP, we examined the levels of platinum in whole cells and cellular fractions of both the SCC-25 and SCC-25/CP cells after 1 h exposure to 100 nM drug. The SCC-25 cells took up 30 pmol of platinum/10<sup>6</sup> cells in 1 h; 64% of the drug was in the nucleus and 21% in the cytosol. The SCC-25/CP cells took up 7 pmol of platinum/10<sup>6</sup> cells; of this, 41% was in the nucleus and 33% in the cytosol. The SCC-25 cell nuclei contained 331 pmol of platinum/mg protein and the cytosol 21 pmol of platinum/mg protein, whereas the SCC-25/CP cell nuclei contained 47 pmol of platinum/mg protein and the cytosol 8.1 pmol/mg protein. The release of drug from both cell lines followed a very similar course and was most rapid over the first 6 h.

There was no difference in the non-protein sulphydryl content of the cell lines. The protein sulphydryl content, as measured by Ellman's procedure, indicated that the SCC-25/CP cell line has approximately a 2-fold increase in protein sulphydryl content compared to the SCC-25 cell line. The SCC-25/CP cell line is about 2-fold resistant to cadmium chloride at 50% cell kill and about 2.5-fold resistant at 1 logarithm kill compared to the SCC-25 cell line. Glutathione transferase activity in crude cytoplasmic extracts was measured and found to be approximately 2- to 3-fold higher in the CDDP resistant cells. The isoelectric point of the glutathione transferase isozyme was 4.8 in both the sensitive and resistant cell lines, suggesting induction of the predominant isozyme present in the parent cell line. By alkaline elution there was greater cross-link formation by CDDP in the SCC-25 cell line than in the SCC-25/CP cell line at the same drug concentrations. In conclusion, the mechanism of resistance of the SCC-25/CP cell line to CDDP is multifactorial, involving plasma membrane changes, increased cytosolic binding, and decreased DNA cross-linking.

INTRODUCTION

CDDP<sup>1</sup> has demonstrated a broad range of activity against several malignancies in humans (1). CDDP is classified with the antitumor alkylating agents because it forms bidentate adducts with DNA (2–4). It is believed that DNA is the critical intracellular target of CDDP and that DNA cross-linking is the lethal lesion caused by this drug (2, 5, 6). Like many antineoplastic agents, CDDP is a potent mutagen, inducing frame shift and base substitution mutations in both bacterial and human cells (7–10). CDDP-sensitive and -resistant L1210 cell lines have been examined by several laboratories. These studies have described changes in the plasma membrane (11–13) and changes at the level of DNA adduct formation (14) in the resistant cell lines.

Experimental studies of other alkylating agents have indicated that resistance to these drugs may occur by a variety of mechanisms. Goldenberg and Begleiter (15) and Goldenberg et al. (16) found resistance to nitrogen mustard to be associated with a transport defect in the choline carrier impeding uptake of nitrogen mustard into the cells. Resistance to PAM has also been attributed to a transport defect, probably involving the leucine carrier (17, 18). In other studies, PAM resistance was associated with an elevation in intracellular glutathione (19–22). Multifactorial resistance to PAM involving rate of drug efflux, sulphydryl levels, and DNA interstrand cross-link formation and repair has been reported in several cell lines (23, 24). Hilton and Colvin (25) have reported that cyclophosphamide-resistant human and rodent cell lines have increased levels of an aldehyde dehydrogenase that inactivates the aldophosphamide metabolite of the drug. Increased repair of DNA monoadducts via the action of guanine O<sup>6</sup>-methyltransferase has been described as the mechanism of resistance in 1,3-bis(2-chloroethyl)-1-nitrosourea-resistant bacterial and human cell lines (26–29).

We have developed a human head and neck squamous cell carcinoma line (SCC-25/CP) which exhibits relatively stable resistance to cis-diaminedichloroplatinum(II) after repeated exposure to escalating doses of the drug (30). The studies reported here were designed to elucidate the mechanism(s) by which the SCC-25/CP cell line are resistant to CDDP.

