Characterization of Resistance Mechanisms to cis-Diaminedichloroplatinum(II) in Three Sublines of the CC531 Colon Adenocarcinoma Cell Line in Vitro


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ABSTRACT

Cisplatin resistance was developed in sublines of the CC531 rat colon adenocarcinoma cell line by continued low level drug exposure. Two relatively stable lines were obtained (RL2 and RL4) which were 6- and 20-fold more resistant to cisplatin. In addition, a subline more sensitive than the parental line by a factor of 2 (RLS) was obtained by subculture from a treated tumor. Mechanisms of resistance to cisplatin were investigated in these four lines, with the aim of determining the relative contributions of different resistance mechanisms at various resistance levels.

Drug accumulation linearly decreased with increasing drug resistance. A 20-fold resistance was associated with only a 5-fold decrease in accumulation, suggesting that other resistance mechanisms may be involved in the total degree of resistance. Intracellular glutathione, measured fluorometrically, also increased with increasing resistance, varying by a factor of 4 between the most and least resistant lines. Reduction of glutathione levels by buthionine sulfoximine to parent line levels increased sensitivity but the cells remained considerably more resistant than parent cells. Resistant lines cultured in the absence of drug became progressively more sensitive, without accompanying changes in total glutathione levels.

DNA-drug adducts, the presumed toxic lesion, were measured immunochromatically. Initial levels decreased with increasing platinum resistance, although not proportional to resistance (factor of 5 decrease for 20-fold resistance). Drug dose ratios for equal initial adducts were similar to dose ratios for equal drug accumulation, implying that intracellular concentrations solely determine DNA addition and that differences in glutathione level had little influence on the proportion of drug which eventually formed adducts. After 48h, a better correlation between remaining adducts and resistance was found (factor 12 less adducts for 20-fold resistance). This implies that repair of adducts was important in determining survival.

These data indicate that decreased drug accumulation played a proportionally greater role in the moderately resistant cell line and that adduct repair played a progressively greater role in the highly resistant cell line.

INTRODUCTION

cDDP3 is one of the most active anticancer drugs and is used to treat a variety of malignancies (1). Unfortunately, the initial response of several tumor types is often followed by a relapse due to either primary or acquired drug resistance. In order to develop strategies for overcoming this resistance more insight into its origins is needed. The various mechanisms involved include: alterations in drug transport (2, 3); enhanced detoxification pathways in the cytoplasm, e.g., increase in cellular glutathione content (4–9) or in expression of metallothionein (10–12); and increased repair of, or tolerance to, DNA damage (13–15). Concerning drug transport, it is still unclear whether cDDP enters the cell by passive diffusion or whether its transport is mediated by active carrier mechanisms (2, 3). Energy and Na+ dependence and inhibition by ouabain of transport point to the presence of active uptake mechanisms. However, changes in membrane phospholipid structure (2, 16) might contribute to decreased passive diffusion. Reduced platinum accumulation could also be the result of increased drug efflux or a combination of both reduced influx and increased efflux (17).

After cDDP has passed through the cell membrane it will interact with proteins, RNA, and DNA. Only 1% of the total intracellular cDDP is bound to DNA (17), while the remainder binds to other cellular components. The extent of the damage induced by cDDP may be limited by detoxifying systems, e.g., glutathione, GSH transferase, or metallothionein. Glutathione can influence adduct formation by binding cDDP to its sulphydryl residue, leading to less DNA platination. In addition, glutathione is able to prevent the formation of bifunctional cDDP-adducts from monooadducts (6, 18).

Upon reaching the DNA, the probable critical target, cDDP will preferentially form cDDP-GG and cDDP-AG intranstrand or interstrand cross-links. One way to reduce the potentially lethal consequences is to remove the DNA lesions by excision repair (19). The importance of an adequate DNA repair system has been demonstrated in cell lines that show a lack of DNA repair. Inadequate removal of cDDP-induced lesions in these cells resulted in a distinct hypersensitivity to the drug (20). Resistant cells might, to some extent, be tolerant to these adducts. Recently there has been growing interest in the detection of genes that might be involved in cDDP resistance. It has been demonstrated that exposure to cytotoxic drugs enhances the expression of these genes (21, 22).

