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Correlation of The Response to Cisplatin of Human Ovarian Cancer Cell Lines, Originating From One Tumor But With Different Sensitivity, With The Recovery of DNA Adducts

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ABSTRACT. Cis-Diamminedichloroplatinum(II) (CDDP) is used in the treatment of various cancers, with or without ionizing radiation. During treatment, resistance may develop, and cross-resistance can also occur. DNA is the main target for CDDP and ionizing radiation, and we therefore evaluated the correlation between the amount of CDDP-DNA adducts and the cytotoxic activity of CDDP in human ovarian cancer cell lines with different platinum sensitivities. DNA-adduct levels were investigated 18 hr after CDDP exposure in three cell lines originating from the same human ovarian cancer. The least sensitive cells appeared to have the largest amounts of CDDP-DNA adducts, while the most sensitive had higher adduct levels than the parental cells. The proportion of the four adducts measured (i.e. Pt-G, Pt-AG, Pt-GG, and G-Pt-G) was comparable in all cell lines, with a preference for Pt-GG adduct formation (>50% of the adducts). Intracellular CDDP concentrations were higher in sensitive than in resistant cells, in contrast to the degree of CDDP adduct formation. Data obtained following continuous exposure of CDDP-resistant cells to CDDP suggest that DNA repair is partly responsible for resistance to CDDP. We conclude that the amount of CDDP-DNA adduct formation in cancer cells is not a predictor of CDDP cytotoxicity. BIOCHEM PHARMACOL 51;5:629–634, 1996.

KEY WORDS. cisplatin; DNA adducts; human cell lines; ovarian cancer; cisplatin resistance

Combined modality treatment including ionizing radiation and cisplatin has been widely used in various treatment protocols. However, the development of drug resistance limits its effectiveness. A change in sensitivity may be induced, not only by the drug itself, but also by irradiation [1–4]. Resistance to CDDP‡ generally develops more slowly and to a much lesser extent than, for example, resistance to anthracyclines. The cisplatin- and carboplatin-resistant human ovarian cancer cells used in the present study initially had resistance factors between 3 and 5 [3, 5] and, like many other platinum-resistant cell lines, were generated *in vitro* by exposure to stepwise increasing concentrations of cisplatin over months or even years [3, 5–9]. We were previously unable to obtain cisplatin- or carboplatin-resistant human ovarian cancer lines by repeated irradiation; in fact, the cells became more sensitive to cisplatin [3]. However, Eicholtz-Wirth and Hirtel were able to induce transient cisplatin resistance in murine fibrosarcoma cells with high radiation doses, in association with an increased intracellular metallothionein content [10]. Subsequently, transient cisplatin resistance was also observed in the same cells after low-dose irradiation *in vitro* and *in vivo* [11]. Various molecular mechanisms have been described in the development of resistance to cisplatin, including increased DNA repair [2, 4, 12], reduced drug accumulation [8, 9], and increased drug detoxification by protein or nonprotein thiols [6, 11, 12]. We have previously described the isolation of cisplatin-resistant clones of a human ovarian cancer cell line following exposure to cisplatin [3, 5] and the isolation of a cell line with transient, increased sensitivity to cisplatin after repeated exposure to ionizing radiation.

The formation of DNA adducts is generally accepted as one of the basic cytotoxic mechanisms of platinum-containing chemotherapeutic agents, such as CDDP. CDDP-DNA adduct formation may be greater in CDDP-sensitive than in CDDP-resistant cell lines, and the same may be true for sensitive and resistant tumor cell populations in a single heterogeneous tumor. The cell lines used herein cannot be regarded as subpopulations of the tumor as such, since it is possible that mutations were generated by exposure to ionizing radiation and CDDP. However, as cancer is a mutation-driven process, the different cell lines may in fact mimic tumor heterogeneity with respect to CDDP activity. It has been suggested that cytotox-

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‡ *Abbreviations:* CDDP, *cis*-Diamminedichloroplatinum(II); Pt-G, Pt(NH3)3-dGMP;Pt-Ag, *cis*-Pt(NH3)2-d(pApg); Pt-GG, *cis*-Pt(NH3)2-d(pGpG); G-Pt-G, *cis*-Pt(NH3)2-d(GMP)2; ID_{50}, 50% inhibiting dose; RF, Resistance Factor; and Gy, Gray.

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icity varies according to the total number of CDDP-DNA adducts generated in cell lines following CDDP exposure. Alternatively, the increased formation of one or two adducts may be important, with the total number of adducts remaining constant.

