Jpn. J. Cancer Res. 84, 83–92, January 1993

The Mechanism of the Difference in Cellular Uptake of Platinum Derivatives in Non-small Cell Lung Cancer Cell Line (PC-14) and Its Cisplatin-resistant Subline (PC-14/CDDP)

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A cisplatin-resistant non-small cell lung cancer cell line, PC-14/CDDP, was established from PC-14 by stepwise escalation of CDDP concentrations *in vitro*. PC-14/CDDP cells were 11.4-fold more resistant to CDDP compared with PC-14 cells. This resistant cell line was cross-resistant to platinum analogues, such as carboplatin (CBDCA) (×3.5), *cis*-diammine(glycolate-O,O′)platinum(II) (254-S) (×5.6) and *cis*-dichloro(ethylenediammine)platinum(II) (cis-DEP) (×4.2). On the other hand, relative resistance value to ormaplatin was only 1.4-fold. To elucidate the mechanism(s) of CDDP resistance and of its circumvention by ormaplatin, we investigated the characteristics of this cell line. Total sulfhydryl content was slightly elevated in PC-14/CDDP cells compared with PC-14 cells. There was no significant difference in the DNA repair ability between the two cell lines. Cellular accumulations of CDDP, CBDCA, 254-S, and cis-DEP in PC-14/CDDP cells were markedly decreased to 23%, 27%, 29%, and 32% of those in PC-14 cells, respectively. However, the accumulation of ormaplatin in PC-14/CDDP was almost the same as that in PC-14. To elucidate the mechanisms of uptake of these platinum analogs in the cells, we studied the effects of ouabain, an Na^+,K^+-ATPase inhibitor, on cellular drug uptake in both cell lines. Preincubation with 300 n*M* ouabain for 1 h inhibited approximately 60% of CDDP accumulation in PC-14. However ouabain preincubation at any concentration up to 300 n*M* did not affect CDDP accumulation in PC-14/CDDP. The accumulation of ormaplatin was not inhibited by ouabain in either of the cell lines. These data suggest that the mechanism of the uptake of ormaplatin is different from that of CDDP, and that ormaplatin exerts a cytotoxic effect in CDDP-resistant cells which have defective cisplatin accumulation.

Key words: Lung cancer — Cisplatin resistance — Ormaplatin — Drug accumulation

cis-Diamminedichloroplatinum(II) (CDDP[6]) remains an important anticancer agent because of its broad-spectrum antitumor activity.[1] However, the development of resistance to CDDP is a significant clinical problem, leading to treatment failure. Studies of cisplatin resistance mechanisms have revealed the following possible mechanisms: (a) decreased cellular accumulation; (b) enhanced inactivation by the cellular detoxication systems such as protein and non-protein thiols; and (c) decreased DNA damage and/or increased repair.[2]

Based on the above categories of resistance mechanism, several strategies for overcoming CDDP resistance have been proposed, including the depletion of glutathione,[3] inhibition of DNA repair,[4] and increasing CDDP accumulation.[5,6] New partially or completely non-cross-resistant CDDP analogues would provide another approach for overcoming CDDP resistance. Tetrachloro-(*d,l-trans*)-1,2-diaminocyclohexaneplatinum(IV) (ormaplatin) is one such second-generation platinum analogue. This drug is known to be rapidly reduced both intraand extracellularly to diaminocyclohexane platinum (II) (PtC12(dach)), which is believed to be the active moiety.[7,8] Ormaplatin has significantly less nephrotoxicity than CDDP[9,10] and increased the life span of L1210-bearing rats significantly more than CDDP did.[11] *In vitro*, it is as or more effective than CDDP against

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[6] The abbreviations used are: CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin); transplatin, *trans*-diamminedichloroplatinum(II); CBDCA, *cis*-diammine(1,1-cyclobutane)dicarboxylatoplatinum(II) (carboplatin); ormaplatin, tetrachloro-(*d,l-trans*)-1,2-diaminocyclohexaneplatinum(IV); PtC12 (dach); dichloro(*d,l-trans*)-1,2-diaminocyclohexaneplatinum (II); cis-DEP, *cis*-dichloro(ethylenediammine)platinum(II); 254-S, *cis*-diammine(glycolato-O,O′)platinum(II); PBS, phosphate-buffered saline; ICL, DNA-interstrand cross-links; GSH, glutathione; IC_{50}, drug concentration that inhibits cell growth by 50%.

several tumor cell lines,[11-13] and it is effective in L1210 and P388 CDDP-resistant cell lines.[12, 13]

In this communication, we demonstrate that a CDDP resistant non-small cell lung cancer cell line (PC-14/CDDP) has defective CDDP uptake and that ormaplatin circumvents this CDDP resistance mechanism.

MATERIALS AND METHODS

Drugs and chemicals CDDP and *cis*-diammine(1,1-cyclobutane) dicarboxylatoplatinum (II) (carboplatin, CBDCA) were donated by Bristol-Myers Research Institute (Tokyo). *cis*-Diammine (glycolato-O,O′) platinum (II) (NSC375101, 254-S) and ormaplatin were obtained from Shionogi Pharmaceutical Co. Ltd. (Osaka) and UpJohn Pharmaceuticals Co. Ltd. (Tokyo), respectively. *cis*-Dichloro(ethylenediammine)platinum(II) (cis-DEP) was kindly provided by Dr. Alan Eastman (Department of Pharmacology, Dartmouth Medical School, Hanover, NH). [methyl-^{12}C]Thymidine (specific activity 52 mCi/mmol) and [^{3}H]inulin (specific activity 1–5 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK), and [^{3}H]ouabain (specific activity 15–30 Ci/mmol) was purchased from DuPont NEN (Boston, MA). [^{195m}Pt]-CDDP (initial specific activity 60,000 μCi/nmol) was made available by Dr. M. Akaboshi at the Research Reactor Institute of Kyoto University (Osaka).

Cell lines and tissue culture The PC-14 cell line was derived from a previously untreated patient with pulmonary adenocarcinoma and kindly provided by Prof. Y. Hayata, Tokyo Medical College. The PC-14/CDDP cell line was obtained by CDDP exposure for 1 year from PC-14/1.5 that was established from PC-14 by stepwise dose escalation of CDDP.[14] PC-14 and PC-14/CDDP cells were grown as half-attached and half-suspension culture in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FBS) (Sigma Chemical Co., St. Louis, MO), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. The degree of CDDP resistance in the PC-14/CDDP cell line was stable for at least 1 year during subculture in CDDP-free medium. The CDDP-resistant cell line was used in this study after at least 1 month of continuous growth in CDDP-free medium. Cells were routinely tested for *Mycoplasma* contamination (Hoechst stain kit for detection of *Mycoplasma* in cell culture; Flow Laboratories, Inc., McLean, VA). The cell size of each cell line was measured by using a Coulter Channalyzer C-256 system (Coulter Electronics, Hialeah, FL).