Despite many studies about cDDP resistance, it is still unclear what the contribution is of each separate mechanism. It is not known whether resistance mechanisms found in highly resistant cells are not or are to a lesser extent involved in low or moderate resistance. To estimate the contribution of different resistance mechanisms at various levels of resistance we characterized several mechanisms in three different sublines of the CC531 colon adenocarcinoma cell line. We have correlated the level of resistance and the associated mechanisms in order to determine the predominant mechanisms of resistance in each cell line ranging from a more sensitive one to a highly resistant one.

MATERIALS AND METHODS

Cell Lines. The CC531 cell line used in these experiments was induced in the colon of a rat exposed to methyloxazomethanol (23). These cells grow well in vitro and were plated at a density of 1 × 104 cells/75-cm² flasks containing 15 ml fresh DMEM with 10% FCS (Gibco, Breda, the Netherlands) and penicillin (20 units/ml), streptomycin (0.02 mg/ml) in a humidified atmosphere with 5% CO₂ at 37°C. The cells were replated twice a week.

The cDDP resistance in the sublines CC531RL2 and CC531RL4 was obtained by continuous incubation of the parent cell line with cDDP. Initial incubation concentrations were 0.05 μg/ml for 8 weeks. For CC531RL4 (hereafter called RL4) cells this used cDDP concentration was raised each 8 weeks to 0.2, 0.3, 0.5, 1, and 2 μg/ml successively. The procedure for CC531RL2 (hereafter called RL2) cells was identical except for the highest cDDP concentration used (0.5 instead of 2 μg/ml). To obtain stable resistance,
RL2 and RL4 cells were cultured in the presence of 0.5 and 2 μg/ml cDDP, respectively. CC531. RLS (hereafter called RLS) is a sensitive subline obtained after inoculation of CC531 cells s.c. in a Wag/Rij rat.

**Chemicals.** cDDP (Platinol) was obtained from Bristol Myers, Weesp, the Netherlands. GSH (reduced glutathione), BSO, α-phthalaldehyde were purchased from Sigma Chemical Co., St Louis, MO.

**cDDP Sensitivity.** cDDP sensitivity was tested using the colony forming assay in 6-well tissue culture plates (Costar, Cambridge, United Kingdom). Cells harvested with trypsin (0.05% w/v)/EDTA (0.02% w/v) were counted and plated into 6-well plates, 150 to 300 cells/well in a volume of 3 ml DMEM containing 10% FCS. Twenty-four h later cells were incubated for 1 h with various cDDP concentrations (0–150 μg/ml). After incubation cells were washed three times with PBS and fresh medium was added. All plates were returned to the incubator and incubated for 7 days for the development of colonies. Colonies were fixed with ethanol, stained with crystal violet for 10 min, and counted (only colonies with more than 50 cells).

**Platinum Analysis.** A model AA40 Atomic Absorption Spectrometer with a GTA 96 Graphite Tube Atomizer (with Zeeman background correction) from Varian (Victoria, Australia) was used for platinum analysis. Sample preparation has been described elsewhere (24).

**Platinum Accumulation Experiments.** A series of experiments was performed to determine alterations in platinum accumulation. To compare platinum accumulation among the sublines, cells were incubated with a standard cDDP dose, irrespective of cytotoxicity. Approximately 2 × 10⁶ cells were incubated for 1 h with cDDP (5 μg/ml). The cells were then harvested, washed three times with cold (4°C) PBS, and counted. This procedure was followed by platinum analysis of the cell pellet as described previously (25). A second series of experiments was performed to determine cellular platinum contents after equitoxic doses. This was attained by incubation of the cells with IC₅₀ concentrations of cDDP (1.3–50 μg/ml, determined from the dose-response curves).

