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Abrogated energy-dependent uptake of cisplatin in a cisplatin-resistant subline of the human ovarian cancer cell line IGROV-1

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Abstract The parental IGROV-1 human ovarian adenocarcinoma cell line was intermittently exposed to increasing concentrations of cisplatin to obtain resistant sublines. A stable resistant subline with a resistance factor of 8.4 had been developed after 9 months and 28 passages, which was denoted $IGROV_{CDDP}$. A high correlation coefficient of 0.97 was found between the log cell survival and the DNA-adduct peak level during the process of resistance development. $IGROV_{CDDP}$ was strongly cross-resistant to carboplatin and doxorubicin and moderately cross-resistant to etoposide, docetaxel, and topotecan. Only minor resistance against 5-fluorouracil was observed, whereas $IGROV_{CDDP}$ was not cross-resistant to methotrexate. Intracellular accumulation of cisplatin was 65% lower in $IGROV_{CDDP}$ as compared with parental IGROV-1 at 37 °C under normal conditions. Coincubation of cisplatin with the Na^+/K^+-ATPase inhibitor ouabain resulted in a more pronounced decrease in platinum accumulation in IGROV-1 (44% decrease) than in $IGROV_{CDDP}$ (26% decrease). Under energy-depleting conditions the accumulation of cisplatin in the parental cell line was approximately 60% lower than that observed under normal (energy [i.e., ATP] rich) culture conditions. In contrast, the accumulation in $IGROV_{CDDP}$ was not affected by ATP-depletion. There appeared to be no significant difference between the intracellular accumulation of platinum in the resistant and sensitive cells under conditions of energy deprivation or when the uptake was studied at 0 °C. In conclusion, abrogation of energy-dependent accumulation in $IGROV_{CDDP}$ seems to be a major mechanism of resistance to cisplatin in this cell line.

Key words Ovarian cancer · Cisplatin · Resistance · Cellular uptake · Energy depletion

Introduction

Cis-Diamminedichloroplatinum(II) (cisplatin) is one of the most widely applied anticancer agents and is particularly effective in the treatment of testicular and ovarian cancer [17]. A major limitation associated with the application of cisplatin is the existence or development of resistance. The cytotoxic activity of cisplatin is most closely correlated with covalent binding to DNA, so-called DNA-adduct formation [7]. Several mechanisms of resistance to cisplatin have been documented, for example, increased repair of the induced adducts, increased intracellular inactivation by glutathione and metallothioneins, increased efflux of cisplatin-glutathione complexes, decreased influx into the cell by not yet fully characterized alterations in active cell-membrane transport mechanisms, and overexpression of oncogenes or tumor-suppressor genes [10, 27, 28]. Resistance to cisplatin overlaps in many cases with resistance to other anticancer agents; hence, mechanisms of resistance are not unique for cisplatin [26]. Resistant cell lines of sensitive parental cell lines have in many cases been established by continuous exposure to the drug [9, 22, 31]. To

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simulate the clinical condition more closely, intermittent exposure seems to be more appropriate. This strategy was followed to develop a resistant cell line that could be used as a tool to study mechanisms of cisplatin resistance. In a preliminary report we described that glutathione levels in the resistant cell line were not increased and that the level of multidrug resistance (MDR)-related P-glycoprotein (P-gp) in resistant and parental cell lines were not significantly different [29]. The aim of the present study was to characterize further the mechanism of resistance to cisplatin in $IGROV_{CDDP}$.

Materials and methods

Chemicals

Platinum standard solution (500 ppm) was obtained from Baker (Deventer, The Netherlands). DNAse I (EC 3.1.21.1), sulforhodamine B(SRB), glucose-free RPMI 1640 medium, 2-deoxy-D-glucose, and sodium azide were supplied by Sigma (St. Louis, Mo., USA). Proteinase K was purchased from Merck (Darmstadt, Germany). RPMI 1640 was obtained from GibcoBRL (Life Technologies B.V., Breda, The Netherlands). Bovine calf serum (BCS) was supplied by Hyclone (Logan, Utah, USA). Cisplatin and methotrexate (MTX) were purchased from Lederle (Wolfratshausen, Germany). Carboplatin, paclitaxel (Taxol), and etoposide (VP-16) were purchased from Bristol Myers (Troisdorf, Germany). Doxorubicin (DOX) was obtained from Pharmacia (Brussels, Belgium), and 5-fluorouracil (5-FU) was supplied by Roche (Mijdrecht, The Netherlands). Ouabain was purchased from Pharmachemie (Haarlem, The Netherlands). Docetaxel (Taxotere) was kindly supplied by Rhône-Poulenc Rorer (Alfortville, France).