Soft agar colony assay Chemosensitivity was determined using the double-layer soft agar colony assay system described previously.[15] PC-14 and PC-14/CDDP cells were plated at 10,000 cells/well, yielding a plating efficiency of 18–20%. Colonies of more than 50 μm in diameter were counted by an automatic particle counter (PC-2000, Shiraimatsu Co. Ltd., Osaka).

Protein assay Protein was measured using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL).

Sulfhydryl determinations Non-protein and total sulfhydryl contents were measured by the Ellman method.[16, 17] The absorbance was measured at 412 nm with a spectrophotometer (U-3210, Hitachi Co. Ltd., Tokyo). Glutathione and cysteine (Sigma) were used to prepare calibration curves.

Drug treatment For drug accumulation and efflux studies, cells were incubated with various platinum analogues at 37°C in a humidified incubator with 5% CO_2. After various incubation times, cells were collected by low-speed centrifugation and washed twice with cold phosphate-buffered saline (PBS). To evaluate efflux rates of these drugs, after 4 h of drug exposure, cells were washed twice with cold PBS and resuspended in drug-free medium. The cells were reincubated for various times and collected using the same procedures as above.

For determination of the effect of ouabain on the cellular accumulation of platinum analogues, cells were incubated at 37°C for 1 h with medium containing various concentrations of ouabain. After the 1 h incubation period, they were treated with the drug and assayed using the same procedure as above. The cell pellets were stored at −70°C until analysis.

Isolation of nuclear fraction For nuclear drug accumulation studies, nuclear fractions from both cell lines were isolated using a modification of Muramatsu's method.[18] Briefly, each cell line was exposed to 50 μM CDDP or ormaplatin for various times. After drug treatment, cells were collected and washed 3 times with cold PBS. The cell pellets were resuspended in 20 volumes of reticulocyte standard buffer (RSB) containing 0.01 M tris HCl, 0.01 M NaCl, 1.5 mM $MgCl_2$, pH 7.4, and allowed to stand for 10 min in an ice bath. The swollen cells were resuspended in the same volume (as employed in the first hypotonic shock) of RSB and solutions of 10% Nonidet P40 (Shell Chemical Co.) and 10% sodium deoxycholate were added to give final concentrations of 0.3%, respectively. The mixture was homogenized in a tightly fitted Potter-Elvehjem type homogenizer with a Teflon pestle giving 5 to 7 strokes. The homogenate was centrifuged at 3000 rpm for 5 min. The nuclear fractions were stored at −70°C until analysis.

Platinum accumulation The cell extracts were analyzed for platinum by atomic absorption spectrophotometry using a polarized Zeeman atomic absorption spectrophotometer (Z-7000 Hitachi Co. Ltd.).

Incorporation of [^{195m}Pt]cisplatin into DNA fractions For the determination of CDDP incorporation into

DNA fraction, cells were exposed to 50 μM [^{195m}Pt]-CDDP for various times. The DNA fraction from each cell line was isolated using the STS method.[19, 20] The DNA content was measured at 254 nm with a spectrophotometer. The radioactivity of the DNA fraction was counted with a Packard auto-gamma scintillation counter. Recovery of DNA binding platinum molecules was more than 91% (data not shown).

CDDP short-term accumulation The short-term CDDP accumulation was determined by a modified oil-stop method.[21] Briefly, 1×10^9 cells of each cell line were preincubated in a volume of 200 ml at 37°C for 1 h, in the presence or absence of 1 μM ouabain. The preincubated cells in the same medium containing [^{14}C]inulin were adjusted to a density of 1.25×10^8 cells/ml. Then 400 μl aliquots of the cell suspensions were placed into 1.5 ml tubes at 37°C and 100 μl aliquots of various concentrations of [^{195m}Pt]CDDP were added and mixed. After various incubation times, the mixtures were transferred to the top layer of 15 ml centrifugation tubes (top layer, 6 ml of PBS; middle layer, 5 ml of dioctyl phthalate: di-*n*-butyl phthalate (1:1.1); under layer, 1 ml of 23% sucrose) and immediately centrifuged. The under layer was sonicated, and radioactivity in the mixture was measured in a Packard auto-gamma scintillation counter. The data were corrected for cellular bound water by subtracting the radioactivity of [^{14}C]inulin.

Alkaline elution assays Cells were radiolabeled by incubation with [methyl-^{14}C]thymidine (0.1 μCi/ml) overnight. Alkaline elution was performed as described by Bungo *et al.*[22] The radioactivity was counted in a liquid scintillation counter (LS3801 Beckman Instruments Inc., Irvine, CA). The frequency of CDDP-induced DNA interstrand cross-links (ICL) was calculated by means of the following formula[23]:

$$CI_{DRUG}=\left(\frac{1-R_{RAD}}{1-R_{DRUG}}\right)^{1/2}-1$$

where CI_{DRUG} is the cross-links index in drug-treated cells, R_{RAD} is the relative retention of 5-Gy-irradiated control cells, and R_{DRUG} is that of drug-treated cells before 5-Gy irradiation.

Ouabain binding For ouabain-binding studies, both cell lines were washed with PBS and adjusted to 4×10^6 cells/ml with a K^+-free buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 3.0 mM $CaCl_2$, 10 mM glucose, 10 mM Tris-HCl (pH 7.4)). Then 0.5 ml of the cell suspension was mixed with an equal volume of the same buffer containing [^{3}H]-ouabain and incubated for 90 min at 37°C. After incubation, cells were washed 3 times with cold PBS and sonicated. Aliquots were collected in scintillation vials and mixed with 4 ml of scintillation fluid. The radioactivity was counted in a liquid scintillation counter (LS6000TA, Beckman Instruments Inc.). Background binding to cells was determined from suspensions that contained 1000-fold excess of cold ouabain and was subtracted from the total count.

Statistics All values are expressed as the means ± SD, and were analyzed by the use of Student's t test; P values of less than 0.05 were considered to be significant.