**Glutathione Assay.** Cellular glutathione was quantified according to the fluorometric method as described by Hissin and Hilf (26). The method can be summarized as follows. Cells in the logarithmic phase of growth were harvested, washed twice with PBS (4°C), sonicated (4°C, 10 min), and precipitated on ice in 10% trichloroacetic acid. The samples were then centrifuged (15 min, 4°C, 15,000 × g). For determination of GSH the resulting supernatant was diluted 1:10 in phosphate-EDTA buffer (pH 8.0). The final assay mixture contained 100 μM of the diluted supernatant, 100 μM α-phthalaldehyde (containing 1 mg/ml α-phthalaldehyde in reagent grade methanol) replenished with phosphate-EDTA buffer (pH 8.0) up to a volume of 2 ml. After thorough mixing and incubation at room temperature for 15 min the solution was transferred to a quartz cuvet. Fluorescence was determined at 420 nm (excitation at 350 nm). Protein measurement was performed using the assay of Lowry et al. (27).

In order to determine the effect of GSH on cDDP sensitivity another series of experiments was carried out. The effect of culturing the resistant RL2 and RL4 cells without cDDP (instead of continued low level drug exposure) was determined. During a period of 12 weeks the resistant cells were cultured in the absence of cDDP. At fixed time points (0, 2, 4, 8, and 12 weeks) colony forming assays and GSH assays were performed as described above.

**Reduction of the Cellular GSH Content.** The possible role that glutathione plays in cDDP resistance was studied by reducing the glutathione content by BSO treatment. In order to find out what role the elevated GSH content played in the resistant cells, the GSH levels were reduced to the GSH level determined in the parent (CC531) cell line. In a series of pilot experiments (data not shown) BSO concentrations of 10 and 50 μM for RL2 and RL4 cells, respectively, resulted in GSH levels similar to that in CC531 cells.

In a second series of experiments the effect of reducing the cellular GSH contents below that of the CC531 parent cells was determined. To achieve this the sensitive cell lines, both RLS and CC531 cells, were incubated with 10 μM BSO. Moreover, the GSH level in the RL4 cells was further reduced by 100 μM BSO.

The effect of glutathione reduction on the cDDP resistance was studied using the colony forming assay. Cells in the logarithmic phase of growth were trypsinized and replated in 6-well tissue culture plates (Costar) as described above. During plating, α-thiobutirone-S-R-sulfoximine (Sigma) was added to one-half of the culture plates. After 24 h the cells were treated with cDDP for 1 h and washed with PBS, and fresh medium (DMEM) was added. Seven days later colonies were fixed and stained and colonies of at least 50 cells were scored.

**DNA Platination Detection.** Cells in log phase were plated at ovalbumin coated (75 μl 0.5% ovalbumin/slide) glass slides (2.6 × 6 cm). After 24 h cDDP was administered in various concentrations for 1 h. Following this incubation period cells were washed three times with PBS and fresh medium was added. This treatment was followed by fixation in cold (−20°C) methanol (10 min) and acetone (2 min), air dried, and stored at −20°C until immunostaining.

