Establishment and Characterization of Acquired Resistance to Platinum Anticancer Drugs in Human Ovarian Carcinoma Cells

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To investigate differences in resistance to *cis*-diamminedichloroplatinum(II) (CDDP) and diammine (1,1-cyclobutanecarboxylato)platinum(II) (CBDCA), and their newly developed derivative, ((-)-(R)-2-aminomethylpyrrolidine (1,1-cyclobutanedicarboxylato)platinum(II)) (DWA2114R), four types of resistant cell lines were established from a parental cell line (NOS2) of a serous cystadenocarcinoma of the ovary. The cross-resistance of CDDP-resistant cells (NOS2CR1 and NOS2CR2) to DWA2114R was slight (only 25% of the resistance to CDDP), and no cross-resistance was observed to anticancer drugs other than the CDDP derivative, except to camptothecin (CPT-11) in the case of NOS2CR2 cells. The cross-resistance of CBDCA-resistant cells (NOS2CBR) to DWA2114R was slight (only about 1/3 of the resistance to CBDCA), and no cross-resistance was observed among anticancer drugs other than the CDDP derivative. On the other hand, DWA2114R-resistant cells (NOS2DR) showed a high cross-resistance to CDDP, CBDCA, etoposide (VP-16), and CPT-11. Intracellular accumulations of CDDP and CBDCA were markedly reduced in NOS2CR1, NOS2CR2, and NOS2CBR cells compared to those in NOS2 cells, but were reduced only slightly in NOS2DR cells. Intracellular accumulation of DWA2114R was reduced somewhat in the four types of resistant cells. Glutathione S-transferase activity was not increased in any of the four types of resistant cells, and intracellular GSH concentration was increased only in NOS2CR2 cells (by 2.6 fold). From these results, we consider that the resistance mechanisms against CDDP and CBDCA are similar, and reduction of intracellular drug accumulations is a significant factor. Resistance to DWA2114R differed from resistance to CDDP and CBDCA in both cross-resistance spectrum and resistance mechanism, indicating that reduction in intracellular drug accumulation is not the major resistance mechanism.

Key words: Cisplatin — DWA2114R — Ovarian cancer — Drug resistance — Glutathione S-transferase

cis-Diamminedichloroplatinum(II) (CDDP) is the most useful anticancer drug used in chemotherapy for ovarian carcinoma, and the response rate in ovarian carcinomas has been improved about 50% by combination chemotherapies which include CDDP (I). However, the development of drug resistance has placed limits on the usefulness of CDDP in treatments, and the long-term survival rate has not improved.^{1,2)} There is the further problem that dosage levels are limited by side effects such as neurotoxicity and nephrotoxicity.

Several mechanisms are believed to be involved in resistance to CDDP. Firstly, a reduction of intracellular CDDP accumulation occurs in the majority of resistant cells.^{3,4)} Secondly, increases in intracellular glutathione (GSH) concentration and glutathione S-transferase (GST) activity have been reported.^{5,6)} GSH and GST are believed to play important roles in drug detoxification.

Thirdly, increased removal of DNA-bound CDDP has been reported.^{7,8)} Based on an improved understanding of the possible resistance mechanisms, several approaches have been tried to overcome resistance, but since the resistance mechanisms are multifactorial and differ for each cell line, these attempts have met with limited success.^{9–11)}

Several CDDP derivatives have been developed recently, and some are being used clinically. Since diammine (1,1-cyclobutanecarboxylato)platinum(II) (CBDCA) has the same clinical response rate as CDDP, with characteristically fewer side effects such as vomiting and nephrotoxicity,^{12,13)} this drug is increasingly being used as a substitute for CDDP. The new CDDP derivative, ((-)-(R)-2-aminomethylpyrrolidine (1,1-cyclobutanedicarboxylato)platinum(II) (DWA2114R), has been shown to have the same antitumor effectiveness as CDDP in *in vivo* tests, and cross-resistance is low in CDDP-resistant cells, so that it is expected to be effective in patients that are CDDP-resistant.^{14,15)}

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Therefore, further fundamental study of the resistance mechanisms to platinum drug is required. We describe here the resistance mechanisms to CDDP, CBDCA, and DWA2114R and the mode of cross-resistance in ovarian carcinoma cells and corresponding resistant cells.

