Transport of Cisplatin and Bis-acetato-ammine-dichlorocyclohexylamine Platinum(IV) (JM216) in Human Ovarian Carcinoma Cell Lines: Identification of a Plasma Membrane Protein Associated with Cisplatin Resistance

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ABSTRACT

The mechanisms by which cis-diaminedichloroplatinum(II) (cisplatin) is transported across the plasma membrane (i.e., passive diffusion versus active transport) were investigated in the 41M and CH1 human ovarian carcinoma cell lines and their acquired cisplatin-resistant variants 41McisR6 and CH1cisR6, respectively. Intracellular cisplatin accumulation was significantly reduced (4.0 ± 1.7-fold) in the parental 41M line at 4°C when compared to incubations at 37°C. However, no significant differences in platinum uptake were observed in the 41McisR6 and in the CH1 pair of lines at 4°C versus 37°C. Similarly, in the presence of ouabain (an inhibitor of Na⁺,K⁺-ATPase), there was a marked reduction (2.0 ± 0.4-fold) in drug accumulation in the sensitive 41M cells only, and no changes in drug uptake were observed in the other cell lines in the absence or presence of ouabain. Platinum accumulation was significantly enhanced in all cell lines in the presence of metabolic inhibitors (NaF and NaN₃). These results suggest that in the parental 41M cell line, cisplatin transport may occur via passive diffusion and active/facilitated transport, whereas in the resistant 41McisR6 variant, cisplatin enters cells by passive diffusion only. The orally active drug bis-acetato-ammine-dichlorocyclohexylamine platinum(IV) (JM216) is a lipophilic platinum(IV) complex that has been shown to circumvent cisplatin resistance in the 41McisR6 by increasing drug uptake. Across the entire range of concentrations used (5–50 μM), intracellular accumulation of JM216 was significantly reduced in 41M and 41McisR6 cells (3.5 ± 0.7-fold; P < 0.01), and in CH1 and CH1cisR6 cells (14.2 ± 6.0-fold; p < 0.01) at 4°C when compared to incubations at 37°C. No significant difference in JM216 uptake was observed in the 41M pair of lines in the absence or presence of ouabain. Additional studies have revealed that the fold reduction observed in cis-ammine(cyclohexylamine)dichloroplatinum(II) (JM118) accumulation in the 41M and 41McisR6 cells at 4°C (3.7 ± 1.9) reflects similar fold reductions to those observed with JM216 uptake at 4°C. These results suggest that the mechanism of JM216 transport across cell membranes is through passive diffusion, predominantly as a result of its enhanced lipophilicity.

Notably, an overexpression of a Mr 36,000 plasma membrane protein was observed in the 41McisR variants when compared to the sensitive 41M line. Increased levels of this Mr 36,000 protein may relate to the observed reduction in active transport of cisplatin in the 41McisR6 variant. Tyrosine phosphorylation of the Mr 36,000 protein appeared to be greater in the resistant 41McisR6 variant than in the parental 41M line. In addition, the constitutive levels of the Mr 36,000 protein in the CH1 pair of lines and in two acquired JM216-resistant variants (41M/JM216R and CH1/JM216R), where resistance in these cell lines is not mediated through reduced drug uptake, were similar to those observed in their respective parental lines. These results suggest that the overexpression of this Mr 36,000 protein in the acquired cisplatin-resistant subline 41McisR6 may play a significant role in cisplatin uptake in resistant cells exhibiting reduced drug accumulation as a major mechanism of cisplatin resistance.

INTRODUCTION

Since the introduction of cisplatin into the clinic in 1971, studies on the development of platinum-based drugs have been aimed at modulating the toxic side effects of cisplatin (primarily nephrotoxicity) and at circumventing both intrinsic and acquired cisplatin resistance. The second generation drug carboplatin has significantly reduced the unfavorable toxicity profile of the parent drug (1). However, both drugs show a similar spectrum of antitumor activity and are i.v. preparations (2). Hence, there is an urgent need to develop a third generation of platinum-based analogues that are superior to cisplatin/carboplatin and are capable of overcoming resistance.

Cisplatin resistance is multifactorial, mainly involving reduced intracellular drug accumulation, enhanced cytoplasmic detoxification (via increased levels of GSH and metallothioneins), and increased repair/tolerance of platinum-DNA adducts.
(for review, see Ref. 3). At present, it is still unclear which is the dominant mechanism responsible for cisplatin resistance. However, impaired cellular accumulation of cisplatin is a commonly observed phenotypic characteristic of cell lines with acquired cisplatin resistance (4–6), and this characteristic can be found during the early stages of selection of cells with cisplatin in vitro (7, 8). The exact mechanisms by which cisplatin enters cells have not been established, but studies have implicated both passive diffusion and active transport (or carrier-mediated transport; Ref. 9). Recently, specific changes in plasma membrane proteins have been reported in association with reduced cisplatin accumulation in resistant cells (10–14).