The present experiments were designed and conducted to answer the following questions:

1. Is the formation of CDDP-DNA adducts predictive of CDDP activity?
2. Is sensitivity to CDDP dependent on the relative amounts of Pt-G, Pt-AG, Pt-GG, and G-Pt-G formed?

MATERIALS AND METHODS

Cell Lines

Three human ovarian cancer cell lines originating from COV413B [5] and displaying different sensitivities to CDDP (Fig. 1a) were used. Here, COV413B cells are denoted as AOvC-M cells, since they were cultured in our laboratory under slightly different circumstances from those published previously [3]. AOvC-R cells were obtained by continuous exposure of the COV413B cells to 1.67 μM CDDP [3, 5]. The CDDP-resistant cells so produced maintained the resistant phenotype [3, 5], as they retained their resistance when no longer exposed to CDDP. AOvC-S, cells showing increased sensitivity to CDDP, were obtained by exposure to ionizing radiation doses of 0.5 or 1.0 Gy every 48 hours for 6 months [3]; continued exposure was then necessary to maintain the increased sensitivity.

The cells were cultured in tissue culture flasks (Falcon) in a monolayer in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco BRL), aspartic acid, and glutamic acid. Antibiotics were not included in the culture medium, and cultures were tested regularly for Mycoplasma infection. The cells were maintained at 37°C in a humidified atmosphere of CO_2/air (5%/95%). The cell lines had a constant doubling time of 24 hr, and were stable with respect to morphology and DNA content. Fifty percent survival (ID_{50}) was noted at concentrations of 4.6, 21, and 107 μM CDDP with an exposure time of 1 hr for AOvC-S, AOvC-M, and AOvC-R cells, respectively.

Cytotoxic Assay for Survival-CDDP Exposure Curves

Following exposure to CDDP, the cell survival curve was determined by the MTT assay, which quantifies the number of surviving cells at a given time after exposure to cytotoxic drugs or irradiation [3]. Cells were exposed to different CDDP concentrations (0–500 μM CDDP) in the culture medium for varying times, according to data obtained in previous studies [13]. From suspension of 200,000 cells/mL, 20,000 cells were seeded in each well of 48-well plates and with 0.5 mL of the