The presence of platinated DNA was determined by quantitative immuno-cytchemistry using an antiserum against cDDP modified DNA. The characteristics of the rabbit antiserum NKI-A59, which was raised against cDDP-modified calf thymus DNA (platinum:nucleotide ratio, 6.7 × 10⁻³) coupled to albumin, have been described previously by Terheggen et al. (15). NKI-A59 (applied without further purification), goat anti-rabbit immunoglobulin and peroxidase-rabbit(anti-peroxidase complex (American Qualex, La Miranda, CA) were used in 1:2300, 1:640, and 1:3200 dilutions, respectively. All sera were diluted in phosphate buffer containing 10 mM KH₂PO₄, 140 mM NaCl, 10% FCS, and 0.04% Triton X-100 (BDH, Poole, United Kingdom). cDDP-DNA adducts bound to NKI-A59 antibody were visualized by double peroxidase-antiperoxidase staining. The nuclear staining density of individual nuclei was quantified with a Knott (Munich, Germany) light measuring device with a beam diameter of 5 μm, which was coupled to a Leitz Orthoplan microscope. An Atari ST computer (Sunnyvale, CA) programmed with a version of the Histochimical Data Acquisition System (Hidacsys; Microscan, Leiden, the Netherlands) was used to analyze the data (28). The integrated absorbance of a selected area was expressed in arbitrary units. In each slide the nuclear staining density of 4 randomly selected areas, corresponding to 20–40 nuclei each, was measured. For background staining determinations in each experiment, a number of untreated but stained cells were measured.

**DNA Platination Experiments.** A series of experiments was performed to determine the cDDP dose required to attain equal initial DNA platination in each cell line and the removal of this platinated DNA in a period of 48 h after incubation. The CC531 sublines were plated on slides as described above. After 24 h, cells in the log phase of growth were incubated for 1 h with cDDP of various concentrations (RL5 25 μM, CC531 50 μM, RL2 100 μM, and RL4 250 μM). The cells were washed three times with PBS and fresh medium was added. At various points (0, 1, 2, 4, 8, 24, and 48 h) cells were fixed for quantification of adduct specific staining density (described above). To evaluate the possible role of cell proliferation in DNA repair, we determined [³H]-thymidine incorporation into DNA, the number of cells and total protein content (27) after cDDP treatment.

Cells in the logarithmic phase of growth were trypsinized, counted, and replated in 6-well tissue plates (Costar) at densities of 5 × 10⁴ and 3.5 × 10⁴/well (resistant and sensitive cells, respectively). Twenty-four h after plating cells were incubated for 1 h with varying cDDP concentrations (RL5 25 μM, CC531 50 μM, RL2 100 μM, and RL4 250 μM). Cellular protein measurements and cell number counts were performed just before and immediately after cDDP treatment at 8, 24, and 48 h after treatment. [³H]Thymidine (0.5 μCi/ml) was added for 2 h prior to these time points (i.e., 2 h before incubation, 6, 22, and 46 h). The cells were lysed with 0.1% NaOH, 15 ml Count Fluid (Ultima Gold) was added, and dpm were determined on a Canberra Packard 1900 CA Tri-Carb liquid scintillation analyzer.

**Statistical Analysis.** Statistical analysis was performed using Student’s t test or analysis of variance (Scheffe’s test). P < 0.05 was considered to indicate significance of the difference between groups.

**RESULTS**

**Sensitivity to cDDP.** Fig. 1 shows the results of the drug sensitivity tests using the colony formation assay. The RLS subline was more sensitive to cDDP than the parent CC531 cells, while the RL2 and RL4 cells were moderately and highly resistant, respectively. The resistance factors (IC₅₀ ratios) were 0.5, 6, and 20 for the RLS, RL2, and RL4 sublines, respectively.

RL2 and RL4 cells cultured in the absence of low level cDDP showed a progressive decrease in the degree of resistance (see below).
**Platinum Accumulation.** In an attempt to determine possible changes in platinum accumulation we determined the cellular platinum concentration after a fixed cDDP dose and after equitoxic doses. Fig. 2A shows the cellular platinum concentrations after cDDP treatment in the CC531 cell line and its three sublines using a standard concentration of 5 μg cDDP/ml for 1 h. Both RLS and CC531 cells showed a high cellular concentration of platinum whereas the two resistant lines, RL2 and RL4, had a significantly reduced platinum accumulation (P values for comparisons with CC531 cells were 0.004 and 0.0003, respectively).