MATERIALS AND METHODS

Drugs. CDDP, diammine [1,1-cyclobutaneedicarboxylato(2-)O,O'-platinum(II)] (carboplatin), and cis-dichloro-trans-dihydroxybis(isopropylamine)platinum(IV) (ifosfamide) were gifts from Johnson Matthey, Inc. (West Chester, PA). [$^{195}$Pt]cis-diaminedichloroplatinum(II) in isotonic saline was made available by Drs. J. D. Hoeschele and F. F. Knapp, Jr. at Oak Ridge National Laboratories (Oak Ridge, TN) (31, 32). CdCl<sub>2</sub> was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Cell Lines. SCC-25 and SCC-25/CP human squamous carcinoma of the head and neck cells grow as monolayers in Dulbecco-Vogt modified Eagle's minimum essential medium supplemented with antibiotics and 5% FBS (30). These cell lines have a plating efficiency of 10–30% and a doubling time of 48–50 h in vitro (33). For cloning, SCC-25 and SCC-25/CP cells were suspended by trypsinization, diluted in complete growth medium, and plated into 60- x 15-mm tissue culture dishes containing 5 ml of complete growth medium. Colonies grow to a countable size (>50 cells) in 2 weeks.

The SCC-25/CP cell line has been maintained for 9 months in the absence of exposure to CDDP and the resistance of this cell line to CDDP has remained stable for that period.

Survival Studies. SCC-25 and SCC-25/CP cells in exponential growth were exposed for 1 h to concentrations of CDDP, carboplatin, or CdCl<sub>2</sub> ranging from 1-1000 µM in media without sera. The cells were then washed three times with PBS and plated for colony formation as described above. Each survival curve was determined in three independent experiments.
Cellular Fractionation. [195mPt]CDDP was received with a specific activity of 145–165 mCi/mmol. The half-life of [195mPt]CDDP was 4.02 days, and therefore experiments were carried out immediately upon receipt of the drug. Two million SCC-25 and SCC-25/CP cells/plate were incubated with 100 μM [195mPt]CDDP in media without sera for 60 min at 37°C in humidified air with 8% CO2. Cells were washed six times with PBS, fresh medium containing serum was added, and the plates were replaced in a 37°C/8% CO2 incubator for 0, 3, 6, or 24 h. At the end of each time period, cells were suspended using 0.5% EDTA/0.125% trypsin, centrifuged at 500 × g, resuspended in water, and lysed by sonication on ice.

The fractionation procedure of Sharma and Edwards (34) was performed at 4°C and the final pellets were resuspended in water. Lysed cells were centrifuged at 1000 × g for 10 min, and the supernatant was centrifuged at 3500 × g for 10 min to obtain the mitochondrial pellet. The second supernatant was centrifuged at 16,000 × g for 20 min to obtain the lysosomal pellet. The third supernatant was centrifuged at 100,000 × g for 60 min to obtain the microsomal pellet. This final supernatant contained the cytosol. The purity of the subcellular fractions from each cell line was checked by electron microscopy. [195mPt]CDDP content was determined using a Beckman Gamma 4000 counter. The amounts of [195mPt]CDDP were determined by comparison to standards. Each point is the mean of duplicate determinations in two independent experiments. The range of duplicate determinations was ±3% (SE) and the range of experimental values was ±8%.

Fluorescence assay, 0.1 ml of the sample was added to 3 ml of 0.1 M potassium phosphate buffer containing 5 mM EDTA (pH 8), then 0.15 ml of an OPT (Alrich Chemical Co., Milwaukee, WI) solution (1 mg/ml OPT in methanol) was added. The derivatization was allowed to continue for 15 min at room temperature in the dark. Total sulfhydryl content was determined by the Ellman method (38-41). SCC-25 and SCC-25/CP cells (107) were suspended using 0.5% EDTA/0.125% trypsin, centrifuged at 500 × g, resuspended in water, and lysed by sonication on ice.

The fractionation procedure of Sharma and Edwards (34) was performed at 4°C and the final pellets were resuspended in water. Lysed cells were centrifuged at 1000 × g for 10 min, the nuclear pellet was obtained. The supernatant from the first 1000 × g spin was centrifuged at 3500 × g for 10 min to obtain the mitochondrial pellet. The second supernatant was centrifuged at 16,000 × g for 20 min to obtain the lysosomal pellet. The third supernatant was centrifuged at 100,000 × g for 60 min to obtain the microsomal pellet. This final supernatant contained the cytosol. The purity of the subcellular fractions from each cell line was checked by electron microscopy. [195mPt]CDDP content was determined using a Beckman Gamma 4000 counter. The amounts of [195mPt]CDDP were determined by comparison to standards. Each point is the mean of duplicate determinations in two independent experiments. The range of duplicate determinations was ±3% (SE) and the range of experimental values was ±8%.