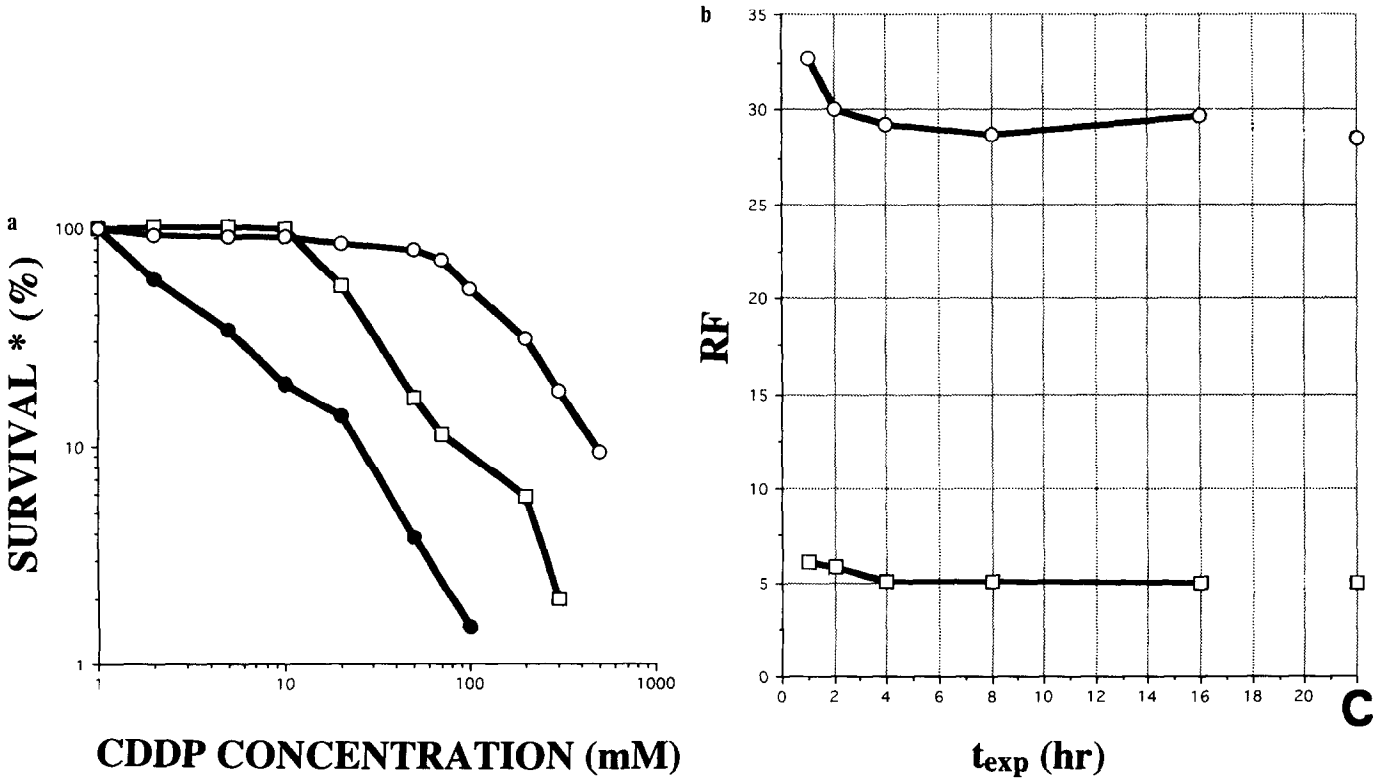

FIG. 1 (a) Dose survival curves of human ovarian cancer cells following 1 hr exposure to cisplatin (CDDP). Sensitive AOvC-S cells (●) obtained by chronic irradiation, AOvC-M (□) or control cells, and AOvC-R (○) cells made resistant to CDDP following continuous exposure. Concentrations at which 50% survival was noted were 4.6, 21, and 107 μM CDDP for AOvC-S, AOvC-M, and AOvC-R cells, respectively. Standard errors of the means were between 8 and 12%. (b) The influence of exposure time (t_{exp}) to CDDP on the resistance factor (RF) of AOvC-R (–○–) and AOvC-M (cells). (C: data obtained with continuous exposure).

culture medium. Cells were incubated at 37°C for 4 days without refeeding. Ten to twelve wells per survival point were plated for each cell line, and each experiment was performed in triplicate.

Determination of CDDP-DNA Adducts

Suspensions containing 20 million AOvC-S, AOvC-M, or AOvC-R cells were incubated with 120 μM CDDP for 1 hr. The cells were then washed, incubated at 37°C for 18 hr in the absence of CDDP, and fixed. The period of 18 hr was required for both intracellular CDDP concentrations and the equilibrium between adduct formation and removal to reach a 'steady-state.'

DNA was isolated and subjected to liquid chromatography after enzymatic digestion to the unmodified mononucleotides dCMP, dAMP, dTMP, and dGMP, and the following platinum-containing (di)nucleotides [14]: Pt-G, derived from CDDP monofunctionally bound to guanine; Pt-AG and Pt-GG, from intrastrand cross-links on neighboring bases in sequences pApG and pGpG, respectively; and G-Pt-G, from intrastrand cross-links on two guanines separated by one or more bases and/or from interstrand cross-links on guanines in opposite strands of DNA [14]. The quantification of the platinum products of interest at identified positions in the column eluate was performed with immunochemical techniques using specific antisera [15, 16]. The dilution of the fractions giving a 50% inhibition of antibody binding in the competitive enzyme-linked immunosorbent assay was determined and used to calculate the amount of CDDP-DNA digestion products of AOvC-S, AOvC-M, and ACvC-R cells exposed to CDDP. Elution times of Pt-G, Pt-AG, Pt-GG, and G-Pt-G were 1.8 min, 3.7 min, 7.9 min, and 8.8 min, respectively. Experiments were carried out in duplicate.

Uptake of CDDP

The cell lines were exposed tc equal concentrations of CDDP in the culture medium for 1 hr at 37°C in 5% CO_2 in a humidified incubator, and washed extensively. Cells were then collected by trypsinization and diluted with 0.9% NaCl to produce a concentration of approximately 7.10^7 cells/mL. The exact number of cells in each sample and the mean cell volume was determined by a Coulter Counter. Each sample was analyzed for platinum content by introducing 40 μM into a Perkin-Elmer model 4000 atomic absorption spectrophotometer. A 0.4 μM $K2PtCl_6$ solution was used to construct a calibration curve [5].