RESULTS

Characteristics of PC-14 and PC-14/CDDP cell lines The characteristics of PC-14 and PC-14/CDDP were examined (Table I). Non-protein sulfhydryl contents of both cell lines were not significantly different. Total sulfhydryl content was increased in PC-14/CDDP compared with PC-14.

The chemosensitivities to CDDP and other platinum analogues of PC-14 and PC-14/CDDP cells were evaluated by clonogenic assay (Table II). The IC_{50} values of

Table I. Characteristics of Human Non-small Cell Lung Cancer Cell Lines

	PC-14	PC-14/CDDP
Doubling time (h)	28.3[a]	21.8
Cell volume (pl)	2.1±0.006[b]	1.8±0.001
Thiol content (nmol/mg protein)		
Total thiol	106.2±11.2	136.4±24.6[c]
Non-protein thiol	63.1±5.2	62.2±8.5

a) Doubling time was determined by MTT assay.
b) Each value is the mean ± SD of the three independent experiments.
c) Significantly different from the corresponding value for thiol content of PC-14 at the $P<0.05$ level, using Student's t test.

Table II. Chemosensitivities to Platinum Analogues in PC-14 and PC-14/CDDP

	IC_{50} values (μM)[a]		
	PC-14	PC-14/CDDP	
CDDP	2.3±0.4[b]	27.0±2.2[d]	(11.8)[c]
CBDCA	21.1±3.4	73.1±14.8[d]	(3.5)
254-S	3.3±0.7	18.6±2.2[d]	(5.6)
cis-DEP	6.6±1.2	27.6±2.0[d]	(4.2)
Transplatin	>100	>100	
Ormaplatin	3.3±0.6	4.6±1.3	(1.4)

a) Drug concentration that inhibits colony formation by 50%.
b) Mean ± SD of three independent experiments.
c) Values in parentheses are relative resistance value (IC_{50} of resistant cell line/IC_{50} of sensitive cell line).
d) Significantly different from the corresponding IC_{50} value of PC-14 at the $P<0.05$ level, using Student's t test.

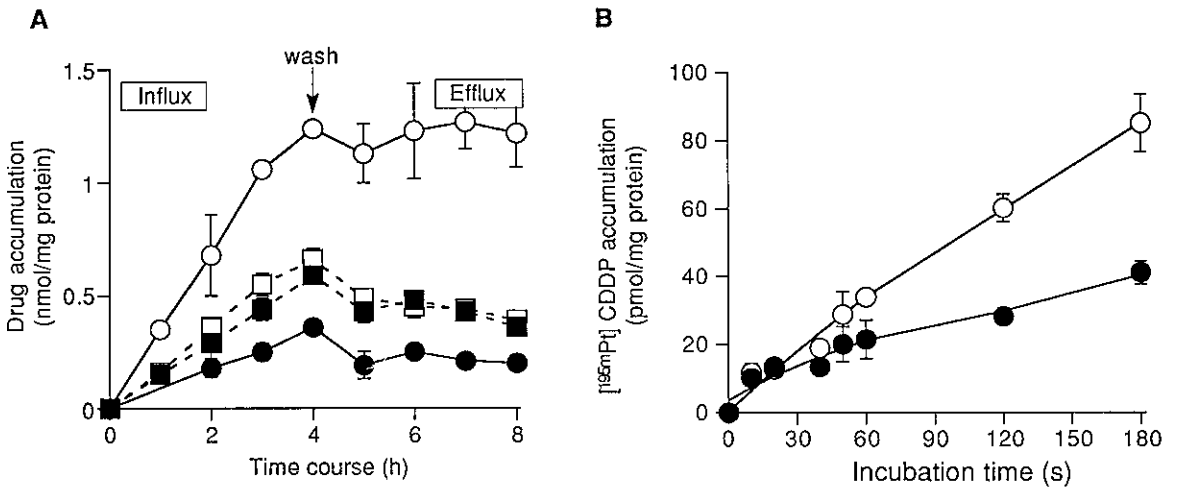

Fig. 1. A, Time course of drug influx and efflux of CDDP and ormaplatin. Cells were incubated with 50 μM CDDP (circles) or ormaplatin (squares) for the indicated time periods. To evaluate efflux rates, after 4 h drug exposure, cells were washed twice with cold PBS(−) and resuspended in drug-free medium. Accumulated platinum was assayed by the atomic absorption method ("Materials and Methods"). B, Short-term [^{195m}Pt]CDDP accumulation was determined by the oil-stop method ("Materials and Methods"). Accumulated platinum was assayed by gamma counting. Points, mean values from more than 3 independent experiments; bars, SD; open symbols, PC-14; closed symbols, PC-14/CDDP.

CDDP in PC-14 and PC-14/CDDP cells were 2.3 μM and 27.0 μM, respectively, for continuous drug exposure. Relative resistance of PC-14/CDDP with respect to PC-14 was 11.8-fold. PC-14/CDDP was cross-resistant to CBDCA, 254-S, and cis-DEP, and the relative resistance values were 3.5-fold, 5.6-fold, and 4.2-fold, respectively. In contrast, the resistant cells show only 1.4-fold resistance to ormaplatin.

Platinum accumulation In order to determine whether the sensitivity to each platinum analogue correlates with the intracellular accumulation of the drug, we evaluated the cellular platinum accumulation using atomic absorption spectroscopy. Fig. 1-A shows the amounts of cellular platinum in sensitive and resistant cell lines exposed to 50 μM CDDP for 4 h. The accumulation of CDDP increased linearly with incubation time in both cell lines. No saturation of the accumulation was observed until after 24 h (data not shown). The cellular accumulation of CDDP in PC-14/CDDP cells was 23% of that in the parental cells as determined from the ratio of the slopes of the regression curves. The decreased CDDP accumulation could be seen as early as 30 s after exposure to CDDP (Fig. 1-B). Most of the cellular CDDP was retained in these cells until 4 h after removal of CDDP from medium and there was no significant difference in efflux of CDDP between the two cell lines (Fig. 1-A).

Decrease of platinum accumulation in PC-14/CDDP was also observed with CBDCA, 254-S, cis-DEP, and transplatin (Table III). There was no significant difference in the accumulation of ormaplatin between PC-14 and PC-14/CDDP (Fig. 1-A).