MATERIALS AND METHODS

Drug CDDP, CBDCA, and VP-16 were provided by Bristol K.K., and DWA2114R was provided by Chugai Seiyaku K.K. Fig. 1 shows the structural formulae of CDDP, CBDCA, and DWA2114R. The DWA2114R "leaving group" is the same as that of CBDCA; DWA2114R is characteristically a carrier ligand, and its molecular weight is 455, 1.5-fold greater than that of CDDP. Adriamycin (ADM), mitomycin C (MMC), and 5-fluorouracil (5-FU) were supplied by Kyowa Hakko K.K., SN-38 (active form of camptothecin (CPT-11)) was supplied by Yakult K.K., and MTT reagent, deoxycytidine, and DMSO (dimethyl sulfoxide) were purchased from Sigma, Inc.

Cells The NOS2 cells were collected from a patient with serous cystadenocarcinoma of the ovary. By continuous exposure to CDDP at slowly escalating concentration (from 0.2 $\mu\text{g/ml}$ to 2.0 $\mu\text{g/ml}$) for 18 months, these cells acquired CDDP resistance and were designated NOS2CR1. The resistance of NOS2CR1 cells was about 7-fold greater than that of NOS2 cells. NOS2CR1 cells exposed to high concentrations of CDDP (from 3.0 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$) for 12 months were designated NOS2CR2 cells, and their resistance was 26-fold greater. NOS2CBR cells were exposed to gradually increasing CBDCA (from 1.0 $\mu\text{g/ml}$ to 25 $\mu\text{g/ml}$) over 18 months

to produce CBDCA-resistant cells whose resistance was about 8-fold greater. NOS2DR cells were likewise exposed to gradually increasing concentrations of DWA2114R (from 2.0 $\mu\text{g/ml}$ to 50 $\mu\text{g/ml}$) over 18 months to produce DWA2114R-resistant cells whose resistance was enhanced 6-fold. These cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cultures were equilibrated with humidified 5% CO_2 in air at 37°C.

MTT assay Sensitivity to anticancer drugs was measured by MTT assay as described in our previous report.¹⁶⁾ Cells were cultured for 4 days in a medium containing drugs at various concentrations. After the addition of tetrazolium (MTT), incubation was continued for a further 4 h. The supernatant was aspirated, and the resulting pigment was dissolved in dimethyl sulfoxide. The absorbance was measured at 550 nm with an easy plate reader (MPR-A4i, Toso). Each point was the mean of six or more values, and the resistance factor was calculated by dividing the IC_{50} value for resistant cells by the IC_{50} value for NOS2 cells.

Measurement of platinum accumulation Exponentially growing cells were incubated in freshly changed RPMI 1640 medium containing 10% fetal calf serum and a platinum anticancer drug at various concentrations. Cells were washed with PBS twice, collected and stored at -20°C until use. Intracellular platinum accumulation was measured by a modification of the method of Pera and Harder.¹⁷⁾ Cell pellets were mixed with concentrated HNO_3 , then evaporated until dry. Each sample was dissolved in 0.1 N HNO_3 and platinum was determined by flameless atomic absorption spectrophotometry (AA-8500 MKII, Nippon Jarrell-Ash Co., Ltd.). The intracellular platinum content was normalized to the cellular protein content estimated by using a BCA protein assay kit.

GSH assay Cellular GSH content was determined in cells grown under conditions identical to those used for the assay of cytotoxicity. The assay for total GSH was conducted by the enzyme-recycling method under the conditions described by Tietze.¹⁸⁾

Measurement of GST activity Cells were washed with phosphate-buffered saline (PBS) three times and suspended in 50 mM sodium phosphate buffer (pH 6.5). The cell suspensions were sonicated on ice (Sonifier 200; Branson, Inc.), then centrifuged at 50,000g for 30 min. GST activity was measured in the supernatant fractions according to the method of Habig *et al.*, using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as the electrophilic substrate.¹⁹⁾ The GST activity was expressed as nmol of GSH-CDNB conjugate formed per min per mg protein. The results are presented as the mean of four individual experiments.