Statistical Analysis

It has been assumed that CDDP concentrations in the culture media were stable during the exposure times used [17]. Analysis of CDDP-DNA adduct data included comparisons of both the amount and percentages of different adducts found in the various cell lines, and was performed by one-way ANOVA with Scheffé's procedure for multiple comparisons. To compare the whole dose-survival curves of the cell lines tested, the Friedman two-way ANOVA test was used. For all tests, $P < 0.01$ was taken as the level of significance.

RESULTS

Cell survival after exposure to CDDP is shown in Fig. 1a. CDDP cytotoxicity after a 1-hr exposure could be observed in AOvC-S cells at concentrations of 1–10 μM CDDP. A decrease in survival of AOvC-M cells was seen at concentrations above 20 μM CDDP, whereas CDDP cytotoxicity in AOvC-R cells occurred only at concentrations higher than 100 μM CDDP. $ID_{s_{50}}$ were 4.6, 21, and 107 μM CDDP for AOvC-S, AOvC-M, and AOvC-R cells, respectively. Under the conditions described above, the RFs of AOvC-M and AOvC-R cells versus AOvC-S cells were 4.6 and 23.3, respectively. The latter is rather high in comparison to RFs reported in the literature [18–20].

The change in RF according to exposure time is presented in Fig. 1b. The RFs of AOvC-M and AOvC-R cells were quite stable in a range of 1–20 hr. Therefore, it is concluded that resistance mechanisms operating in the cells are independent of exposure time, in contrast to data found with rat CC531 colon cancer cell lines resistant to CDDP [21] under the conditions described for CDDP-DNA adduct analysis, most of the AOvC-R cells survived, whereas almost all AOvC-S and AOvC-M cells died (Fig. 1a).

The extent of CDDP-DNA adduct formation in the various cell lines was not indicative of cell death (Fig. 2). Cells were exposed to identical conditions for 1 hr and then cultured for 18 hr in the absence of the drug. During this period some adduct formation may still occur, but—unless deficient—repair processes will dominate. Decreased sensitivity of the CDDP-resistant cells to CDDP was not associated with a decreased level of platinum-DNA adducts. On the contrary, the total amount of adducts was more than three times greater in

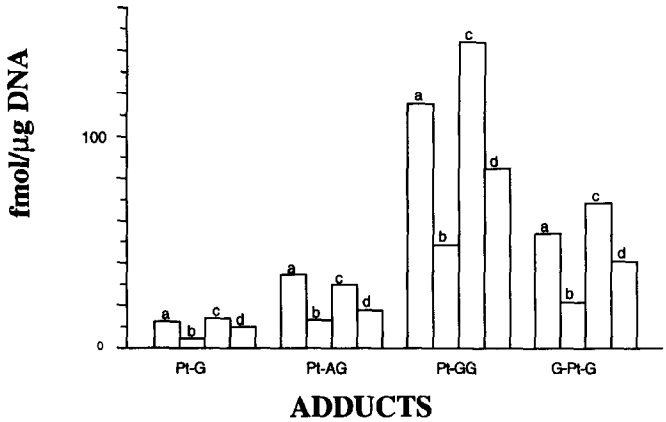

FIG. 2. CDDP-DNA adducts of (a) AOvC-S, (b) AOvC-M, and (c) AOvC-R cells 18 hr after a 1 hr exposure to CDDP. DNA adducts in AOvC-R cells cultured for 6+ months at 1.67 μM are presented in lanes d. Adducts in AOvC-M cells were significantly lower than those in AOvC-S and AOvC-R cells (I-ANOVA, $P < 0.01$). Standard errors of the means were in the range 8–13%.

the AOvC-R cells than in the AOvC-M cells (Fig. 2, lanes b versus c). There was no significant difference in the proportion of the different adducts. In all cell lines, the Pt-G represented 4–5% of the total adduct formation; Pt-AG, 11–14%; G-Pt-G, 17–27%; and the most abundant Pt-GG adduct, 57–67%. In the sensitive cells (AOvC-S), produced by chronic irradiations, the total amount of all four adducts was twice that in the control cells (AOvC-M). Therefore, it is concluded that in this particular case, increased sensitivity to CDDP is associated with increased levels of platinum-DNA adducts.

The cells most sensitive to CDDP (i.e. AOvC-S cells prepared by irradiation) contained higher levels of all four CDDP-DNA adducts 18 hr after a single CDDP treatment (Fig. 2, lanes a). Surprisingly, AOvC-R cells also demonstrated elevated levels of all adducts, although they were the least sensitive to CDDP (Fig. 2, lanes c). AOvC-R cells do not require continuous CDDP exposure to maintain the resistant phenotype, and are therefore classified as stable resistant [3]. Consequently, the experimental results were not influenced by residual levels of CDDP adducts.