Instruments

A Perkin-Elmer 3030B atomic absorption spectrophotometer (AAS) equipped with an HGA600 flameless system and an AS 60 autosampler (Uberlingen, Germany) was applied for determination of intracellular cisplatin (measured as platinum) and cisplatin-DNA adducts. A Beckman DU62 UV spectrophotometer (Fullerton, Calif., USA) was used for spectrophotometric quantitation of DNA. A Perkin-Elmer LS-3B fluorescence spectrophotometer (Uberlingen, Germany) was used for the determination of DNA interstrand cross-links. Cell-cycle analyses were performed on a FACScan flow cytometer (Becton Dickinson, Etten-Leur, The Netherlands).

Cell lines and development of resistance

The sensitive IGROV-1 human ovarian cancer cell line was originated by Dr. J. Benard et al. [6]. Cells were cultured in RPMI 1640 medium with HEPES and phenol red supplemented with 10% BCS, 10 m*M* $NaHCO_3$, 2 m*M* glutamine, gentamycin (45 μg/ml), penicillin (110 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells were frequently monitored for mycoplasma contamination.

The cells were exposed to cisplatin for 1 h once a week or once every 2 weeks, depending on the recovery. The treated cells were cultured in fresh medium until the surviving cells had recovered and showed a normal exponential growth rate. The incubation concentration was gradually increased from 3.33 μ*M* (1 μg/ml, IC_{90} of the sensitive cell line) to 33.3 μ*M*. After 20 passages the cells were split and a part was repeatedly exposed to the concentration of 33.3 μ*M*, whereas the other part was exposed to further increasing concentrations of cisplatin. The resistant cell line, which was developed after 28 passages, was denoted $IGROV_{CDDP}$.

Cloning efficacy

The colony formation was determined after plating of 1×10^3 exponentially growing cells of the sensitive and resistant cell lines. Colonies were counted at 10–14 days after the start of the experiment. The experiment was carried out four times in duplicate.

Assessment of cytotoxicity

Cytotoxicity was assessed using the SRB assay as previously described [24]. By this assay the level of cross-resistance in the resistant cell line for different antitumor agents, i.e., carboplatin, DOX, 5-FU, MTX, topotecan (TPT), paclitaxel, and docetaxel was determined.

Cell survival was tested every four passages during the development of resistance, and the cells were used in experiments after culturing in cisplatin-free medium for a period of 2 weeks.

Intracellular platinum accumulation

The intracellular platinum accumulation was studied using 6 T175 flasks, each containing approximately $1–1.5 \times 10^7$ cells (50–70% confluent). The parental and resistant cells were used at 3–4 days after plating. The cells were incubated with cisplatin for 2 h at a concentration of 0, 8.3, 16.7, 33.3, 83.3, and 166.7 μ*M*. Immediately afterward the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested by scraping. The cells were collected in 2 vols of 1.5 ml ice-cold PBS, centrifuged, and resuspended in 1.0 ml of deionized (Milli-Q) water. A volume of 50 μl was taken for protein determination using the Lowry method [19], and another 800-μl volume was dried by centrifugation under vacuum for 2 h at 70 °C. The cell pellet was digested in 200 μl of 65% nitric acid at 75 °C for 2 h. After dilution with water, platinum was analyzed by AAS [21]. The cellular platinum level was expressed as in nanograms of platinum (Pt) per microgram of protein.

The effects of coincubation with ouabain, temperature reduction, and energy deprivation on the intracellular accumulation of platinum were investigated as previously described [4, 32]. In the ouabain experiment, $IGROV_{CDDP}$ and IGROV-1 cells were exposed to 33.3 μm cisplatin for 2 h with or without ouabain at 0.5 μg/ml. No toxicity was noted when cells were incubated with this concentration of ouabain alone for 2 h.