Western blotting Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed

Fig. 1. Chemical structures of platinum anticancer drugs.

according to the method of Schagger and von Jagow.²⁰⁾ Western blots were carried out according to the method described by Towbin *et al.*²¹⁾ Cytosol protein fractions were run on 15% SDS-PAGE (40 mg protein per lane), and were electrophoretically transferred onto nitrocellulose filters. These filters were incubated with antibody against human GST- π for 1 h at room temperature, then treated with biotinylated secondary antibody, and developed according to the manufacturer's specifications (Vectastain ABC kit; Vector Laboratories, Inc.).

RESULTS

NOS2, NOS2CR1, NOS2CR2, NOS2CBR, and NOS2DR cells were grown as monolayers in flasks; no morphological changes were observed. The IC_{50} values of each anticancer drug in the 5 cell types are shown in Table I. NOS2CR1 cells exhibited a cross-resistance of 3-fold for CBDCA and 1.6-fold for DWA2114R, but no cross-resistance was observed to other drug. NOS2CR2 cells expressed cross-resistances of 19-fold for CBDCA, 7.1-fold for DWA2114R, and 1.6-fold for CPT-11. The NOS2CBR cells had cross-resistances of 5.7-fold for CDDP and 2.8-fold for DWA2114R. NOS2DR had cross-resistance of 6.9-fold for CDDP, 5.4-fold for CBDCA, 4.3-fold for VP-16, and 5.6-fold for CPT-11. These findings indicate that NOS2DR has a strong cross-resistance to VP-16 and CPT-11 as well as to platinum drugs, unlike NOS2CR and NOS2CBR. Although strong cross-resistances were found for CDDP and CBDCA, the NOS2CR and NOS2CBR cells had only slight cross-resistances to DWA2114R.

Intracellular platinum accumulation of each cell type was measured by atomic absorption spectrophotometry after 2 h exposure to various concentrations of CDDP, CBDCA, and DWA2114R (Fig. 2). Intracellular platinum accumulation increased dose-dependently in each cell type after 2 h exposure to CDDP at concentrations

Fig. 2. Intracellular platinum accumulations of CDDP (A), CBDCA (B), and DWA2114R (C) after 2 h exposure to the respective drugs at various concentrations in NOS2 (■), NOS2CR1 (▨), NOS2CR2 (▩), NOS2CBR (▧), NOS2DR (□), as measured by atomic absorption. Points are the mean values for 4 independent experiments and bars indicate the SD.

Table I. Sensitivity of NOS2, NOS2CR1, NOS2CR2, NOS2CBR, and NOS2DR to Anticancer Drugs

	IC_{50} (μ g/ml)				
	NOS2	NOS2CR1	NOS2CR2	NOS2CBR	NOS2DR
CDDP	1.6	11.2 (7.0)	41.0 (25.6)	9.1 (5.7)	11.0 (6.9)
CBDCA	28.0	82.8 (3.0)	524 (18.7)	224 (8.0)	151 (5.4)
DWA2114R	23.0	36.8 (1.6)	163 (7.1)	64.4 (2.8)	138 (6.0)
Adriamycin	0.33	0.29 (0.9)	0.26 (0.8)	0.38 (1.2)	0.35 (1.1)
Etoposide	1.2	1.2 (1.2)	1.3 (1.1)	1.3 (1.1)	5.0 (4.2)
Camptothecin	0.010	0.012 (1.2)	0.016 (1.6)	0.012 (1.2)	0.056 (5.6)
Mitomycin C	0.11	0.11 (1.0)	0.11 (1.0)	0.11 (1.0)	0.1 (0.9)
5-Fluorouracil	1.0	1.0 (1.0)	0.9 (0.9)	1.2 (1.2)	0.9 (0.9)

Values in parenthesis are resistance factors, defined as IC_{50} of the respective cells/ IC_{50} of NOS2 cells.

