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Characterization of Acquired Resistance to *cis*-Diamminedichloroplatinum(II) in Mouse Leukemia Cell Lines

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We have established *in vivo* cisplatin-resistant mouse leukemia cell lines, L-1210/DDP and P388/DDP, in order to elucidate the mechanism of acquired resistance to cisplatin. Resistance indices were 22 and 14, respectively, when the cells were exposed to cisplatin for 48 h. Uptake of cisplatin by both resistant lines was significantly reduced, compared with values for the respective parent lines (17% for L-1210/DDP and 27% for P388/DDP, at 100 μM for 1 h). While glutathione contents in the resistant cells were 1.7–1.9 times higher than those in the sensitive ones, their reduction by preincubation with buthionine sulfoximine did not influence the sensitivity of the cells to cisplatin. In addition, the resistant lines did not show lower sensitivity to $CdCl_2$ than the respective sensitive ones, suggesting that intracellular SH groups might contribute little to the mechanism of cisplatin resistance in these cells. Postincubation with DNA repair inhibitors, caffeine and aphidicolon, did not selectively enhance the sensitivity of the resistant cells to cisplatin. These results suggested that reduced drug uptake would be a primary mechanism of cisplatin resistance in L-1210/DDP and P388/DDP. Cross-resistance patterns to platinum complexes were quite different between L-1210/DDP and P388/DDP. Colon 26/DDP, another cisplatin-resistant mouse tumor showed a different pattern from those observed with L-1210/DDP and P388/DDP. In the development of new platinum complexes we should use plural resistant lines for examining cross-resistance patterns to candidate platinum complexes.

Key word: Cisplatin resistance — L-1210 — P388 — Cisplatin uptake — Cross-resistance

Cisplatin (DDP) is one of the most prominent antitumor agents for the treatment of various solid cancers.[1,2] Severe nephrotoxicity associated with DDP treatment has been ameliorated in most of the second-generation platinum complexes.[3,4] Even with them, however, it is difficult to overcome acquired resistance after repeated treatment, which is another problem encountered in DDP therapy. A number of reports concerning the mechanism of cisplatin resistance have merely revealed its complexity; there does not seem to be a single mechanism common to almost all resistant lines, but rather several factors might influence resistance to this drug.[5–9] Possible mechanisms are reduced drug uptake, increased intracellular sulfhydryl groups such as glutathione and metallothionein, decreased cross-linking with DNA, enhanced DNA repair, and so on.

We have developed DDP-resistant sublines of mouse leukemias L-1210 and P388[10] and a mouse colon adenocarcinoma Colon 26, to help clarify the mechanism of cisplatin resistance and to devise means to overcome the resistance.

Platinum complexes consist of chemically stable carrier ligands and susceptible leaving groups. Retrospective study of the characteristics of various platinum complexes revealed that the latter generally defined the solubility and toxicity of complexes, whereas their antitumor activities and antitumor spectra were rather influenced by the former.[11–13] Therefore, we tried to search for carrier ligands to which DDP-resistant lines would lack cross-resistance in the hope that a derivative containing such a carrier ligand might escape cisplatin resistance.

MATERIALS AND METHODS

Tumor cells Mice bearing leukemias L-1210 and P388 and a colon adenocarcinoma Colon 26 were kindly supplied by the National Cancer Institute, Bethesda, MD and were kept by serial transplantation in the respective syngeneic mice, purchased from Charles River Japan, Inc., Atsugi, Kanagawa. DDP-resistant sublines of L-1210 and P388 (L-1210/DDP and P388/DDP) were established by intraperitoneal injections of a single dose of DDP (6–8 mg/kg) given 2 days after the passage of 10^6 cells over successive generations in $CD2F_1$ mice.[10] The treatments with DDP were continued for 230 and 80 generations, respectively. A DDP-resistant subline of Colon 26 (Colon 26/DDP) was established *in vitro* by repeated 1-h exposure to DDP 1 day after each subculture, the concentration being gradually increased to

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300 μM. Six months later, cloning of the cells was carried out.

Platinum complexes Platinum complexes used in the experiment were kindly supplied by Prof. Kidani, Nagoya City University, and Dr. Miyamoto, University of Tokyo. The chemical structures of the carrier ligands are presented in Fig. 2. Most of the leaving groups are chloride ions and the others are shown under the respective chemical structures in parentheses.