The energy-depletion experiment was performed by replacement of the standard medium with glucose-free RPMI 1640 medium containing 2-deoxy-D-glucose at 1 mg/ml and 10 m*M* sodium azide at 15 min prior to exposure of the cells to 33.3 μ*M* cisplatin for 2 h. The intracellular concentration of cisplatin was measured by AAS as described above.

The influence of temperature reduction on the accumulation of platinum was studied by incubation of cells with cisplatin at 0 °C. Experiments at 0 °C were performed simultaneously with incubations at 37 °C of the same batch of cells. After exposure of the cells to cisplatin for 2 h, cells were washed and collected. Subsequently, platinum accumulation was determined by AAS as described above.

Efflux of platinum

IGROV-1 and $IGROV_{CDDP}$ cells were incubated in culture flasks for 30 min with 100 and 300 μ*M* cisplatin, respectively, to achieve equal levels of intracellular platinum. Subsequently, the cells were washed twice with PBS and cultured in drug-free medium. After 0, 10, 30, 60, and 120 min a flask was withdrawn and the cells were washed with ice-cold PBS and collected by scraping. Cellular platinum was analyzed by AAS.

Total cisplatin-DNA adducts and interstrand cross-link kinetics

Total cisplatin-DNA adducts were determined in the parental cell line and during the development of drug resistance. Exponentially

growing cells ($1–1.5 \times 10^7$) in 6 T175 flasks were exposed to 13.3 μ*M* cisplatin for 2 h. Subsequently, the cells were washed twice with PBS and fresh cisplatin-free medium was added. At time −1, 0, 6, 24, 48, and 72 h after the withdrawal of cisplatin, one flask was taken and cells were harvested by trypsinization. Cells were washed once with nuclear buffer [400 m*M* NaCl; 2 m*M* ethylenediaminetetraacetic acid (EDTA); 10m*M* TRIS; pH 7.3], resuspended in 3 ml of nuclear buffer, and subsequently stored at −20 °C until DNA isolation within 2 weeks. DNA isolation was carried out according to a previously published method [20, 23]. Total cisplatin-DNA adducts were determined according to the method of Reed et al. [25], with modifications according to Ma et al. [20]. DNA-adduct levels were expressed in femtomoles of Pt per microgram of DNA.

The relationship between the incubation concentration and the total DNA adducts and intrastrand cross-links (ISCs) was studied using two series of six T175 flasks containing the sensitive and resistant cell lines under the same incubation conditions described for the intracellular platinum-accumulation experiment. Cells were exposed to cisplatin at a concentration of 3.33, 6.67, 13.3, 33.3, 66.7, or 100 μ*M* for 2 h. Subsequently, cells were washed twice with PBS, collected by trypsinization, and washed once more with nuclear buffer. The cells were resuspended in 3 ml of the nuclear buffer and stored at −20 °C until the isolation of DNA for determination of total DNA adducts and ISCs. ISCs were analyzed using the ethidium bromide (EB) method as described by de Jong et al. [12] and Ali-Osman et al. [1], with some modifications. In brief 5–10 μg DNA (isolated as outlined above) was dissolved in 200 μl of nuclear buffer. Each sample was divided into two portions. To each sample, 3 ml of an EB solution (10 μg/ml, pH 12) was added. The first sample was immediately analyzed for the native fluorescence of DNA using an excitation wavelength of 525 nm and an emission wavelength of 580 nm. The second sample was heated to 100 °C and kept at that temperature for 5 min. Subsequently, the sample was immediately cooled down to room temperature, the fluorescence was analyzed, and the ISCs were calculated as previously reported [12]. Experiments were carried out in duplicate and in at least three independent experiments.

Flow-cytometry analysis

Determination of the cell-cycle distribution was carried out after incubation of IGROV-1 and $IGROV_{CDDP}$ with equitoxic levels of cisplatin (the IC_{50}). Cells were trypsinized and washed twice with RPMI medium without phenol red. Subsequently, cells were stained with propidium iodide and the DNA content was analyzed as described elsewhere [15]. Cisplatin was continuously incubated for 2 days and samples were analyzed after 24 and 48 h.

Statistical analysis

Student's *t*-test and Pearson's correlation analysis were used, and $P < 0.05$ was applied as the significance level.