Table III. Cellular Accumulation of Platinum Analogues

	Drug accumulation (nmol/mg protein)		
	PC-14	PC-14/CDDP	
CDDP[a]	1.24±0.003[c]	0.36±0.020	(29.2)[d]
CBDCA[b]	0.22±0.024	0.05±0.003	(27.2)
254-S[b]	0.43±0.043	0.13±0.007	(28.6)
cis-DEP[a]	0.68±0.006	0.22±0.012	(31.8)
Transplatin[a]	0.63±0.067	0.33±0.043	(52.6)
Ormaplatin[a]	0.66±0.051	0.59±0.004	(88.9)

a) Tumor cells were treated with 50 μM drugs for 4 h.
b) Tumor cells were treated with 100 μM drugs for 4 h.
c) Mean±SD of the three independent experiments.
d) Values in parentheses are reduction of drug accumulation (drug accumulation value of resistant cell line/drug accumulation value of parental cell line).

Next, we examined the intracellular distribution of CDDP. Of the net accumulations, 24% and 25% were distributed into the nuclear fractions and 2.4% and 2.5% into the DNA fractions in PC-14 and PC-14/CDDP, respectively. The platinum content of CDDP in the nuclear and DNA fractions correlated with that in whole cells, and the distribution ratio of CDDP into the intracellular components was almost the same in both cell lines (Table IV).

Table IV. Cellular Distribution of Platinum Analogues into Nuclear and DNA Fractions

		Drug accumulation ($\mu g/1\times10^7$ cells)[a]			
		PC-14		PC-14/CDDP	
CDDP	whole cell	0.92±0.03[b]		0.26±0.03	
	nuclear fraction	0.22±0.14	(23.9)[c]	0.07±0.07	(24.7)
Ormaplatin	whole cell	0.84±0.05		0.64±0.06	
	nuclear fraction	0.16±0.04	(18.8)	0.12±0.10	(18.0)
[^{195m}Pt]CDDP	whole cell	1.01±0.03		0.31±0.02	
	DNA fraction	0.025±0.002	(2.4)	0.008±0.001	(2.5)

a) Cells were exposed to 50 μM drug for 4 h.
b) Mean±SD of 3 independent experiments.
c) Values in parentheses are distribution ratio of drug (drug accumulation of the cellular fraction/whole cell drug accumulation).

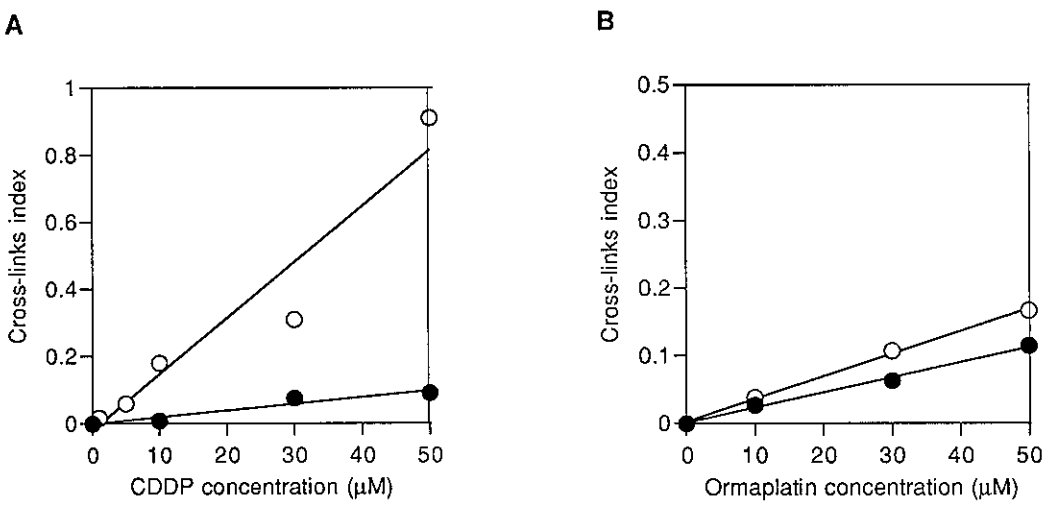

Fig. 2. Dose dependency of ICL formation by CDDP (A) and ormaplatin (B). Cells were incubated with various concentrations of drugs for 4 h and ICL formation was determined by the alkaline elution technique. The frequency of ICL expressed as cross-links index ("Materials and Methods"). Points, mean values from 3 independent experiments (○) PC-14; (●), PC-14/CDDP.

The rates of removal of [^{195m}Pt]CDDP from DNA fraction were also determined at doses of this drug giving equivalent cytotoxic effect in the two cell lines. At 15 h after removal of the drug, CDDP retained in DNA fractions had decreased to 30.7% and 41.8% of the initial incorporations into DNA fractions in PC-14 and PC-14/CDDP, respectively.

Formation of ICL Formation of ICL is considered to play a critical role in the cytotoxicities of platinum analogues.[22, 24, 25] To elucidate whether the accumulations of CDDP and ormaplatin are related to the cellular DNA damage induced by these drugs, ICL was evaluated as one of the parameters of DNA damage by using the filter elution technique. CDDP and ormaplatin did not cause DNA single-strand breaks or DNA double-strand breaks in PC-14 and PC-14/CDDP (data not shown). Fig. 2-A shows the amount of CDDP-induced ICL, expressed as "cross-links index," in both cell lines when the cells were exposed to various concentrations of CDDP for 4 h. The ICL formation in PC-14/CDDP was 12% of that in PC-14 as determined from the ratio of the slopes of regression curves. The results are consistent with the relative chemosensitivity to CDDP. The amounts of ormaplatin-induced ICL were not significantly different between PC-14 and PC-14/CDDP (Fig. 2-B). Although the incorporation of ormaplatin into nuclear fraction was approximately equal to that of CDDP, the amount of ormaplatin-induced ICL was significantly lower than the

amount of CDDP-induced ICL at equitoxic drug concentrations in each cell line.

The repair of ICL after 4 h exposure to CDDP was also determined by the alkaline elution method. PC-14 and PC-14/CDDP cells were exposed to 7.5 μM and 50 μM CDDP, respectively, to induce the same peak frequency of ICL. The percentages of CDDP-induced ICL retained after 48 h were 38.8% and 42.3% of the peak frequency of ICL in PC-14 and PC-14/CDDP, respectively. The results suggest that there is no significant difference in ICL repair ability between the two cell lines.