Determination of drug sensitivity *in vitro* L-1210 and P388 cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) and 100 μg/ml kanamycin. When DDP-resistant cells were cultured, 5 μM 2-hydroxyethyldisulfide (Aldrich Chemical Co., Inc., Milwaukee, MI) was added to the culture medium. Exponentially growing leukemic cells (5×10^4 cells/ml) were incubated with various concentrations of platinum complexes for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. Cells were counted using a ZBI Coulter counter and the concentration of a complex which produced 50% inhibition of cell growth (IC_{50}) was determined by plotting T/C% vs. drug concentration.

For mouse Colon 26 cells, Eagle's MEM supplemented with 10% FBS and 100 μg/ml kanamycin was used. Cells were plated into 96-well microtiter plates at 5,000 cells/well in 100 μl of the medium and were incubated at 37°C. Twenty-four h after plating, various concentrations of complexes in 100 μl were added to each well and incubated for 72 h. After an addition of 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/ml) to each well, the plate was further incubated for 4 h. The medium was removed by aspiration and the MTT formazan formed was dissolved in 150 μl of DMSO. The optical density at 540 nm of each well was determined using a Microplate Reader (Model 2550, Bio Rad Lab., Tokyo).

Uptake of cisplatin DDP-sensitive and -resistant leukemic cells were harvested from the peritoneal cavity of tumor-bearing $CD2F_1$ mice. If necessary, erythrocytes were removed by gentle centrifugation at 1000 rpm for 1 min, and the leukemic cells were washed twice with RPMI 1640 medium. One hundred million cells were suspended in 50 ml of the medium containing an appropriate concentration of DDP and incubated at 37°C under gentle agitation for a specified time. The cells were washed twice with cold PBS and digested with 3 ml of concentrated HNO_3 at 80°C in a water bath for 5 h. After cooling, the aqueous phase was transferred to another tube, and neutralized with 3.5 ml of 40% NaOH and 2 ml of 25% Na_2CO_3 to pH 7–9. Three hundred mg of sodium diethyldithiocarbamate (DDTC) was added and the solution was heated at 80°C for 1 h. Pt $(DDTC)_2$ formed was extracted three times with chloroform, evaporated and dissolved in 0.5 ml of methanol. Pt contents was determined at 269.5 nm by using a Hitachi atomic absorption spectrometer (Z-8000).

Determination and modulation of intracellular glutathione Intracellular glutathione contents were determined by Suzukake's modification of Tietze's method.[14, 15] Briefly, the assay was based on the measurement of the change of absorbancy at 412 nm following an incubation of protein-free supernatant of cell lysate in the presence of NADPH, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and glutathione reductase at 30°C and pH 8.0. Modulation of intracellular glutathione levels was carried out by preincubation of cells for 24 h with 50 μM D,L-buthionine sulfoximine (BSO).

DNA repair inhibitors Caffeine and aphidicolin were purchased from Sigma Chemical Co., St. Louis, Mo. The concentrations of inhibitors were chosen so as not to inhibit the growth of the cells by more than 20% alone. Cells were exposed to DDP for 1 h and changes in IC_{50} values were examined by adding the inhibitors to post-incubation medium. When caffeine was used, after washing with cold PBS, cells were incubated for 48 h in the culture medium with or without 1 mM caffeine. In the experiment with aphidicolin, cells were grown to confluence, then the culture medium was replaced with arginine-deficient RPMI 1640 containing 5% dialyzed FBS to minimize semiconservative DNA synthesis.[16] Twenty-four h later, cells ($1\sim3\times10^5$ cells/ml) were exposed to DDP for 1 h. After being washed with PBS, the cells were incubated with or without 4 μM aphidicolin for 72 h. For the first 24 h postincubation, arginine was removed from the medium, followed by incubation in the complete medium for a further 48 h.

RESULTS

Uptake of DDP by mouse leukemia cells Fig. 1 shows the uptake of DDP by DDP-sensitive and -resistant leukemia cells, when exposed to 100 μM drug. Both L-1210 and P388 lines (Fig. 1a and 1b, respectively) accumulated DDP (determined as Pt) almost linearly for at least 60 min. The Pt contents in both resistant lines were significantly low, compared with the sensitive lines. The uptake ratios of DDP by the resistant lines to the respective sensitive ones are shown in Table I. At 100 μM the uptake ratios by L-1210/DDP and P388/DDP were 16.6 and 27.1%, respectively.