Results

During 9 months of incubation and five stepwise increases in the incubation concentration of cisplatin from 3.33 to 33.3 μ*M* a stable cisplatin-resistant cell line, denoted $IGROV_{CDDP}$, was established with a resistance factor of 8.4 (Table 1). No significant change in the doubling time or cloning efficacy was observed (with doubling times of 28 ± 3.4 and 26 ± 4.5 h and cloning efficacies of 19 ± 8% and 23 ± 4% being noted for IGROV-1 and $IGROV_{CDDP}$, respectively). The average diameters of IGROV-1 and $IGROV_{CDDP}$ were 14.09 ± 0.97 and 14.34 ± 0.78 μm, respectively. Without cisplatin exposure the $IGROV_{CDDP}$ remained resistant during the follow-up period of 4 months.

Table 1 Cross-resistance of $IGROV_{CDDP}$ to other anticancer drugs (*NS* Not significant)

Drugs	IGROV-1	$IGROV_{CDDP}$	R_f[a]	*P*
	(IC_{50} μ*M*)			
Cisplatin	0.53	4.46	8.4	< 0.001
Carboplatin	5.04	32.24	6.4	< 0.001
DOX	0.13	0.77	6.0	< 0.001
Paclitaxel	0.07	0.22	3.1	< 0.001
Docetaxel	0.02	0.06	3.2	0.01
VP-16	1.33	3.55	2.7	0.002
TPT	0.02	0.06	2.7	< 0.05
5-FU	2.48	4.14	1.7	< 0.05
MTX	0.04	0.04	1.1	NS

[a] R_f Resistance factor, determined with the SRB method in which resistant and sensitive cell lines were exposed to the drug for 5 days

$IGROV_{CDDP}$ was highly cross-resistant to carboplatin and DOX (Table 1). The cell line was also moderately cross-resistant to paclitaxel, docetaxel, VP-16, and TPT. Minor or nonsignificant cross-resistance with 5-FU and MTX was observed.

The resistance of IGROV-1 to cisplatin increased, and the DNA-adduct levels after cisplatin exposure decreased with increasing passage number (Fig. 1). High levels of correlation were found between the log cell survival and the DNA-adduct level ($R = 0.97$) after cisplatin exposure. The DNA-adduct levels decreased 3-fold during the development of resistance (Fig. 1). There was a linear and apparently nonsaturable relationship between the incubation concentration and the intracellular platinum concentration in IGROV-1 and $IGROV_{CDDP}$ over the studied range of 3.33–167 μ*M* (Fig. 2). The accumulation of cisplatin observed in $IGROV_{CDDP}$, as determined from the slope of the regression line, was approximately one-third of that seen

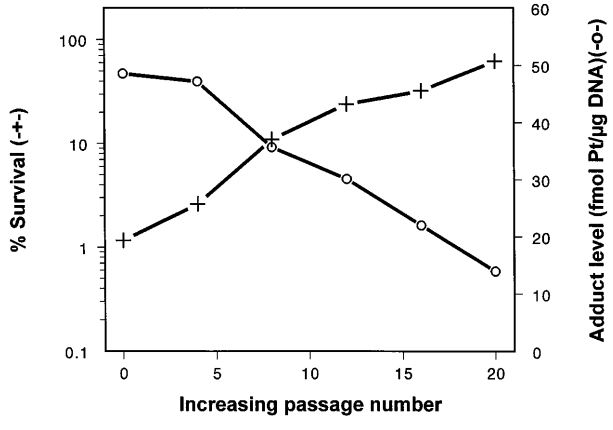

Fig. 1 Log-linear relationship between increasing passage number and cell survival (clonogenic assay) [+] and DNA adduct level [○] as determined by AAS. Determinations were performed after incubation with 13.3 μ*M* cisplatin for 2 h

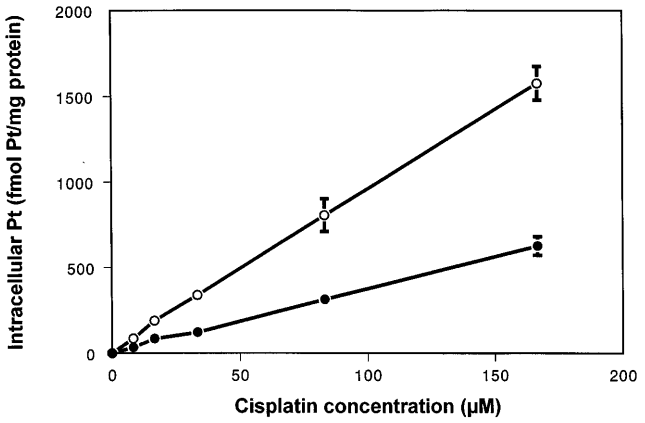

Fig. 2 Intracellular platinum concentration determined after exposure of parental IGROV-1 [○] and $IGROV_{CDDP}$ [●] cell lines to increasing concentrations of cisplatin (exposure time 2 h). Intracellular concentrations of platinum were measured by AAS as described in Materials and methods. Data are mean values ± SD from at least 3 independent experiments