Fig. 2B shows incubation with equitoxic doses. Treatment with IC_{50} doses (1.3, 2.5, 15, and 50 μg/ml, 1 h, respectively) confirms the results described with the fixed cDDP dose, although with one exception. The RLS cell line had a much lower intracellular platinum concentration than the CC531 cell line (4-fold). RLS cells contained a significantly lower platinum concentration than CC531 (P = 0.003), whereas the RL2 and RL4 cells contained 1.9- and 2.6-fold more platinum, respectively (P = 0.03 and 0.0004, respectively).

Fig. 3A shows platinum accumulation after a 1-h incubation with a fixed dose and after an equitoxic dose for each cell line. cDDP doses required for a given amount of platinum accumulation (chosen at 2.5 ng/10^6 cells) were read off the curve fits through these data (Fig. 3A) and ratios of doses relative to CC531 were calculated. These ratios were 1.1, 1.0, 2.4, and 5.5 for RLS, CC531, RL2, and RL4, respectively. In Fig. 3B the dose ratios for equal accumulation were compared with the degree of resistance. The dashed line shows the expected curve if accumulation is totally responsible for resistance. These lower ratios than found for cell killing suggest that either cDDP is in some way compartmentalized within the cell (e.g., less in the nucleus) or other mechanisms of cDDP resistance besides reduced platinum accumulation are also involved in these two sublines.

**Glutathione Levels.** Cellular glutathione levels were determined in the log phase of cell growth. In the two cDDP sensitive cell lines, RLS and CC531, the glutathione levels were similar (P = 0.16) whereas an increase was observed in the two resistant sublines, RL2 and RL4 (P = 0.001 and P < 0.0001, respectively, compared with CC531 cells) (Fig. 4A).

In Fig. 4, B and C, the degree of resistance and accompanying cellular GSH contents of the RL2 and RL4 sublines cultured for 12 weeks without a continuous low level drug exposure is shown. Both RL2 and RL4 cells showed a progressive decrease in drug resistance. In the RL4 cells, there was an immediate fall in the degree of resistance, whereas the resistance decreased only after 4 weeks in RL2 cells. During this 12-week drug free period the degree of resistance decreased by a factors of 3.5 and 4.5 for RL2 and RL4 cells, respectively. In neither cell line was there a tendency for the GSH levels to decrease, implying little role for GSH in determining resistance. Both cell lines remained more resistant than the parent cell line after culturing in drug free medium (by factors of approximately 1.2 and 4.0 for RL2 and RL4, respectively). However, the trend for increasing sensitivity showed no sign of slowing, suggesting that further passages without drug may lead to the parent levels of resistance being approached.

To further investigate the role of this thiol, cell survival experiments were carried out after manipulating cellular GSH. Glutathione levels in RL2 and RL4 cells were reduced by BSO (10 and 50 μM BSO for 24 h) to glutathione levels similar to those in CC531 parent cells. The sensitivity of the resistant cell lines increased significantly as a result of this treatment (Fig. 5A). The DMF at the IC_{50} level in RL4 cells was 2.2 (P < 0.05). The DMF of the RL2 cells was 1.3 (P < 0.05). Decreasing the glutathione levels further by means of a higher BSO concentration (100 μM) did not result in any further sensitization of the RL4 cells. The sensitive RLS and CC531 cells were also treated with BSO (10 μM) to detect whether a reduction of glutathione levels below their usual levels would also sensitize these cells. BSO treat-
was reduced to 106 ± 12%, i.e., 50% of the maximal adduct formation. A similar pattern was seen for CC531 cells showing a rapid increase to 192 ± 20% in the first 8 h followed by a decrease to 82 ± 5%, i.e., 42% of the peak level.