Intracellular glutathione contents of mouse leukemia cells Glutathione contents of DDP-sensitive and -resistant mouse leukemia cells are listed in Table II. L-1210/DDP and P388/DDP contained 1.7 and 1.9 times more glutathione than the respective sensitive cells. Incubation of the resistant cells with 50 μM BSO for 24 h reduced the glutathione contents to 11.7 and 7.6%, respectively.

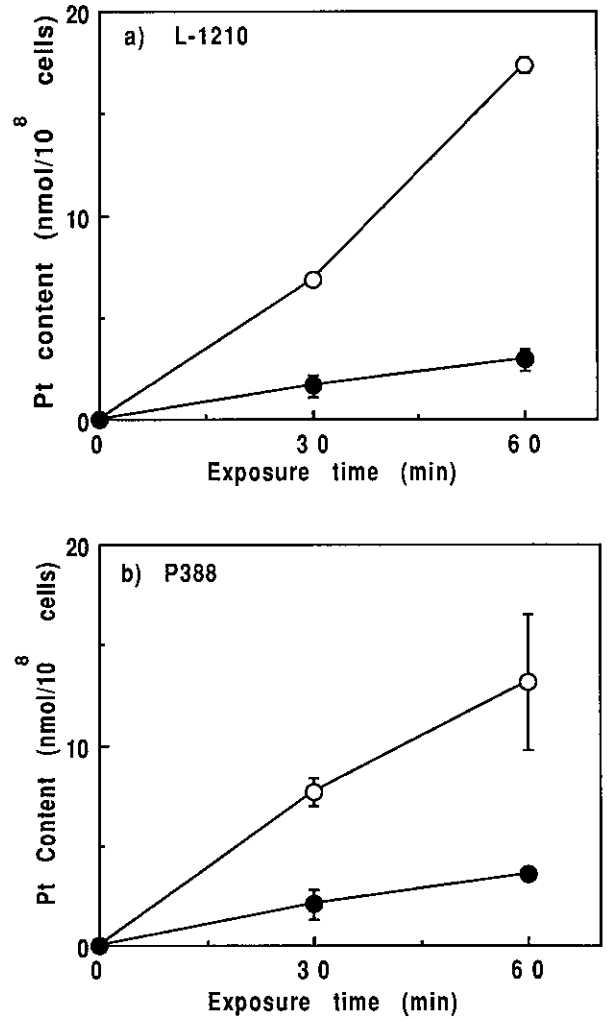

Fig. 1. Uptake of DDP by mouse leukemia cells sensitive and resistant to DDP. Cells were exposed to 100 μM DDP at 37°C. a) L-1210, b) P388; (○), sensitive; (●), resistant line. Bars, SD.

Table I. Uptake of DDP by Mouse Leukemia Cells Sensitive and Resistant to DDP

Tumor	DDP concentration[a] (μM)	Pt uptake (nmol/10^8 cells)		R/S (%)
		Sensitive (S)	Resistant (R)	
L-1210	100	17.40±0.38[b]	2.89±0.559	16.6
	25	1.95±0	<0.33	<17
	10	0.98±0.27	<0.33	<34
P388	100	13.16±3.39	3.57±0.231	27.1
	25	3.08±0.93	1.28±0.356	42.0
	10	0.77±0.15	<0.33	<43

a) Cells were exposed to DDP for 1 h.
b) Mean±SD.

Table II. Contribution of Intracellular Glutathione Contents to DDP Resistance in Mouse Leukemia Cells

Cell line	Glutathione content (nmol/10^8 cells)			IC_{50} (μM)	
	−BSO	+BSO	(%)[a]	−BSO	+BSO
L-1210	74.5±18.4			0.10	0.18
L-1210/DDP	138±18.6[b]	14.2±0.050	10.3	5.5	4.3
P388	108±10.4			0.17	0.16
P388/DDP	183±38.8[b]	13.7±0.037	7.6	5.8	4.3

a) +BSO/−BSO.
b) Significantly different from value of the sensitive line ($P<0.05$).