in IGROV-1 ($P < 0.01$). This 3-fold difference in the intracellular accumulation of cisplatin is in agreement with the observed decrease in DNA-adduct levels. Also, the relationship between the total DNA adducts or ISC and the incubation concentration was linear over the studied range, with the difference in slope between the two cell lines being approximately one-third (Fig. 3a,b).

Coincubation of cisplatin with the Na^+/K^+ATPase inhibitor ouabain resulted in a decrease in platinum accumulation by 44% in the IGROV-1 cell line and by 26% in the resistant $IGROV_{CDDP}$ line (data not shown). Lowering of the temperature to 0 °C resulted in a significantly reduced intracellular accumulation of cisplatin in both IGROV-1 (by 95% as compared with 37 °C) and resistant $IGROV_{CDDP}$ cells (by 91% as compared with 37 °C; Fig. 4). At 0 °C there was no significant difference between the intracellular concentration of platinum seen in the IGROV-1 and $IGROV_{CDDP}$ cell lines. Under energy-deprived conditions the intracellular platinum concentration in IGROV-1 was significantly reduced to 35% of the level obtained under standard energy-rich culture conditions (Fig. 4). However, in the $IGROV_{CDDP}$ cell line, depletion of energy did not result in a significantly decreased accumulation of platinum. Interestingly, under energy-deprived conditions the difference between the sensitive and resistant cell lines was no longer significant (Fig. 4).

No significant efflux of platinum from cisplatin-loaded IGROV-1 or $IGROV_{CDDP}$ cells was noted after incubation of the preloaded cells in drug-free medium for 30, 60, or 120 min.

Without exposure to cisplatin, only small differences in cell-cycle distribution were observed between IGROV-1 and $IGROV_{CDDP}$ (Table 2). However, following a 2-day exposure to equitoxic (IC_{50}) concentrations of cisplatin, the increase in the number of cells in the G_2/M phase at 48 h after the start of incubation with cisplatin was much higher in $IGROV_{CDDP}$ (from 12% to 56%) than in IGROV-1 (from 14% to 32%). Due to this arrest in G_2/M the numbers of cells in the G_1 and S phases decreased.