The objective of this study was to obtain an insight into the process by which cisplatin is transported across the plasma membrane and how this has changed in the resistant cells. Two human ovarian carcinoma cell lines, 41M and CH1, and their acquired cisplatin-resistant variants 41McisR6 and CH1cisR6, respectively, were selected. Previous results have shown that although these acquired cisplatin-resistant sublines were generated from their parent cells using the same protocol, resistance has occurred through contrasting mechanisms. In the 41McisR6 line, reduced drug accumulation (with no alteration in drug efflux) plays a major role in the mechanism of cisplatin resistance (8). However, the resistance in the CH1cisR6 variant appears to have occurred at the DNA level (15). The role of passive diffusion versus active transport was investigated by examining the effects of temperature, ouabain (an inhibitor of Na⁺,K⁺-ATPase), and metabolic inhibitors (NaF and NaN₃) on cisplatin transport in these cell lines.

The orally active platinum agent JM216, which is currently in Phase II clinical trial, is a lipophilic platinum(IV) complex with in vitro activity against a wide range of sensitive and cisplatin-resistant cell lines (16–18). We have shown that JM216 is capable of circumventing cisplatin resistance in the 41McisR6 variant by enhancing platinum accumulation (8). The effects of temperature and ouabain on JM216 transport were also investigated. Furthermore, identification of any specific changes in plasma membrane proteins that may be involved in the transport of cisplatin and their phosphorylation status were examined. This study describes the overexpression of a plasma membrane protein (Mr, 36,000) in the resistant 41McisR6 line. The constitutive levels of this protein were also determined in the CH1 pair of lines and in two acquired JM216-resistant sublines, 41M/JM216R and CH1/JM216R. The biochemical mechanisms of acquired resistance to JM216 have been identified by Mellish and Kelland (19); resistance in the 41M/JM216R was mainly due to elevated GSH levels, and increased DNA repair appeared to be the major mechanism of resistance in the CH1/JM216R.

MATERIALS AND METHODS

Drugs and Chemicals. Cisplatin, JM216, and JM118 (structures are shown in Fig. 1) were synthesized by and obtained from the Johnson Matthey Technology Centre (Reading, United Kingdom). The majority of the chemicals used in this study were purchased from Sigma Chemical (Poole, United Kingdom), unless otherwise stated.

Cell Lines. The biological properties of the two "parent" human ovarian carcinoma cells 41M and CH1 have been described previously (20). 41M was derived from a previously untreated patient, and CH1 was established from a patient who...
had prior cisplatin and carboplatin therapy. Acquired cisplatin-resistant variants to these cell lines were generated as described previously (8, 15). Cell lines (41McisR2, 41McisR4, and 41McisR6) with approximately 2-, 4-, and 6-fold resistance, respectively [as assessed by the sulforhodamine B assay (21)] were generated in the 41M line. A 6-fold resistance to cisplatin (CH1cisR6) was achieved in the CH1 cells. Acquired JM216-resistant cell lines (41JM/JM216R and CH1/JM216R) were generated in the same two parent lines using a similar protocol for the generation of cisplatin resistance (19). Resistance factors (IC50 resistant/IC50 parent) to JM216 of 1.9 and 6.2 were obtained for 41M/JM216R and CH1/JM216R cells, respectively.

All cell lines grew as monolayers in DMEM containing 10% heat-inactivated FCS (Imperial Laboratories, Andover, United Kingdom), 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 2 mM l-glutamine, 10 μg/ml insulin, and 0.5 μg/ml hydrocortisone in a 10% carbon dioxide–90% air atmosphere. There was no contamination with Mycoplasma throughout the course of the study.