Table II. Glutathione and GST Activity in NOS2 and Resistant Cells

	Total glutathione ($\mu\text{g}/\text{mg}$ protein)	Fold difference	GST activity (mU/mg protein)	Fold difference
NOS2	1.62 ± 0.51^a		137.0 ± 2.8^a	
NOS2CR1	1.30 ± 0.21	0.8	100.9 ± 2.2	0.74
NOS2CR2	4.14 ± 1.4	2.6	ND	0
NOS2CBR	1.55 ± 0.46	0.96	86.1 ± 2.3	0.63
NOS2DR	1.94 ± 0.32	1.2	ND	0

a) Mean $\pm$ SD; $n > 3$. ND: not detectable.

ranging from 100 to 300 μM , and decreased to 25, 26, and 26% of that of NOS2 cells, respectively, in NOS2CR1, NOS2CR2, and NOS2CBR cells at 300 μM concentration. However, the platinum accumulation only decreased to 63% in NOS2DR cells. Intracellular platinum accumulation also increased dose-dependently in each cell type after 2 h exposure to CBDCA at concentrations ranging from 500 to 2,000 μM , and decreased to 58, 38, and 30% of that of NOS2 cells, respectively, in NOS2CR1, NOS2CR2, and NOS2CBR cells at 2,000 μM concentration. NOS2DR cells, however, showed only a slight decrease to 68% of that of NOS2 cells. Intracellular platinum accumulation increased dose-dependently in each cell type after 2 h exposure to DWA2114R at concentrations ranging from 500 to 2,000 μM , but the rate of reduction in the cases of CDDP and CBDCA was comparatively slight in all resistant cells. NOS2DR cells had an intracellular accumulation of 72% at 2,000 μM , compared to NOS2 cells.

Although NOS2CR1, NOS2CR2, and NOS2CBR cells exhibited similar trends of reducing intracellular drug accumulations, the reductions in CDDP, CBDCA, and DWA2114R drug accumulations were only slight in NOS2DR cells, so that they might be considered as different from the other resistant cells. The reduction in intracellular drug accumulations does appear to be an important mechanism of CDDP resistance and CBDCA resistance. However, NOS2DR cells showed only a slight decrease in intracellular platinum drug accumulations, and the participation of other resistance mechanisms must be considered.

Intracellular GSH concentration in NOS2CR2 cells was increased 2.6-fold compared to that of NOS2 cells, but no such increase was observed in NOS2CR1, NOS2CBR, or NOS2DR cells (Table II). Among the four types of resistant cells, none exhibited an increase in GST activity compared to NOS2 cells. GST activity was particularly poor in NOS2CR2 and NOS2DR cells by our assay methods. Western blotting with anti-GST- π antibody showed a clear band of about 22 kDa in NOS2, NOS2CR1, and NOS2CBR cells but only a weak band was observed in NOS2CR2 and NOS2DR cells (Fig. 3).

Fig. 3. Western blot analysis of GST π in 5 types of cells. Molecular markers are indicated to the left. WT, NOS2; CR1, NOS2CR1; CR2, NOS2CR2; CBR, NOS2CBR; DR, NOS2DR.

Thus, GST activity is thought to make only a minimal contribution, if any, to the resistance in those 4 types of resistant cells.

DISCUSSION

Cross-resistance to both CBDCA and DWA2114R was observed in NOS2CR1 cells and NOS2CR2 cells. However, cross-resistance to DWA2114R was low in these cells compared to CDDP-resistance, although there was a strong cross-resistance to CBDCA. It has been reported that P388/DDP cells derived from leukemia cells P388 and having a 75-fold resistance to DWA2114R, exhibit a high 100-fold cross-resistance to CBDCA.¹⁵⁾ CBDCA cross-resistance in CDDP-resistant cells has been reported for a majority of cell types, and it is likely that the resistance mechanism is the same for both CDDP and CBDCA.^{5, 22, 23)}