In contrast, not only did the two resistant cell lines RL2 and RL4 reach their peak values much sooner but they also showed lower peak values. The highest adduct levels for both the RL2 and RL4 cells were reached 1 h after incubation (125 ± 12% and 144 ± 15%, respectively). Removal of adducts was rapid during the first 8 h in both cell lines amounting to a 41 ± 3% and 67 ± 9% decrease (RL2 and RL4 cells, respectively). After a rapid decrease the removal was much slower. The level of platinated DNA at 48 h in RL2 cells was 45 ± 2.4%, 36% of the peak level, compared to 35 ± 1.5%, 24% of the peak level in RL4 cells. The “final” degree of DNA platination at

![Diagram](image)

**Fig. 3.** A. platinum accumulation after fixed and equitoxic doses from Fig. 2. ——— accumulation level used to calculate dose ratios (1.1, 1.0, 2.4, and 5.5 for RL5, CC531, RL2, and RL4, respectively; see text). B. ratios of cisplatin doses required for equal platinum accumulation compared with cisplatin dose ratios for equal toxicity (degree of resistance, IC50 dose ratio). ——— equal ratio.

ment (10 μM) resulted in a small but significant increase in sensitivity of RLS cells (DMF 1.2, P < 0.05) but not in the parent cell line (Fig. 5B).

**DNA Platination.** Removal of cisplatin adducts from DNA was detected immunohistochemically. The degree of adduct specific nuclear staining was determined at fixed time points up to 48 h after incubation. The four cell lines were incubated with CDDP doses which, based on pilot experiments, would give equal adduct staining. These doses (CC531 50 μM, RLS 25 μM, RL2 100 μM, and RL4 250 μM) resulted in an average adduct specific nuclear staining intensity of 1.1 ± 0.1 (SD; expressed as arbitrary units) which was more than 10 times the background staining. The Y-axis values in Fig. 6A are expressed as a percentage. For each cell line the nuclear staining density at various time points was divided by the staining density measured immediately after the CDDP incubation period (t = 0).

Since all sublines showed an equal nuclear staining immediately after incubation, the percentage changes in staining density among the several sublines could be adequately compared.

The most sensitive cell line (RLS) showed a phase of rapid increase in adduct formation during the first 4 h after incubation. The peak adduct level of the RLS cells was the highest of the four cell lines, i.e., an increase to 211 ± 13%. The amount of platinated DNA after 48 h

![Diagram](image)

**Fig. 4.** A. total cellular glutathione levels of CC531 and sublines. CC531 levels were significantly different from RL2 and RL4 (P = 0.001 and 0.0001), but not from RLS cells (P = 0.16). Errors are ±1 SD. B. degree of resistance (IC50 dose) and GSH contents (μg/mg protein) in RL2 cells cultured for 12 weeks in the absence of CDDP. C. degree of resistance and GSH contents in RL4 cells cultured for 12 weeks in the absence of CDDP.
48 h, expressed as a ratio to that in CC531 cells, was 1.29, 0.55, and 0.42 for RLS, RL2, and RL4 cells, respectively. The extent of removal of platinated DNA can be expressed as the inverse of the above ratios. These were 0.8, 1.8, and 2.4 for RLS, RL2, and RL4, respectively. A good correlation was found between these ratios and the degree of resistance expressed as log of relative resistance \( r = 0.99 \) (Fig. 6B).

To obtain a better insight into the role which DNA platination plays, dose ratios required to attain the same extent of DNA platination compared with the parent cell line were calculated. This was performed for initial, peak, and final (48 h) DNA platination levels. The dose ratios were 0.5, 1.0, 2.0, and 5.0 (Fig. 6) for initial DNA platination, 0.45, 1.0, 3.1, 6.7 for peak DNA platination, and 0.4, 1.0, 3.6 and 11.7 for final (48 h) DNA platination for RLS, CC531, RL2, and RL4, respectively. Fig. 7A shows these dose ratios compared with dose ratios for equal toxicity. It can be seen that as the degree of resistance increases, the required cDDP dose for equal cDDP-DNA formation also increases. The dashed line shows where DNA platination ratios are equal to resistance ratios. The “final” adduct levels lie closest to this line, implying that these are more important for survival than initial or peak levels.