The proportion of the various CDDP adducts was comparable in the different cell lines tested (Fig. 2, lanes a–c). The Pt-GG and G-Pt-G adducts accounted for the majority of CDDP-DNA adducts (i.e. approximately 75%). In order to estimate repair activity in the least sensitive cells, AOvC-R cells were cultured for 6+ months in 1.67 μM CDDP. Adduct levels were then determined for 18 hr following a 1-hr exposure to 120 μM CDDP, and levels of all adducts were reduced by approximately 40% (Fig. 2, lanes d).

DISCUSSION

CDDP is a cytotoxic agent effective in the treatment of various malignancies, such as ovarian and testicular cancer. CDDP-induced biochemical alterations in the cell are known to be complex [22, 23]. The processes involved in the cytotoxic action of CDDP remain uncertain, perhaps varying between tumors and even between tumor cell lines within one tumor. However, it is generally believed that the antitumor activity of platinum-containing drugs such as CDDP results from their interaction with DNA, leading to inhibition of DNA replication. Differences in sensitivity of various tumor cell lines to treatment with CDDP may be due to a combination of factors, such as alterations of cell membrane permeability, differences in accumulation of drugs within the cell, an increase in the intracellular glutathione level in resistant cells, or differences in the repair of CDDP adducts [22–24]. A number of CDDP-resistant experimental and human tumor cell lines exhibit a reduced uptake of CDDP, whereas the efflux of the drug is similar, if not higher, in the resistant lines [24]. Decreased accumulation as a mechanism of resistance to CDDP in human non-small cell lung cancer cell lines and its relation to DNA damage and repair has been described by Bungo *et al.* [25]. As no enhancement of repair was demonstrated, it was concluded that the decrease in accumulation was the most important mechanism of resistance. However, Eastman *et al.* suggested that reduced accumulation could also be the result of an increased efficiency of repair mechanisms in resistant cells [26]. L1210 cell lines were made resistant to CDDP by stepwise exposure of the cells to increasing CDDP concentrations; a selection of a 100-fold resistant cell line resulted [27]. The resistant cells exhibited a decrease in drug accumulation of about 40%, but this reduction was not proportional to the degree of resistance [27]. Analysis of the individual nucleoside-bound adducts demonstrated that the rate of repair of adducts at GG sequences was markedly enhanced in the resistant cells. Studies of the formation and repair of CDDP-induced adducts to DNA in cultured normal and repair-deficient human fibroblasts have illustrated the impact of the DNA repair capacity of cells on their survival following CDDP exposure [28]. Discrepancies in the effect of CDDP on survival of human testicular and bladder cancer cell lines can be explained by levels of adducts formed and alterations in DNA repair [29]. Fichtinger-Schepman *et al.* were not able to find differences, 1 hr after the administration of 10 mg/kg CDDP, in the platinum levels in CDDP-sensitive or -resistant IgM immunocytomas implanted in LOU/M rats [30]. The formation of CDDP-DNA adducts in the CDDP-sensitive and -resistant tumors appeared to be similar, indicating that the acquired CDDP resistance was not attributable to a decrease in adduct formation [30]. Induced resistance to CDDP in human ovarian cancer cell lines has been found in different studies to be similar to that induced by other DNA-damaging agents such as melphalan and ionizing radiation [3, 5, 31]. It has been demonstrated that CDDP-DNA adducts in leukocyte cells may be predictive of the therapeutic outcome in CDDP-treated ovarian cancer patients [32, 33]. It is therefore of interest to study DNA adduct levels in tumor cell lines originating from a single tumor but with different CDDP sensitivities in order to define the predictive power of CDDP-DNA adduct formation for CDDP cytotoxicity in the target cells themselves. The cell lines used in this study, analysed for CDDP-DNA adduct levels 18 hr after CDDP exposure, may reflect tumor heterogeneity with respect to CDDP sensitivity. The three cell lines originate from the same tumor (i.e. COV413B [5, 34], and exhibit a wide variation in CDDP-sensitivity (Fig. 1). Data concerning CDDP-DNA adduct formation in different cell lines originating from one solid tumor are rare and limited to two cell lines. The high number of adducts found in AOvC-R cells is remarkable when the reduced cellular Pt concentrations are taken into account. Intracellular Pt concentrations were markedly lower than those in the AOvC-M cells, as determined by flameless atomic absorption spectrophotometry. With 25 μM CDDP in the culture medium, the cellular concentrations were 1.8 μM and 0.3 μM in AOvC-M and AOvC-R cells, respectively. With 120 μM extracellular CDDP, the cellular CDDP concentrations increased to 7.1 μM and 2.2 μM in AOvC-M and AOvC-R cells, respectively. The lower cellular concentrations in AOvC-R cells, compatible with the outcome of the CDDP cytotoxicity tests (Fig. 1), appeared to be associated with higher CDDP-DNA adduct levels than in AOvC-M cells. Therefore, we conclude that the formation and repair of CDDP-DNA adducts is a separate process from that of CDDP