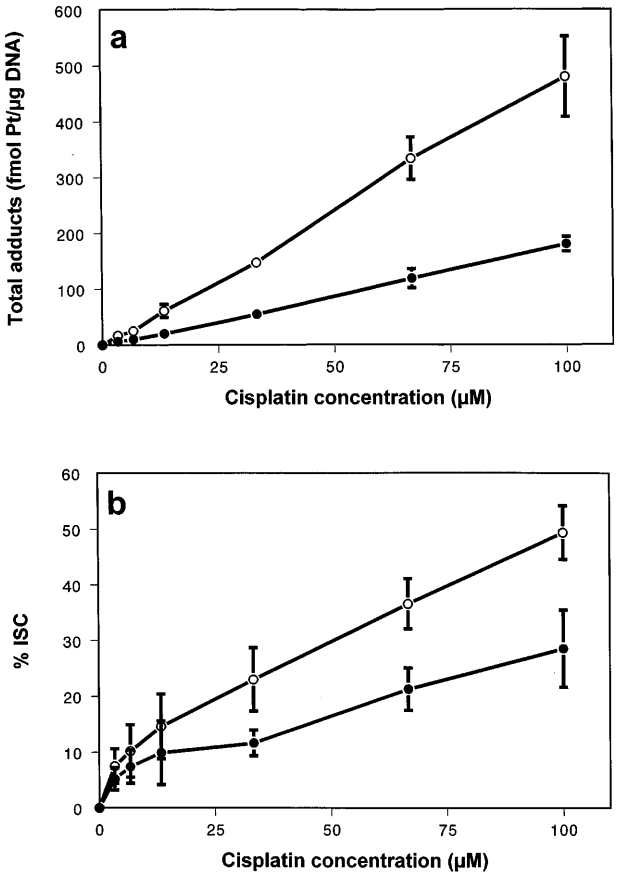

Fig. 3a,b Total DNA adducts (**a**) and ISC (**b**) determined after exposure of IGROV-1 [○] and $IGROV_{CDDP}$ [●] cells to increasing concentrations of cisplatin for 2 h. Total adduct and ISC levels were determined as described in Materials and methods. Data are mean values ± SD from at least 3 independent experiments

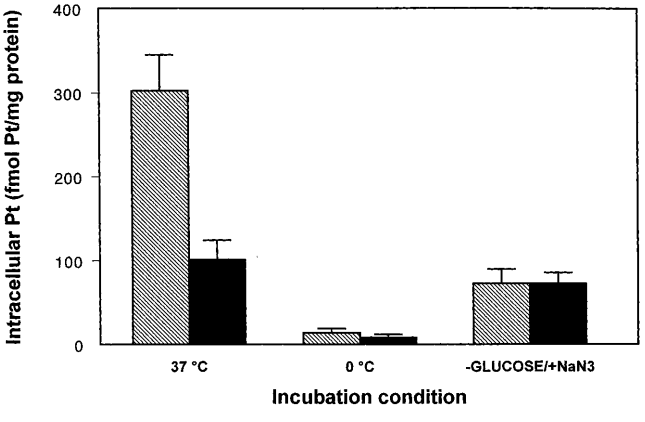

Fig. 4 Intracellular concentrations of cisplatin as determined in the IGROV-1 [▨] and $IGROV_{CDDP}$ [■] cell lines following 2 h of incubation with 33.3 μ*M* cisplatin. Furthermore, the accumulation at 0 °C and under energy-depleted conditions (as described in Materials and methods) is displayed. Data are mean values ± SD from at least 3 independent experiments

Table 2 Cell-cycle distribution in IGROV-1 and $IGROV_{CDDP}$ without cisplatin and at 48 h after treatment with SRB-determined IC_{50} doses of cisplatin

	Blank			IC_{50} cisplatin		
	% Cells in			% Cells in		
	G_1	S	G_2/M	G_1	S	G_2/M
IGROV-1	74	12	14	60	8	32
$IGROV_{CDDP}$	78	10	12	35	9	56

Discussion

Both intrinsic and acquired resistance to cisplatin limit the clinical utility of this anticancer agent. Previous studies have shown that decreased accumulation of cisplatin is an important factor in in vitro and in vivo acquisition of resistance [1, 16]. In the present study we developed and characterized a resistant subline of the IGROV-1 human ovarian adenocarcinoma cell line. The cell line was intermittently exposed to mimic the clinical application of cisplatin. Intermittent exposure of the cisplatin-sensitive IGROV-1 ovarian cancer cell line resulted in a stable resistant cell line, denoted $IGROV_{CDDP}$, with a resistance factor of 8.4 in the SRB assay.

Accumulation of cisplatin decreased significantly in $IGROV_{CDDP}$ as compared with the parental cell line. Consequently, platinum-DNA adduct levels in $IGROV_{CDDP}$ were lower at equal extracellular cisplatin concentrations. Ouabain, an Na^+/K^+-ATPase inhibitor [2, 5, 13], applied in combination with cisplatin resulted in a significant decrease in the intracellular concentration of platinum. The uptake of cisplatin in IGROV-1 was reduced by 44%, which is in accordance with the results of other studies in other tumor cell lines [30]. Upon treatment with ouabain, the cisplatin uptake was decreased more pronouncedly in IGROV-1 than in $IGROV_{CDDP}$. This observation suggests that the functional activity of Na^+/K^+-ATPase has changed in the resistant $IGROV_{CDDP}$ cell line. The reduced influence of ouabain observed in the resistant cell line is also suggestive of a role for K^+ homeostasis and Na^+/K^+-ATPase in the mechanism underlying the active uptake of cisplatin.