Table III. Sensitivity of Mouse Leukemia Cell Lines to $CdCl_2$

Tumor line	IC_{50} (μM)	Resistant/Sensitive (%)
L-1210	5.05±1.63[a]	
L-1210/DDP	4.31±0.84	85.3
P388	7.49±1.62	
P388/DDP	1.22±0.03	16.3

Cells were exposed to $CdCl_2$ for 48 h.
a) Mean±SD.

Table IV. Effect of Postincubation with Caffeine on the Sensitivity of DDP-Sensitive and -Resistant Leukemia Cells to DDP

Cell line	IC_{50} (μM)		
	(−) Caffeine	(+) Caffeine	(%)[a]
L-1210	12±4.2[b]	5.4±1.7[c]	45.0
L-1210/DDP	162±38	92±16[c]	56.8
P388	4.1±1.3	2.7±0.84	65.3
P388/DDP	140±33	80±29	57.1

After exposure to DDP for 1 h, cells were incubated with or without 1 mM caffeine for 48 h.
a) (+) Caffeine/(−) caffeine.
b) Mean±SD.
c) Significantly different from the value without caffeine ($P<0.05$).

However, the pretreatment with BSO had little effect on the IC_{50}s of DDP against the sensitive as well as resistant lines, suggesting that glutathione content is not correlated with cellular sensitivity to DDP.

Sensitivity of cells to $CdCl_2$ To estimate whether metallothionein contributes to the cisplatin resistance of these cell lines or not, the sensitivity to $CdCl_2$ was compared between sensitive and resistant lines. The results after exposure to $CdCl_2$ for 48 h are shown in Table III. The

Table V. Effect of Aphidicolin on Sensitivity of DDP-Sensitive and -Resistant Mouse Leukemia Cell Lines to DDP

Tumor	IC_{50} (μM)		
	(−) Aphidicolin	(+) Aphidicolin[a]	(%)[b]
L-1210	14±4.6[c]	9.0±2.8	62.9
L-1210/DDP	255±64	180±42	70.6
P388	9.2±1.2	5.5±0.84[d]	60.3
P388/DDP	270±71	243±74	90.0

a) After exposure to DDP for 1 h, cells were incubated with or without 4 μM aphidicolin for 72 h.
b) (+) Aphidicolin/(−) aphidicolin.
c) Mean±SD.
d) Significantly different from the value without aphidicolin ($P<0.05$).

Table VI. Resistance Indices of DDP-Resistant Mouse Tumor Lines to Various Platinum Complexes

JCI No. of Pt complex	Resistance index		
	L-1210/DDP	P388/DDP	Colon 26/DDP
DDP	22.3	13.9	16.2
729	11.7	33.1	11.0
1990	12.4	6.3	12.5
1914	1.6	5.0	5.9
1885	1.8	13.3	11.9
1639	2.3	34.9	8.0
4999	1.0	10.3	6.5
1499	1.9	47.8	3.9
2016	1.3	5.1	9.5
2416	1.9	59.0	4.8
1465	1.9	3.5	6.0
810	1.0	8.0	2.7
2745	0.8	8.2	3.8
3388	2.8	>60	4.6
4801	1.4	>30	3.7
3894	1.0	30.3	4.6
3890	3.4	46.5	3.3
4196	1.3	22.7	2.8