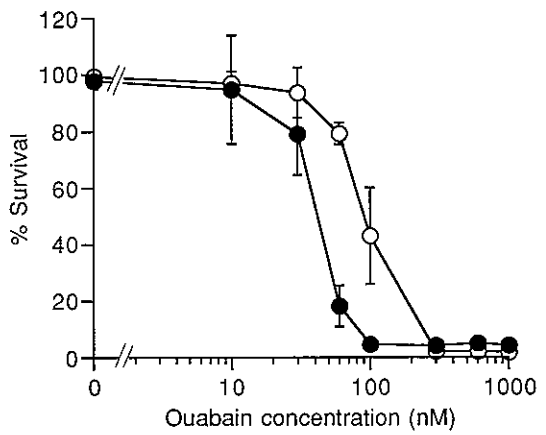

Fig. 3. Growth inhibition curves of PC-14 (○) and PC-14/CDDP (●) against ouabain concentration. Cytotoxicity was determined by clonogenic assay with continuous exposure to ouabain. Points, mean values from 3 independent experiments; bars, SD.

Effect of ouabain on CDDP and ormaplatin accumulation

The different cellular accumulation patterns of CDDP and ormaplatin led us to question whether the circumvention of CDDP resistance was due to the operation of different uptake mechanisms of the drugs. Andrews *et al.* reported that CDDP uptake was inhibitable by ouabain in human ovarian carcinoma cells.[26] Accordingly, we examined the effect of ouabain on CDDP and ormaplatin accumulation in PC-14 and PC-14/CDDP.

We determined the direct cytotoxic effect of ouabain by clonogenic assay in both cell lines with continuous exposure (Fig. 3). PC-14/CDDP is 2.1-fold more sensitive to ouabain than PC-14.

Fig. 4-A shows CDDP accumulation in PC-14 and PC-14/CDDP after exposure to 50 μM CDDP for 4 h and the effects of pretreatment with various concentrations of ouabain. A marked decrease in the CDDP accumulation was observed in PC-14 treated with ouabain in a concentration-dependent manner. Approximately 60% of CDDP accumulation was inhibited by 300 nM ouabain in PC-14. In contrast, pretreatment with ouabain at concentrations up to 300 nM did not influence the CDDP accumulation in PC-14/CDDP. The same result was obtained in short-term CDDP accumulation experiments (Fig. 4-B). Ouabain inhibited CDDP accumulation as

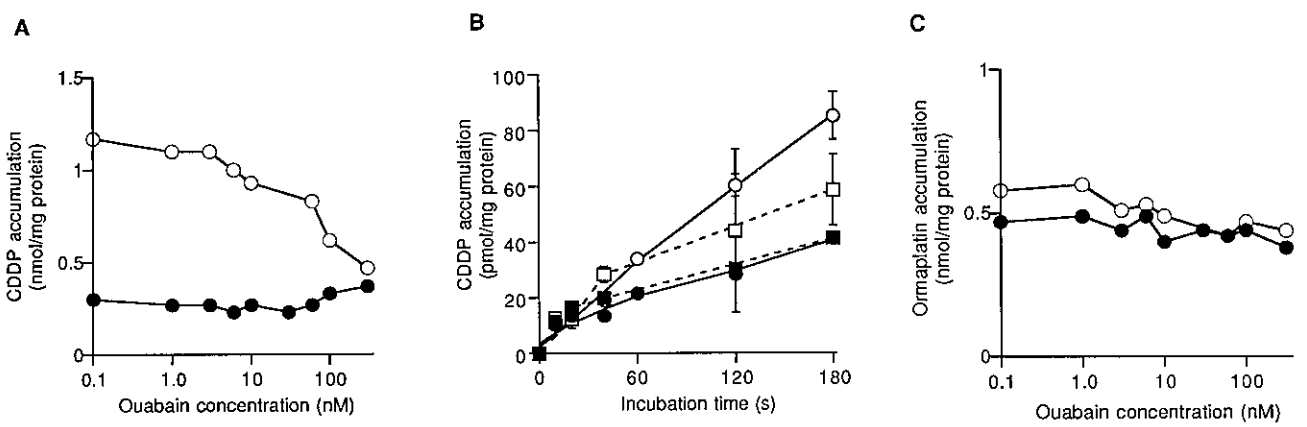

Fig. 4. A, Effect of ouabain concentration on CDDP accumulation in PC-14 and PC-14/CDDP. Cells were incubated for 1 h with the indicated concentration of ouabain prior to the addition of 50 μM CDDP. Incubations were then continued for an additional 4 h with ouabain present. Accumulated platinum was assayed by the atomic absorption method ("Materials and Methods"). B, Effect of ouabain on short-term [^{195m}Pt]CDDP accumulation. Cells were incubated for 1 h with (squares) or without (circles) 100 nM ouabain prior to addition of 100 μM [^{195m}Pt]CDDP. The short-term [^{195m}Pt]CDDP accumulation was determined by the oil-stop method ("Materials and Methods"). Accumulated platinum was assayed by gamma counting. Points, mean values from 3 independent experiments; bars, SD. C, Effect of ouabain concentration on ormaplatin accumulation in PC-14 and PC-14/CDDP. Cells were incubated for 1 h with the indicated concentration of ouabain prior to the addition of 50 μM ormaplatin and assayed as described above. Open symbols, PC-14; closed symbols, PC-14/CDDP.

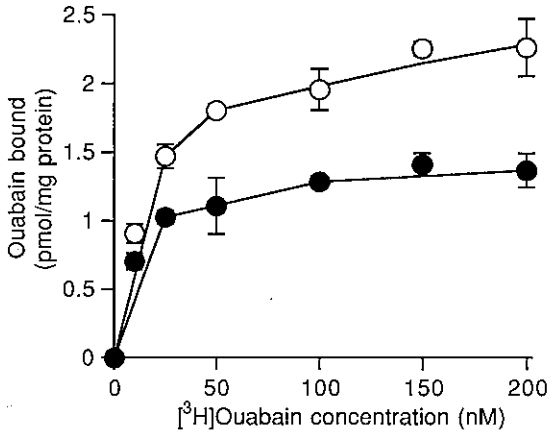

Fig. 5. Ouabain binding to PC-14 and PC-14/CDDP. Cells were incubated with various concentrations of [^{3}H]ouabain for 90 min in a K^+-free buffer ("Materials and Methods"). Bound ouabain was assayed by using a liquid scintillation counter. Points, mean values from 2 independent experiments; bars, SD. (○) PC-14; (●), PC-14/CDDP.

early as 120 s after the exposure of PC-14 to [^{195m}Pt]-CDDP.

In the case of ormaplatin, inhibition of drug accumulation by 300 n*M* ouabain in PC-14 and PC-14/CDDP amounted to approximately 19% and 14%, respectively (Fig. 4-C). The inhibition of ormaplatin accumulation by ouabain was almost the same in the two cell lines and was less than that of CDDP accumulation in PC-14.