NOS2CBR cells exhibited cross-resistance to CDDP and DWA2114R, but the degree of resistance was low for DWA2114R compared to that for CBDCA. This tendency is similar to that observed for NOS2CR1 and NOS2CR2 cells. Cross-resistance to anticancer drugs other than platinum anticancer drugs was not evident in NOS2CR1 cells and NOS2CBR cells. NOS2CR2 cells exhibited a weak cross-resistance to CPT-11 (1.6-fold), suggesting the possibility of additional new resistance mechanisms coincidental with the increase in CDDP resistance. It has been reported that among two types of CDDP-resistant cells, L1210PtR4 cells (13.5-fold resistance) and L1210DDP5 cells (25.8-fold resistance) derived from mouse leukemia L1210 cells, only the latter type exhibits a weak resistance (1.4-fold) to ADM.²⁴⁾ This again suggests the possible development of a new cross-resistance mechanism when CDDP resistance increases. NOS2DR cells showed a strong cross-resistance to VP-16 and CPT-11 (90% and 115%, respectively), and, from the mode of this cross-resistance, it is clear the NOS2DR cells are different in character from NOS2CR and NOS2CBR cells.

Changes in intracellular platinum accumulation showed the same trend in NOS2CR1, NOS2CR2, and NOS2CBR cells. The reduction in intracellular accumulations of CDDP and CBDCA is thought to be an important factor indicating that CDDP-resistant cells and CBDCA-resistant cells are mutually cross-resistant. The degree of resistance of NOS2CR2 cells to CDDP and the degree of their cross-resistance to CBDCA were increased compared to those of NOS2CR1 cells, but the decrease in intracellular drug concentrations is almost the same, suggesting that other additional resistance mechanisms may be operating coincidentally with the increase in resistance. The slight reduction in intracellular DWA2114R accumulation in NOS2CR1 and NOS2CBR cells may be the reason why only slight cross-resistance to DWA2114R was observed in these cells. P388/DDP cells exhibited a reduction of intracellular platinum accumulation to 38% when exposed to CDDP, and a reduction to 42% when exposed to CBDCA, but no reduction was observed when they were exposed to DWA2114R. Cross-resistance to DWA2108R, whose "leaving group" changes to chloric residues in the same

way as that of CDDP, was similar to that to CDDP; intracellular platinum accumulation was reduced to 50%.¹⁵⁾

NOS2DR cells showed a cross-resistance to CDDP and CBDCA to the same degree as DWA2114R, but the reduction in intracellular platinum accumulation when the cells were exposed to CDDP and CBDCA was slight compared to those of NOS2CR1, NOS2CR2, and NOS2CBR cells. That is, there is little possibility that cross-resistance in NOS2DR cells arises from a reduction in intracellular drug accumulation. The intracellular platinum accumulation in NOS2DR cells exposed to DWA2114R was 72% of that of NOS2 cells. It is therefore likely that the reduction in intracellular drug accumulation is not the main mechanism of resistance to DWA2114R.

Increases of GSH and GST have been reported in many CDDP-resistant cell lines, but other authors have found no change in GSH and GST.^{22, 23, 25, 26)} Among GST isozymes, GST- π is considered to have a central role in anticancer drug detoxification, but its participation in CDDP resistance was considered doubtful on the basis of genetic experiments, and even if it does participate, such participation is inferred to be minimal.^{27, 28)} In the present study, an increase in GSH was observed only in NOS2CR2 cells. Conversely, GST activity was decreased in NOS2CR2 and NOS2DR cells, suggesting that the GSH detoxification mechanism may not participate as a resistance mechanism in the resistant cells used in this study. Thus, an additional CDDP resistance mechanism in NOS2CR2 cells might be an increase in removal of DNA-bound CDDP, since the decrease in intracellular CDDP concentration is at almost the same level.

The number of patients treated with CDDP derivatives is expected to increase. It is therefore important that DWA2114R's main resistance mechanism differs from that of CDDP, and because DWA2114R suffers only slight cross-resistance in CDDP-resistant cells, it can be expected to be useful in patients who have developed CDDP resistance. The possibility must also be borne in mind that CDDP, CBDCA, VP-16, and CPT-11 will be ineffective in patients who have developed DWA2114R resistance.

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