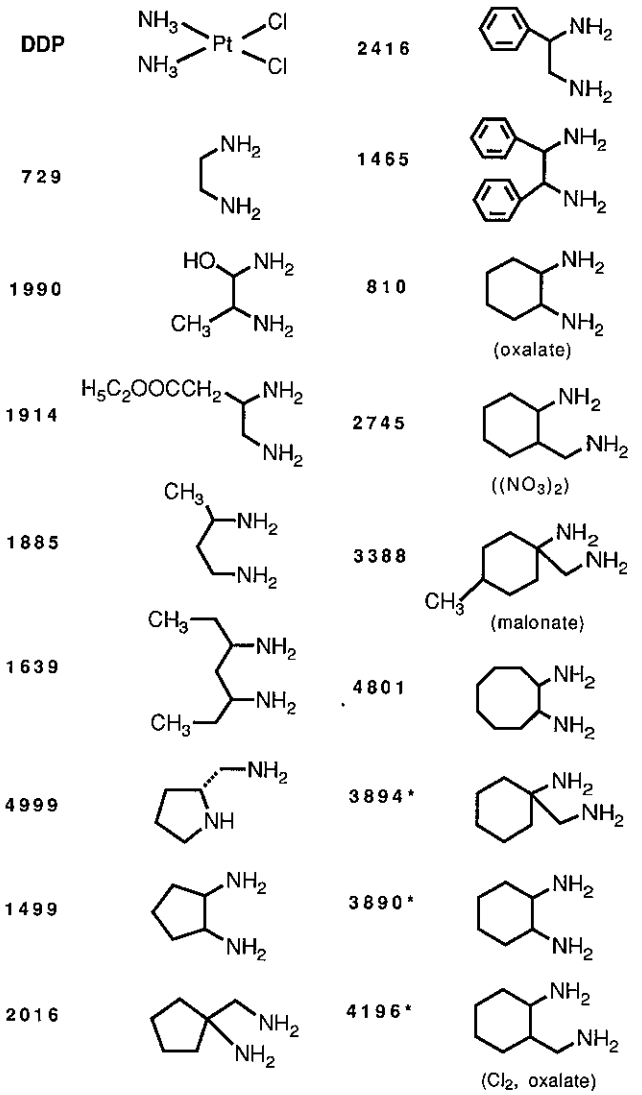

Fig. 2. Chemical structures of DDP and carrier ligands of its derivatives. Each chemical structure expresses the carrier ligand of a platinum complex, the registration number of which is indicated on the left side. Most of the leaving groups of complexes are chloride ions and the others are indicated under the respective structures in parentheses. Pt(IV) complexes are marked with an asterisk (★).

IC_{50}s to L-1210/DDP and P388/DDP were not higher than those to the sensitive lines and were 85 and 16% of those to sensitive L-1210 and P388, respectively.

Effect of postincubation with caffeine and aphidicolin on the sensitivity of cells to DDP In order to examine the possible role of enhanced DNA repair as a mechanism for DDP resistance, the effects of postincubation with DNA repair inhibitors, caffeine and aphidicolin, on the sensitivity to DDP were tested. The sensitivity of L-1210 and L-1210/DDP to DDP was significantly enhanced by postincubation with 1 mM caffeine (Table IV). In the experiment to examine the effect of aphidicolin, after 1-h exposure to DDP, cells were incubated for 72 h in the presence of 4 μM aphidicolin. As shown in Table V, a significant enhancement of growth inhibition was observed only in the sensitive P388 line.

Cross-resistance patterns of DDP-resistant mouse tumors to several platinum complexes In Table VI resistance indices of L-1210/DDP, P388/DDP and Colon 26/DDP

to several platinum complexes are compared. The carrier ligands are not the same (Fig. 2). The resistance indices (RI's) of these cell lines to the parent drug, DDP, were 22.3, 13.9 and 16.2, respectively. All resistant lines were clearly cross-resistant to JCI 729 and 1990. L-1210/DDP showed only a little cross-resistance to the other 15 derivatives and RI's to them were 0.8 to 3.4. On the other hand, P388/DDP was cross-resistant to all derivatives tested and RI's to some complexes were 50 to 60. As for Colon 26/DDP, resistance indices to the derivatives mostly lay between those of L-1210/DDP and P388/DDP, and the cells showed relatively higher resistance to aliphatic amine-carrier ligands (upper part in Table VI).

DISCUSSION

The mechanisms of cisplatin resistance have not yet been clearly elucidated, in part due to the difficulty in obtaining a stable radioisotope. Thus, comparison of the uptake of DDP between sensitive and resistant cells has to be carried out by atomic absorbance spectrophotometry, though its detection limit of Pt is not sufficiently good to permit analysis of the mechanism in detail. Several reports using murine and human resistant lines have indicated that the mechanisms of cisplatin resistance could be multifactorial and the degree of resistance is not determined by a single mechanism.[5-9] Indeed, even several resistant lines originating from the same tumor line often exhibited different characteristics.[9,17] Therefore, we have also established resistant lines of mouse leukemias, L-1210 and P388, and studied their resistance mechanisms.