[^{3}H]Ouabain binding to PC-14 and PC-14/CDDP cells

We hypothesized that the difference in the effect of ouabain on CDDP accumulation between PC-14 and PC-14/CDDP might be due to a change in the activity or number of ouabain-binding sites. Therefore, we quantitated [^{3}H]ouabain binding to these cells (Fig. 5). The binding of ouabain to PC-14/CDDP was less than that to PC-14, and the maximal binding at saturation was approximately 57% of that in the parental cell line.

DISCUSSION

The amount of CDDP accumulated in PC-14/CDDP cells was significantly lower than that in PC-14 cells. Incorporation of platinum into the nuclei and the DNA fractions correlated well with the net cellular accumulation of CDDP. Cellular thiols, such as glutathione and metallothionein, can bind to CDDP and prevent it from binding to DNA. An increase in thiol content has been reported in several CDDP-resistant cancer cells.[25, 27, 28] In our study, although elevation of protein sulfhydryl content was observed in the resistant subline (Table II), the distribution ratios of CDDP into nuclei and DNA fractions were the same in both cell lines (Table IV). In fact, similar incorporation of CDDP into the DNA fraction was observed at doses giving equivalent cellular CDDP accumulation in the two cell lines (data not shown). Accordingly, it is unlikely that a change of thiol content participates in the CDDP resistance in PC-14/CDDP, because thiol content changes would change the distribution ratios of CDDP, especially in the DNA fraction.

The repair of CDDP-induced DNA damage is also an important factor in cellular resistance to CDDP. Therefore, we evaluated the ability to repair DNA damage in terms of the removal of [^{195m}Pt]CDDP from DNA fractions and the repair of CDDP-induced ICL. Equivalent amounts of CDDP removal or repair of ICL were observed in both cell lines at equitoxic concentrations of CDDP. These findings suggest that the decrease of cellular CDDP accumulation is the dominant mechanism of CDDP resistance in PC-14/CDDP.

Although the mechanism of CDDP resistance is thought to be multifactorial, the decreased accumulation of CDDP is a consistent finding in a number of cell lines with acquired CDDP resistance.[2] A recent study indicated that decreased CDDP accumulation occurs at an early stage in the development of resistance.[29] Based on these findings, decreased intracellular accumulation of CDDP may be the primary factor for CDDP resistance.

Possible mechanisms of decreased CDDP accumulation include decreased influx, decreased intracellular binding or sequestration, and increased efflux of the drug. There was no significant difference in the efflux or in the distribution of the drug between PC-14 and PC-14/CDDP. The decrease of CDDP accumulation in PC-14/CDDP was observed as early as 30 s (Fig. 1-B). These results suggest that the decreased accumulation of CDDP was due to a change of drug influx step. This finding is in agreement with previous reports.[30, 31]

The mechanism by which CDDP enters the cell membrane is not well understood. Based on the evidence that CDDP accumulation is not saturable up to 3 m*M* CDDP[32] and is not inhibitable by structural analogues,[31] it has long been assumed that CDDP moves through the cell membrane by passive diffusion. However, CDDP accumulation can be modulated by ouabain,[26] osmotic strength and pH,[33] and cAMP levels,[5] which suggests that multiple CDDP uptake mechanisms may exist.

Andrews *et al.* reported that CDDP accumulation was inhibited by ouabain treatment and suggested that the alterations of Na^+,K^+-ATPase in CDDP-resistant ovarian carcinoma cells, 2008/DDP, may be linked to the decreased CDDP accumulation.[26] In our study, the inhibitory effect of ouabain on CDDP accumulation was observed in PC-14 cells. This effect was also observed in short-term accumulation studies. However, ouabain at any concentration up to 300 n*M* did not affect CDDP

accumulation in PC-14/CDDP cells. The amount of ouabain binding in PC-14/CDDP cells was reduced to 57% of that in PC-14. These results suggest that a CDDP uptake mechanism inhibitable by ouabain exists in PC-14 cells. This mechanism might be quantitatively or qualitatively defective in PC-14/CDDP cells.

PC-14/CDDP cells were more sensitive to ouabain-induced cytotoxicity than PC-14 cells. We have no evidence to show why PC-14/CDDP cells exhibit collateral sensitivity to ouabain, but we hypothesize that decreased activity of Na^+,K^+-ATPase, which plays an important role in the maintenance of transmembrane Na^+ and K^+ balance, at lower concentrations of ouabain induces an imbalance of the ions in PC-14/CDDP, but not in PC-14 cells. We are now evaluating the activity and the expression level of mRNA of Na^+,K^+-ATPase in PC-14 and PC-14/CDDP cells.

Several reports show that CDDP-resistant cells are cross-resistant to CBDCA,[34,35] 254-S,[36] and cis-DEP,[35] but there are few reports of CDDP-resistant cells being cross-resistant to the PtC12(dach) derivative ormaplatin.[28,37] In our cell line as well, ormaplatin was non-cross-resistant to other platinum analogues. Ormaplatin accumulated in resistant cells at the same concentrations and rates as the parental cell line (Table III). Several laboratories have found identical accumulation of PtC12(dach) in both parent and CDDP-resistant cell lines.[38,39] Panneerselvam and Rahman found higher levels of platinum in CDDP-resistant human ovarian carcinoma cell lines treated with ormaplatin.[40] Our results were consistent with these findings. The possible mechanisms for the equivalent accumulation of ormaplatin in both PC-14 and PC-14/CDDP include the following: 1) the cellular uptake mechanism of ormaplatin is different from that of CDDP; 2) the intracellular pharmacokinetics of this drug is different from that of CDDP. We found no difference in the cellular accumulation (Fig. 1-A) or in the distribution ratio into nuclear fraction (Table IV) between ormaplatin and CDDP. In addition, ouabain pretreatment decreased CDDP accumulation but not ormaplatin accumulation in PC-14 (Fig. 4-A, C). Accordingly, the cellular uptake mechanism of ormaplatin is likely to be different from that of CDDP. This supports the concept of differential drug uptakes as a key mechanism of CDDP resistance. If the decrease of CDDP accumulation is the dominant mechanism of CDDP resistance, ormaplatin is likely to have substantial clinical potential.