Since platinum accumulation in cells is likely to be a major factor involved in the extent of DNA platination, we compared these two parameters (Fig. 7B). The dashed line shows where DNA platination ratios are equal to accumulation ratios. Fig. 7 shows that the degree of initial adduct levels lies closest to this line, implying that drug accumulation determines initial adduct levels.

To evaluate a possible influence of cell growth in diluting platinum-DNA adducts and giving the artificial appearance of DNA repair, \(^{3}H\)thymidine incorporation, protein levels, and cell numbers were determined in the cell lines, and the changes in these parameters were expressed as a ratio relative to immediate pretreatment levels. The average ratio for the three parameters was used as a “proliferation factor.” The results of these experiments indicated that cell proliferation occurred after treatment to some extent in all lines. At 48 h after incubation these factors were 2.6, 2.3, 2.3, and 2.5 for RLS, CC531, RL2, and RL4, respectively. The fact that no major differences were demonstrated suggests that any possible artifacts were approximately equal in all cell lines, indicating that changes in adduct level reflected repair differences.

**DISCUSSION**

cDDP resistance in patients is usually of a low or moderate degree (29). The question of whether this acquired resistance corresponds to the resistance investigated in various in vitro cell models (relatively high resistance) remains a matter for discussion (16, 30–32). We developed an in vitro model of the CC531 colon adenocarcinoma cells and three sublines, one more sensitive (RLS), one with a moderate
degree of resistance (RL2), and another with a relatively high degree of resistance to cDDP (RL4). Our aim was to characterize the various mechanisms of resistance in these cell lines and to correlate them with the degrees of resistance.

A decrease in drug accumulation has been observed in many cell lines with induced resistance for many chemotherapeutic agents. For cisplatin, a decrease in total cellular platinum content compared to the parent line has been found in a variety of resistant cell lines, e.g., L1210, A2780/CP70, and other resistant human ovarian carcinoma cell lines (14, 17, 33, 34), although a decrease in platinum accumulation in resistant cells has not been found in all studies (12, 35, 36).

Alterations in platinum accumulation can be brought about by diminished uptake as well as by increased drug efflux, in particular by the rapid efflux phase in the first 10 minutes after incubation (3, 37). This altered cDDP membrane transport might be the result of changes in membrane lipid composition and fluidity (2), increased efflux due to altered intracellular biotransformation (3) causing a shift of the equilibrium between the slow and rapid effluxing pool. Since our resistant sublines showed reduced drug accumulation, at least one of these mechanisms might be involved. If we compare the contribution of decreased platinum accumulation with the degree of resistance, it is apparent that as the degree of resistance increases, the contribution of this mechanism decreases (Fig. 3B). This implies that in highly resistant cells the contribution of other resistance mechanisms (e.g., repair) is increased. This was supported by the present data on platinum accumulation at IC50 doses, where the resistant sublines required a considerably higher cDDP concentration to achieve the same level of cell kill. An alternative explanation is that the resistant cells in some way compartmentalize the intracellular drug, affording protection from vital targets. No subcellular distribution studies were done to support or deny this hypothesis, although given the free diffusibility of free cDDP, we regard this as less likely than the presence of other resistance mechanisms. Another factor that might influence the accumulation results is the proportion of free in contrast to macromolecular bound cDDP. If a considerable percentage of intracellular cisplatinum is bound this might result in an underestimation of accumulation.

One way of diminishing DNA platination is detoxification by glutathione. In spite of numerous studies, the role that glutathione plays in cDDP resistance is still unclear. Some studies show equal or hardly elevated levels of glutathione in sensitive and resistant cells (12), while other studies show increased cellular glutathione levels but no sensitization after glutathione depletion with BSO (33, 38). The glutathione levels detected in our experiments showed a 2.4- and a 4.3-fold increase in the two resistant cell lines, and BSO treatment resulted in sensitization by DMFs of 1.3 and 2.2, respectively. These data are similar to the findings of Meijer et al. (32) who found a 2.5-fold elevation in glutathione level and 2.8- and 1.7-fold increase in sensitivity to cDDP in resistant and sensitive lung cancer cells, respectively.