uptake. It must be remembered that the amount of CDDP-DNA adduct formation is determined after exposure of the total number of cells, including those that have been killed. Cell survival at the CDDP concentration used for the determination of CDDP-DNA adducts was 0%, 9%, and 81% for the AOvC-S, AOvC-M, and AOvC-R cells, respectively (Fig. 1). Thus, AOvC-R cells are able to survive adduct levels comparable to those monitored in the CDDP-sensitive AOvC-S cells. It has been postulated that AOvC-R cells are able to withstand high adduct levels by an enhanced repair mechanism. Continuous exposure to low CDDP concentrations might induce repair; indeed, in AOvC-R cells continuously exposed to CDDP (6+ months at 1.67 μM), decreased CDDP-DNA adduct levels were found 18 hr after a 1-hr exposure to 120 μM CDDP (Fig. 2, lanes d) with no change in the proportion of the different adducts. Assuming that the rate of repair reaches a steady state at 6+ months of 5 μM CDDP exposure, it is probable that AOvC-R cells can maintain significant CDDP-DNA adduct levels during massive replication, as survival at 5 μM CDDP is not affected in these cells (Fig. 1a). The continuing growth of resistant tumor cells, despite the presence of significant amounts of DNA adducts, may be related to increased tolerance of unrepaired DNA adducts during the DNA replication process.

Increased DNA repair and reduced intracellular CDDP uptake may not be the only mechanisms of CDDP resistance operating in AOvC-R cells. Measuring CDDP-DNA levels at different time points would provide further information concerning repair mechanisms. However, this could not be performed properly for the cell lines in the present study, owing to the variation of cell kill/μM CDDP observed in the cell lines of interest (Fig. 1a).

Hill *et al.* [35] showed that the repair of cisplatin-DNA adducts in human testicular teratoma cell lines established from untreated patients were deficient, in keeping with the fact that germ cell tumor cell lines appear generally more sensitive to CDDP. The data illustrated the apparent inability of the cell lines to repair the major platinum-DNA intrastrand crosslinks, and so provided a biological basis for their hypersensitivity to CDDP. Johnson *et al.* [36] studied a series of CDDP-resistant cell lines, and their results also support a role for DNA repair and alterations in interstrand cross-link formation in CDDP resistance. In another interesting study on two CDDP-selected resistant human testicular teratoma sublines, Hill *et al.* [37] revealed the importance of differential formation and enhanced removal of specific CDDP-DNA adducts. In our study, however, there was no difference in the relative proportion of adducts in cells with different CDDP sensitivities. In addition, Hill *et al.* [38] demonstrated that there was no association between sensitivity and cellular uptake of CDDP, total glutathione levels, or associated enzyme activities. Jekunen *et al.* [39] recently concluded that a CDDP-resistant phenotype in human ovarian cancer cells was accounted for primarily by impaired uptake and decreased interaction of cisplatin with DNA, rather than by changes in efflux or DNA repair. We have found that impaired uptake does not necessarily result in an impaired interaction of cisplatin with DNA. On the contrary, impaired uptake was found to be associated with an increased interaction of cisplatin with DNA.

In conclusion, data of the present study demonstrate that levels of CDDP-DNA adducts in human ovarian tumor cells with different sensitivities to CDDP do not necessarily predict the cytotoxic activity of CDDP. Pt-GG and G-Pt-G were found to be the most frequently occurring CDDP-DNA adducts, both in CDDP-sensitive and -resistant tumor cells. As the three tumor cell lines tested originate from one and the same human ovarian cancer, the data of CDDP adduct formation demonstrate that within one tumor, CDDP-DNA adducts can markedly differ among cell lines, and that one cell line can survive high adduct levels compared to another.

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