Under normal conditions the intracellular concentration of platinum in IGROV-1 was 3-fold that in $IGROV_{CDDP}$ cell line (Fig. 2, 4). However, the difference was not significant under energy-depleting conditions (Fig. 4). It has been suggested by other investigators that cisplatin enters the cells partly by passive diffusion and partly by active transport [8]. Our energy-depletion experiments illustrate that in the IGROV-1 cells the major part of cisplatin (approximately 65%) is taken up by energy-dependent processes, whereas only a minor part enters the cell via passive diffusion. Furthermore, our results indicate that the energy-dependent uptake in this resistant cell is abrogated and, therefore, this abrogation may be a major factor determining the resistance of this cell line of cisplatin. The importance of both active and passive transport of cisplatin in the parental IGROV-1 line is also indicated by the strongly diminished cisplatin accumulation observed at 0 °C. Lowering of the temperature to 0 °C has two effects: energy-dependent processes are abrogated, and, due to a changed membrane fluidity at lower temperatures, passive diffusion of cisplatin is affected. The reduction of cisplatin accumulation at this low temperature to values below those observed upon energy depletion provides additional support for the hypothesis that both active and passive transport of cisplatin are important in IGROV-1. Importantly, at 0 °C the accumulation of cisplatin in IGROV-1 and $IGROV_{CDDP}$ is equal. These results strongly suggest the presence of both active and passive uptake mechanisms for cisplatin in IGROV-1 and of only passive diffusion of cisplatin in the $IGROV_{CDDP}$ cell line.

No saturation of the uptake of cisplatin up to the highest tested concentration of 167 μM was observed. This unsaturable influx of cisplatin has also been demonstrated in other cisplatin-resistant cell lines [3, 4]. Although this seems to argue against energy dependence of cisplatin uptake, it is possible that cisplatin is accumulated by facilitated diffusion of cisplatin through gated channels [8]. These channels may be dependent on the energy status of the cells, and this feature may explain the combined energy dependence and unsaturability of cisplatin uptake [8]. In other cisplatin-resistant human ovarian cancer cell lines, reduced influx of cisplatin has also been reported [4, 18, 30]. The explanation for this reduced influx, however, remains unclear. Shar et al. [30] identified a plasma membrane protein (M_r 36,000) that was overexpressed in the cisplatin-resistant cell line. However, it is not known whether this protein is involved in the reduced accumulation of cisplatin. As reduced accumulation is a well-known phenomenon in in vivo acquired resistance to cisplatin, extended research into the mechanism of this reduced accumulation is warranted. The $IGROV_{CDDP}$ cell line may be a good model for such studies.

The MDR-associated protein (MRP) has been suggested to be involved in cisplatin resistance [10, 14]. Importantly, cell lines expressing MRP but lacking resistance to cisplatin have also been described [11]. Apparently, MRP alone is not sufficient to yield cisplatin resistance [11], and high glutathione levels may be an important additional factor needed for MRP to result in this type of resistance [11, 33]. We have previously demonstrated that this second prerequisite is not met in $IGROV_{CDDP}$ [29], and MRP is therefore not expected to be involved in the mechanism of cisplatin resistance in $IGROV_{CDDP}$. However, as based on the cross-resistance pattern of this cell line, overexpression MRP in $IGROV_{CDDP}$ cannot be excluded. By immunocytochemical detection, however, no overexpression of MRP was noted in $IGROV_{CDDP}$ as compared with IGROV-1

(unpublished results). This result, together with the previously reported absence of increased P-gp levels in $IGROV_{CDDP}$ [29], indicates that resistance in $IGROV_{CDDP}$ is not related to one of the known MDR mechanisms. However, the observed difference in cell-cycle distribution upon exposure to equitoxic concentrations of cisplatin suggests that changes other than cellular membrane alterations have also developed in the resistant $IGROV_{CDDP}$ cell line. The indication of multifactorial adaptations is supported by the observed pattern of cross-resistance with the tested anticancer agents. The identity of these additional alterations is not yet clear.

In conclusion, $IGROV_{CDDP}$ is 8.4-fold resistant to cisplatin as compared with its parental cell line IGROV-1. The main mechanism of resistance appears to be altered uptake kinetics of the drug, possibly caused by abrogated active uptake of cisplatin. However, other cellular adaptations may have occurred. The cell lines that have been and are currently being developed can be used as a model in future studies directed at elucidating cell-uptake-related mechanisms of cisplatin resistance.

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