Culture of the resistant RL2 and RL4 cells without cDDP resulted in a decreased level of resistance with no accompanying decrease in cellular GSH contents. This fall in resistance may therefore be mediated by a decreased function of other resistance mechanisms, e.g., increased repair or decreased drug accumulation. Although the RL2 and RL4 cells indeed showed a progressive increase in sensitivity, they still remained resistant in comparison with the CC531 parent line. The elevated GSH levels may be involved in this basal resistance. Increased GSH synthesis as a consequence of cDDP therapy may not be rapidly switched off and remain high for a relatively long time after interruption of cDDP therapy.

Simple depletion of glutathione may not be sufficient to decrease resistance to cDDP (8). In the absence of BSO, glutathione synthesis is rapidly switched on and the effect of glutathione depletion is diminished. This is unlikely to have been a factor in the present experiments, however, since BSO was not removed during cDDP incubation. The effect of BSO on intracellular compartments, e.g., nucleus and mitochondria, is not fully understood. An increase in the BSO concentration is needed to lower the mitochondrial glutathione to a level similar to that in cytosol (39). It may be that elevated nuclear glutathione levels (32) are not affected, or are only partially affected, by BSO treatment; this may result in an underestimation of the resistance mechanism by glutathione.

The results of our experiments in highly and moderately cDDP resistant cells suggest that glutathione may be involved in some way in cDDP resistance. To determine this contribution, the DMFs for BSO were compared with the glutathione ratios. In moderately resistant RL2 cells this resulted in 1.3 (DMF):2.4 (GSH) = 1.8, and 2.2 (DMF):4.3 (GSH) = 1.9 in RL4 cells. This indicates that the elevated glutathione content contributes equally to the increased resistance in RL2 and RL4 cells. In other studies, markedly elevated glutathione levels in highly resistant cells have also occasionally been demonstrated (40).

It is generally accepted that DNA is the critical target of cDDP therapy (41), and therefore changes in platinum-DNA interactions are
likely to be involved in CDDP resistance. The total amount of platinated DNA is the result of an equilibrium between platinum-DNA adduct formation and removal. CDDP resistance can be mediated by decreased initial platinum-DNA adduct formation, by a better DNA repair or a combination of the two. The initial platinum levels could be adequately explained by accumulation differences, implying that glutathione had no effect on initial DNA platinatation. Glutathione may, however, mediate resistance by stimulating DNA repair (5). This is consistent with the greater adduct removal found here. The importance of repair in these cell lines is indicated by the finding of increased repair (final versus peak levels) with increasing resistance and that the final equi-adduct dose ratios were in closer agreement with resistance ratios than those for initial or peak adducts.

The fact that 48-h adduct level ratios did not correspond exactly with resistance ratios (Fig. 7A), may have resulted from a number of factors. These include: lesions other than those recognized by the antibody used here could affect survival; repair may be incomplete at 48 h; repair of some areas of the genome (critical genes) may be more important than others and such areas may have a different repair rate (42). Whichever mechanism is involved, the data support the idea that adduct repair plays a crucial role in cell survival, especially in the resistant lines.

In the last several years it has become clear that CDDP resistance is multifactorial. Our study indicates that, although all possible resistance mechanisms were not investigated, the contribution of each separate mechanism varies according to the degree of resistance. Reduced platinum accumulation appeared to play a greater role in moderate CDDP resistance whereas higher resistance appeared to involve a greater component of repair. The sensitive RLS cells showed increased initial DNA platinatation for a given level of drug accumulation and a decreased DNA repair as the main factors determining sensitivity to CDDP.

REFERENCES