A clear difference between sensitive and resistant lines of both L-1210 and P388 was the significant reduction of cisplatin uptake by the resistant lines, compared with the respective sensitive ones. Similar phenomena have been observed in many resistant lines, but what kind of intracellular changes would result in the reduction has not been elucidated.

The increase in intracellular sulfhydryl groups could contribute to DDP resistance, as DDP would be inactivated by reacting with them. Indeed, levels of glutathione, which is a primary intracellular non-protein SH, in L-1210/DDP and P388/DDP were 1.7 to 1.9 times higher than those in the respective parent lines. Pretreatment of the resistant cells with BSO, however, did not influence the cellular sensitivity to DDP, though after BSO treatment the glutathione contents in these cells decreased to levels below those in sensitive cells. In a DDP-resistant subline of human ovarian cancer, 2780^{CP}, Hamilton *et al.* observed the enhancement of the sensitivity to DDP after BSO treatment, though the enhancement was also seen in the parent line, A2780.[18] Since little difference in drug uptake between A2780 and 2780^{CP} was reported by Newman *et al.*,[19] a unique resistance mechanism might function in this resistant line.

Another sulfhydryl compound possibly relevant to DDP resistance is metallothionein, which is an SH-rich protein and plays a role in detoxifying heavy metals such as Cd and Hg. Metallothionein also binds Pt.[20] If metallothionein contents were increased in resistant sublines, the sensitivity of the cells to $CdCl_2$ would be lowered. Teicher *et al.* reported that the sensitivity of SCC 25/DDP to Cd was 2–2.5-fold less than that of sensitive SCC 25[5] and further, the metallothionein contents in several human resistant lines including the former were increased 2–5-fold.[21] As shown in Table III, however, IC_{50} values of $CdCl_2$ against L-1210/DDP and P388/DDP were not increased but rather decreased, suggesting that metallothionein could contribute little to the mechanisms of resistance of these cell lines.

Enhancement of DNA repair as a mechanism for DDP resistance was reported in DDP-resistant lines of L-1210,[22] A2780,[16] etc. We expected that if DNA repair was enhanced in the resistant cells, their sensitivity to DDP would be increased in the presence of DNA repair inhibitors. When L-1210/DDP and P388/DDP were incubated with caffeine[23] (Table IV) or aphidicolin[16] (Table V) after exposure to DDP, only small enhancements of the sensitivity to the drug were seen in both the sensitive and resistant lines. From these results, enhanced DNA repair does not appear to be the mechanism of DDP resistance.

In developing new derivatives of antitumor agents already clinically used, the degree of cross-resistance to a candidate derivative in tumor lines resistant to the parent agent is an important factor.[24] In an early stage of development of second-generation platinum complexes, L-1210/DDP showed a lack of cross-resistance to platinum complexes containing 1,2-cyclohexanediamine as a carrier ligand.[25] Thus, this subline has been often used as a model resistant tumor to examine cross-resistance to a new derivative. In our present experiment (Table VI) L-1210/DDP showed cross-resistance only to a few Pt complexes. On the other hand, P388/DDP exhibited resistance to all complexes tested, the resistance indices of some being more than 50. Both L-1210 and P388 are mouse leukemias and their resistant sublines were induced *in vivo* by similar treatments. Nevertheless their cross-resistance patterns to several platinum complexes were completely different. Colon 26/DDP, another DDP-resistant line of mouse tumor, also showed a different cross-resistance pattern from those observed with both L-1210/DDP and P388/DDP. Although the reason for the difference in cross-resistance patterns is not clear at present, one possibility is a contribution of another mechanism, in addition to reduced drug uptake, by which the mode of drug penetration through plasma

membrane of the cells or drug binding to DNA strands would be altered in each resistant line. These differences might hold the key to elucidation of the resistance mechanism.

Considering that the final targets of chemotherapy are not experimental tumors but human patients, we should reconsider whether L-1210/DDP would be the best model tumor to examine cross-resistance patterns to platinum complexes. Characteristics of human tumors would probably be diverse, suggesting that some of them might resemble L-1210, while others might resemble P388. Therefore, as many DDP-resistant cell lines as possible, and human lines, if possible, should be used for examining cross-resistance to candidate platinum complexes.

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