In order to evaluate the correlation between drug accumulation and DNA damage, we examined ormaplatin-induced ICL formation by the filter elution technique. There was no significant difference of ICL formation by this drug between PC-14 and PC-14/CDDP (Fig. 2-B). Nevertheless, in PC-14, the level of ICL formation by ormaplatin was significantly low, being only about 10% of that by CDDP at equitoxic concentrations. Jennerwein *et al.* demonstrated that PtC12(dach) and CDDP produced adducts at similar sites in DNA[41] and Page *et al.* demonstrated a slower conversion from dach-Pt monoadducts to diadducts than with CDDP in a platinated DNA template.[42] To determine whether the slower conversion causes lower ICL formation of this drug, we followed the time course of ormaplatin-induced ICL formation in cells after 4 h exposure to this drug. However, ICL formation by ormaplatin was much lower than that by CDDP up to 24 h after drug exposure (data not shown). These data suggest that ormaplatin causes less ICL than CDDP does in PC-14. It is still unclear what kinds of CDDP-DNA adducts are predominantly related to the cytotoxicity of CDDP. However, it has been reported that CDDP-induced ICL formation is well correlated to the cytotoxic effect of this drug. CDDP-induced ICL formation is thought to be an important parameter of the cytotoxic effect of CDDP.[22,25,37] Thus, we hypothesize that ormaplatin may have different platinum adduct lethality from that of CDDP. The precise effect of ormaplatin on platinum adduct lethality requires further study.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, and from the Ministry of Education, Science and Culture. The authors gratefully acknowledge the support of the Fund of Bristol Myers Squibb Co. Thanks are due to Dr. Dean Brenner Associate Professor of the University of Michigan for a critical review of the manuscript, and to Dr. K. Komiya (Department of Radiopharmacy, Hoshi University, Tokyo, Japan) for his assistance and advice during the [^{195m}Pt]CDDP accumulation study.

(Received July 13, 1992/Accepted October 17, 1992)

REFERENCES

1) Loether, P. J. and Einhorn, L. H. Cisplatin. *Ann. Intern. Med.*, **100**, 704–713 (1984).
2) Andrews, P. A. and Howell, S. B. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. *Cancer Cells*, **2**, 35–43 (1990).
3) Hromas, R. A., Andrews, P. A., Murphy, M. P. and Burns, C. P. Glutathione depletion reverses cisplatin resistance in murine L1210 leukemia cells. *Cancer Lett.*,

34, 9–13 (1987).
4) Roberts, J. J. and Kotsaki-Koabtsi, V. P. Potentiation of sulphur mustard or cisplatin-induced toxicity by caffeine in Chinese hamster cells correlates with formation of DNA double-strand breaks during replication on a damaged template. *Mutat. Res.*, **165,** 207–220 (1986).
5) Mann, S. C., Andrews, P. A. and Howell, S. B. Modulation of *cis*-diamminedichloroplatinum(II) accumulation and sensitivity by forskolin and 3-isobutyl-1-methylxanthine in sensitive and resistant human ovarian carcinoma cells. *Int. J. Cancer,* **48,** 866–872 (1991).
6) Morikage, T., Bungo, M., Inomata, M., Yoshida, M., Ohmori, T., Fujiwara, Y., Nishio, K. and Saijo, N. Reversal of cisplatin resistance with amphotericin B in a non-small cell lung cancer cell line. *Jpn. J. Cancer Res.*, **82,** 747–751 (1991).
7) Mauldin, S. K., Gibbons, G., Wyrick, S. D. and Chaney, S. G. Intracellular biotransformation of platinum compounds with the 1,2-diaminocyclohexane carrier ligand in the L1210 cell line. *Cancer Res.*, **48,** 5136–5144 (1988).
8) Chaney, S. G., Wyrick, S. and Till, G. K. *In vitro* biotransformations of tetrachloro(*d,l-trans*)-1,2-diaminocyclohexaneplatinum(IV) (tetra platin) in rat plasma. *Cancer Res.*, **50,** 4539–4545 (1990).
9) Smith, J. H., Smith, M. A. and Litterst, C. L. Comparative toxicity and renal distribution of the platinum analogs tetraplatin, CHIP, and cisplatin at equimolar doses in the Fischer 344 rat. *Fundam. Appl. Toxicol.*, **10,** 45–61 (1988).
10) Smith, M. A., Smith, J. H. and Litterst, C. L. *In vivo* biochemical indices of nephrotoxicity of platinum analogs tetraplatinum, CHIP and cisplatin in the Fischer 344 rat. *Fundam. Appl. Toxicol.*, **10,** 62–72 (1988).
11) Rahman, A., Roh, J. K., Wolpert-Defilippes, M. K., Goldin, A., Venditti, J. M. and Wooley, P. V. Therapeutic and pharmacological studies of tetrachloro(*d,l-trans*)-1,2-diaminocyclohexane platinum(IV) (tetraplatin), a new platinum analogue. *Cancer Res.*, **48,** 1745–1752 (1988).
12) Anderson, W. K., Quagllato, D. A., Haugwitz, R. D., Narayanan, V. L. and Wolpert-Defilippes, M. K. Synthesis, physical properties, and antitumor activity of tetraplatin and related tetrachloroplatinum(IV) stereoisomers of 1,2-diaminocyclohexane. *Cancer Treat. Rep.*, **70,** 997–1002 (1986).
13) Wilkoff, L. J., Dulamadge, E. A., Trader, M. W., Harrison, S. D., Jr. and Griswold, D. P., Jr. Evaluation of *trans*-tetrachloro-1,2-diaminocyclohexane platinum(IV) in murine leukemia L1210 resistant and sensitive to *cis*-diamminedichloroplatinum(II). *Cancer Chemother. Pharmacol.*,**20,** 96–100 (1987).
14) Hong, W. S., Saijo, N., Sasaki, Y., Minato, K., Nakano, H., Nakagawa, K., Fujiwara, Y., Nomura, K. and Twentyman, P. R. Establishment and characterization of cisplatin-resistant sublines of human lung cancer cell lines. *Int. J. Cancer,* **41,** 462–467 (1988).
15) Ohe, Y., Nakagawa, K., Fujiwara, Y., Sasaki, Y., Minato, K., Bungo, M., Niimi, S., Horichi, N., Fukuda, M. and Saijo, N. *In vitro* evaluation of the new anticancer agents KT61149, MX-2, SM5887, Menogaril, and Libromaycin using cisplatin- or adriamycin-resistant human cancer cell lines. *Cancer Res.*, **49,** 4098–4102 (1989).
16) Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82,** 70–77 (1959).
17) Sedlak, J. and Lindsay, R. H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.*, **25,** 192–205 (1968).
18) Muramatsu, M., Hayashi, Y., Onishi, T., Sakai, M., Takai, K. and Kashiyama, T. Rapid isolation of nucleoli from detergent purified nuclei of various tumor and tissue culture cells. *Exp. Cell Res.*, **88,** 345–351 (1974).
19) Schimidt, G. and Thannhuser, S. J. A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. *J. Biol. Chem.*, **1612,** 83–89 (1945).
20) Schneider, W. C. Phosphorus compounds in animal tissues. *J. Biol. Chem.*, **164,** 747–751 (1946).
21) Plagemann, P. G. W. and Woffendin, C. Na^+-dependent and -independent transport of uridine and its phosphorylation in mouse spleen cells. *Biochim. Biophys. Acta,* **981,** 315–325 (1989).
22) Bungo, M., Fujiwara, Y., Kasahara, K., Nakagawa, K., Ohe, Y., Sasaki, Y., Irino, S. and Saijo, N. Decreased accumulation as a mechanism of resistance to *cis*-diamminedichloroplatinum(II) in human non-small cell lung cancer cell lines: relation to DNA damage and repair. *Cancer Res.*, **50,** 2549–2553 (1990).
23) Zwelling, L. A., Anderson, T. and Kohn, K. W. DNA-protein and DNA interstrand cross-linking by *cis*- and *trans*-platinum(II) diammine dichloride in L1210 mouse leukemia cells and relation to cytotoxicity. *Cancer Res.*, **39,** 365–369 (1979).
24) Wallner, K. E., DeGregorio, M. W. and Li, G. C. Hyperthermic potentiation of cis-diamminedichloroplatinum(II) cytotoxicity in Chinese hamster ovary cells resistant to the drug. *Cancer Res.*, **46,** 6242–6245 (1986).
25) Kasahara, K., Fujiwara, Y., Nishio, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matsuda, T. and Saijo, N. Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer Res.*, **51,** 3237–3242 (1991).
26) Andrews, P. A., Mann, S. C., Huynh, H. H. and Albright, K. D. Role of the Na^+,K^+-adenosine triphosphatase in the accumulation of *cis*-diamminedichloroplatinum(II) in human ovarian carcinoma cells. *Cancer Res.*, **51,** 3677–3681 (1991).
27) Fujiwara, Y., Sugimoto, Y., Kasahara, K., Bungo, M., Yamakido, M., Tew, K. D. and Saijo, N. Determinants of drug response in a cisplatin-resistant human lung cancer cell line. *Jpn. J. Cancer Res.*, **81,** 527–535 (1990).
28) Hospers, G. A. P., Mulder, N. H., de Jong, B., de Ley, L., Uges, D. R. A., Fichtinger-Schepman, A. M. J., Scheper, R. J., and Vries, E. G. E. Characterization of a human small cell lung carcinoma cell line with acquired resistance