RESULTS

The SCC-25/CP cell line was developed by repeated treatment with escalating concentrations of CDDP and is generally not cross-resistant to alkylating agents of the nitrogen mustard and nitrosourea classes as determined by median inhibitory concentration ratios (30). However, it was not known whether the SCC-25/CP line would be resistant to other platinum complexes. The survival of SCC-25 and SCC-25/CP cells exposed to various concentrations of CDDP and two second-generation platinum complexes, carboplatin and ifroplatin, is shown in Fig. 1. A 30-fold higher concentration of CDDP is required to kill 90% of SCC-25/CP cells compared to SCC-25 cells. Carboplatin and ifroplatin are less potent cytotoxic agents.
The release of drug from both cell lines followed a similar pattern to these platinum complexes which are structurally related to CDDP. Using \[^{195mPt}\]CDDP, we examined the levels of platinum in various times over the following 24 h. The SCC-25 cells took up 30 pmol of platinum/10⁶ cells in 1 h; 64% of the drug was in the nucleus and 21% in the cytosol. Each of the other cellular fractions (lysosomes, microsomes, and mitochondria) contained 5% of the total platinum taken up. The SCC-25/CP cells took up 7 pmol of platinum/10⁶ cells; of this, 41% was in the nucleus and 33% in the cytosol. Each of the other cellular fractions from this cell line contained 8–9% of the platinum taken up. The SCC-25/CP cells (0.391 mg protein/10⁶ cells) are some 30% cysteine, can comprise a large percentage of cellular protein sulfhydryl content. Resistance to the cytotoxic effects of cadmium chloride has been used as an indicator for an increased metallothionein content (46–49). The survival of SCC-25 and SCC-25/CP cells exposed to various concentrations of cadmium chloride for 1 h is shown in Fig. 3. The SCC-25/CP cell line is about 2-fold resistant to cadmium chloride at 50% cell kill and about 2.5-fold resistant at 1 log kill compared to the SCC-25 cell line. This finding correlates very well with the increase in protein sulfhydryl content of the SCC-25/CP cells.

In addition to metallothionein binding, conjugation of CDDP to GSH by glutathione-S-transferase could account for the reduced nuclear content of CDDP. GST activity in crude cytoplasmic extracts of both cell lines was measured and GST activity was found to be 2- to 3-fold higher in the SCC-25/CP cells. The isoelectric point of the principal GST isozyme was about 30% cysteine, can comprise a large percentage of cellular protein sulfhydryl content. Resistance to the cytotoxic effects of cadmium chloride has been used as an indicator for an increased metallothionein content (46–49). The survival of SCC-25 and SCC-25/CP cells exposed to various concentrations of cadmium chloride for 1 h is shown in Fig. 3. The SCC-25/CP cell line is about 2-fold resistant to cadmium chloride at 50% cell kill and about 2.5-fold resistant at 1 log kill compared to the SCC-25 cell line. This finding correlates very well with the increase in protein sulfhydryl content of the SCC-25/CP cells.

**Fig. 2.** Subcellular distribution of \[^{195mPt}\]in SCC-25 and SCC-25/CP cells. Cells were exposed to 100 µM \[^{195mPt}\]CDDP for 1 h. The zero time is immediately after drug removal. ▲, cytosol; ●, nucleus; ○, mitochondria; ■, lysosomes; □, microsomes.

**DISCUSSION**

CDDP has gained wide clinical use as an antineoplastic alkylating agent (1, 50). The goal of this study is to understand the mechanism(s) by which the SCC-25/CP human squamous carcinoma cell line is resistant to CDDP. These mechanisms may also account for the cross-resistance to carboplatin and iroplatin in this cell line. Cross-resistance with CDDP analogues has previously been reported in a human ovarian cancer model.
**CELLULAR RESISTANCE TO CDDP**

Table 1  *Protein and non-protein sulfhydryl content and glutathione transferase activity in SCC-25 and SCC-25/CP cells*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Non-protein sulfhydryl*</th>
<th>Protein sulfhydryl*</th>
<th>Glutathione transferase activity*</th>
<th>Isoelectric point*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC-25</td>
<td>25 ± 2 (55 ± 5)</td>
<td>23 ± 3 (48 ± 7)</td>
<td>231 ± 25 (503 ± 55)</td>
<td>4.8</td>
</tr>
<tr>
<td>SCC-25/CP</td>
<td>21 ± 3 (54 ± 7)</td>
<td>39 ± 2 (100 ± 5)</td>
<td>447 ± 73 (1145 ± 186)</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Measured by fluorescence emission at 420 nm of an OPT derivative.