to *cis*-diamminedichloroplatinum(II) *in vitro*. *Cancer Res.*, **48**, 6803–6807 (1988).
29) Andrews, P. A., Jones, J. A., Varki, N. M. and Howell, S. B. Rapid emergence of acquired *cis*-diamminedichloroplatinum(II) resistance in an *in vivo* model of human ovarian carcinoma. *Cancer Commun.*, **2**, 93–100 (1990).
30) Wand, W. R. Differential uptake of *cis*-diamminedichloroplatinum(II) by sensitive and resistant murine L1210 leukemia cells. *Cancer Res.*, **47**, 6549–6555 (1987).
31) Andrews, P. A., Mann, S. C., Velury, S. and Howell, S. B. Cisplatin uptake mediated cisplatin-resistance in human ovarian carcinoma cells. *In* "Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy," pp. 248–254 (1988). Martinus Nijhoff, Boston.
32) Mann, S. C., Andrews, P. A. and Howell, S. B. Short-term *cis*-diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Chemother. Pharmacol.*, **25**, 236–240 (1990).
33) Smith, E. and Brock, A. P. The effect of reduced osmolarity on platinum drug toxicity. *Br. J. Cancer*, **59**, 873–875 (1989).
34) Behrens, B. C., Hamilton, T. C., Masuda, H., Grotzinger, K. R., Whang-Peng, J., Loui, K. G., Knutsen, T., McKoy, W. M., Young, R. C. and Ozols, R. F. Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res.*, **47**, 414–418 (1987).
35) Kuppen, P. J. K., Schuitemaker, H., van't Veer, L. J., de Bruijn, E. A., van Oosterom, A. T. and Schrier, P. I. *cis*-Diamminedichloroplatinum(II)-resistant sublines derived from two human ovarian tumor cell lines. *Cancer Res.*, **48**, 3355–3359 (1988).
36) Kikuchi, Y., Iwano, I. and Miyauchi, M. Reduced cisplatin-uptake by cisplatin-resistant human ovarian cancer cells. *Gan To Kagaku Ryoho*, **15**, 2896–2898 (1988) (in Japanese).
37) Fram, R. J., Woda, B. A., Wilson, J. M. and Robichaud, N. Characterization of acquired resistance to *cis*-diamminedichloroplatinum(II) in BE human colon carcinoma cells. *Cancer Res.*, **50**, 72–77 (1990).
38) Kraker, A. J. and Moore, C. W. Accumulation of *cis*-diamminedichloroplatinum(II) and platinum analogues by platinum-resistant murine leukemia cells *in vitro*. *Cancer Res.*, **48**, 9–13 (1988).
39) Gibbons, G. R., Page, J. D., Mauldin, S. K., Husain, I. and Chaney, S. G. Role of carrier ligand in platinum resistance in L1210 cells. *Cancer Res.*, **50**, 6497–6501 (1990).
40) Panneerselvam, M. and Rahman, A. Molecular mechanisms of resistance of human ovarian cancer cells to cisplatin and tetraplatin. *Proc. Am. Assoc. Cancer Res.*, **30**, 523 (1989).
41) Jennerwein, M. M., Eastman, A. and Khokhar, A. Characterization of adducts produced in DNA by isomeric 1,2-diaminocyclohexaneplatinum(II) complexes. *Chem.-Biol. Interact.*, **70**, 39–49 (1989).
42) Page, J. D., Husain, I., Sancar, A. and Chaney, S. G. Effect of the diaminocyclohexane carrier ligand on platinum adduct formation, repair and lethality. *Biochemistry*, **29**, 1016–1024 (1990).