**Fig. 3. Survival of SCC-25 (•) and SCC-25/CP (O) cells treated with various doses of cadmium chloride. Points, means of three independent determinations ± SE (bars).**

**Fig. 4. DNA cross-linking in SCC-25 and SCC-25/CP cells at various times after drug exposure. The concentrations of CDDP used were: •, 50; O, 10; and □, 2 μM. The cross-linking factor was calculated according to the method of Kohn et al. (43). Data were derived from three independent experiments ± SE (bars).**

The cellular distribution of platinum following treatment with CDDP has been described for cells in culture (52, 53) and in several tissues in vivo (34, 54, 55). Using analytical electron microscopy, Khan and Sadler (52) found that CDDP was predominantly localized in the nucleolus and the inner side of the nuclear membrane. In nucleosomes, using a fluorescent probe, histone H3 as well as DNA were found to be targets for CDDP (53). The intracellular localization of platinum in liver, kidney, and other tissues has been measured by atomic absorption (34, 55) and scanning transmission electron microscopy in conjunction with X-ray probe microanalysis (54). In these tissues, high percentages of platinum were found in the cytosol bound to metallothionein-like proteins and in lysosomes in the kidney tubules. Studies of L1210 cells resistant to CDDP have provided evidence that the drug is transported into the cells by an amino acid transport system (11-13). Plasma membrane alteration is a common mechanism of cellular resistance to alkylating agents (11-13, 15-18) and to a wide variety of other drugs and toxins (56-58). We have found that both SCC-25 and SCC-25/CP cells concentrate platinum in the nuclei to some degree; however, there is a difference in platinum levels in the parent line compared to the CDDP resistant line, implying an alteration in the plasma membrane of the SCC-25/CP cells leading to decreased platinum levels in that cell line under the same exposure conditions as the parent line.

There also appears to be cytosolic changes in the SCC-25/CP cell line. Although the levels of the non-protein sulfhydryl, GSH, are the same in both cell lines, there is an increase in protein sulfhydryl content and an increase in GST in the SCC-25/CP cell line when the SCC-25 and SCC-25/CP cell lines were treated with BSO, the levels of GSH in both cell lines decreased similarly. The response of both cell lines to the cytotoxic actions of CDDP remained unaffected by the BSO treatment. Similar results with BSO have been reported recently in two human ovarian carcinoma cell lines 2.5- to 3-fold resistant to CDDP (59). Increased protein sulfhydryl content and cadmium chloride resistance indicate that the SCC-25/CP cell line may have increased levels of metallothionein-like proteins. These proteins have been induced acutely by exposure to cadmium, zinc, and glucocorticoid hormones through increased transcription (60, 61) or chronically through gene amplification (46). Although CDDP has not been shown to induce metallothionein (47, 48), cells which have a high content of metallothionein are resistant to CDDP (49), and CDDP has been shown to bind to metallothioneins both in vitro and in vivo (62, 63).

In conclusion, the mechanism of resistance of the SCC-25/CP cell line to CDDP is multifactorial: (a) there are reduced intracellular levels of platinum in the SCC-25/CP compared to SCC-25 cells, implying altered plasma membrane properties in the resistant line; (b) there is a 2-fold increase in protein sulfhydryl content, a 2-fold increase in GST, and a 2-fold reduction in sensitivity to CdCl₂, implying altered cytosolic binding and metabolism of CDDP in the resistant line; and (c) there is a reduced level of DNA cross-linking by CDDP in the SCC-25/CP line compared to the parent line. In addition to these changes which we have measured, there may be other alterations in the SCC-25/CP cell line which result in the 30-fold resistance observed.

* E. Frei III, B. A. Teicher, and C. A. Cucchi, unpublished observations.
REFERENCES


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