**Intracellular Platinum Accumulation.** All platinum agents were dissolved before use in 0.9% saline at 500 μM. Ouabain octahydrate (at 1 mM), NaF (at 50 mM), and NaN3 (at 50 mM) were dissolved in sterile water. The platinum drugs were added to approximately 1–4 × 10⁶ exponentially growing cells (in triplicate 25-cm² flasks) at various concentrations (5–100 μM) for 2 h. Immediately after drug exposure, the medium was aspirated, and the cells were washed three times with 25 ml ice-cold PBS. Subsequently, the cells were scraped, harvested in 0.5 ml PBS, and sonicated (Soniprep 150; Soniprep, Loughborough, United Kingdom) at 4°C. The intracellular platinum content was determined using flameless atomic absorption spectrometry (Perkin Elmer 1100B and HGA 700; Perkin Elmer/Cetus, Beaconsfield, United Kingdom) using a 50-μl aliquot of cell sonicate that had been digested overnight in 200 μl 1 N sodium hydroxide at 37°C. Cellular platinum levels were then expressed as nmol platinum/mg protein.

The effects of ouabain and metabolic inhibitors were examined by exposing the cells to various concentrations of the platinum agent in the absence or presence of the modulator for 2 h. Cells were treated with ouabain or metabolic inhibitors for 1 h prior to the addition of cisplatin. Immediately after exposure, cells were processed as described above.

**Estimation of the Integrity of the Plasma Membrane Using Flow Cytometry.** Exponentially growing cells (1–4 × 10⁶) were exposed to cisplatin (25 μM) in the absence or presence of metabolic inhibitors (10 mM NaF and 10 mM NaN₃) for 2 h. Cells that were treated with cisplatin and metabolic inhibitors had been preincubated with the inhibitors for 1 h. After the exposure period, cells were washed twice with 10 ml medium, and harvested by trypsinization. To a suspension (500 μl) of single cells at 10⁶/ml in medium, 50 μl fluorescein diacetate (100 ng/ml), and 50 μl PI (100 μg/ml) were added, and incubated at room temperature for 10 min. Cell viability was estimated by using PI, which is excluded from viable cells, but when dead cells or dying cells take up PI, it binds to nuclei acid and fluoresces red (22). Fluorescein diacetate itself is not fluorescent, but when taken up by cells is converted to fluorescein by intracellular esterases. Fluorescein is retained by the cell if the plasma membrane is intact. The samples were then analyzed by flow cytometry (Coulter Elite ESP; Coulter Corporation, Hialeah, FL). In the cytogram of red versus green fluorescence, the viable cells were counted as those which were positive for green. Results were expressed as the percentage of control (untreated) cells.

**Crude Plasma Membrane Extract.** Exponentially growing cells (1–1.5 × 10⁶) were washed three times with 25 ml PBS, scraped, and harvested twice with 0.5 ml lysis buffer containing 10 ml 150 mM sodium chloride and 50 mM Tris (pH 7.5), 500 μl phenylmethylsulphonylfluoride (from 20 mM stock), 2 μl aprotinin (10 mg/ml stock), 2 μl leupeptin (10 mg/ml stock), 100 μl sodium orthovanadate (10 mM stock), 100 μl NP40 (100% stock), and 100 μl SDS (20% stock). Cells were left on ice for 1 h. Nuclei and unbroken cells were removed by centrifugation (450 × g, 10 min, 4°C). The resultant supernatant was then centrifuged (60,000 × g, 1 h, 4°C). The pellet (i.e., crude plasma membrane extract) was resuspended in the lysis buffer and stored at −70°C until electrophoresis was performed. Protein was assayed using the bicinchoninic acid protein assay (supplied as a kit; Pierce, Rockford, IL).

**Immunoblotting for Antiphosphotyrosine.** Membrane proteins (60 μg/well) were resolved by electrophoresis in an 8–16% SDS-PAGE gradient, and electroblotted onto a polyvinylidene difluoride filter (Millipore, Watford, United Kingdom), as described by Towbin et al. (23), in transfer buffer containing 10% methanol at 300 mA for 2 h at 4°C. The polyvinylidene difluoride filter was first blocked for 1 h at room temperature or overnight at 4°C in buffer containing 1% BSA in 0.1% Tris, 0.1 M sodium chloride, and 0.1% Tween 20 (the use of milk as a blocking agent should be stringently avoided). The filter was then exposed for 1 h to a horseradish peroxidase-labeled mouse antiphosphotyrosine antibody (RC2OH; Transduction Laboratories, Nottingham, United Kingdom) diluted 1:2500 in blocking buffer. The filter was washed thoroughly in PBS containing 0.1% Tween 20. Detection was achieved with enhanced chemiluminescence reagents (Amersham, Buckinghamshire, United Kingdom) and exposure to autoradiography film (Hyperfilm-ECL, Amersham).

**Statistical Analysis.** Statistical significance was tested using a two-tailed Student’s t test; a P value <0.05 was considered significant.

**RESULTS**

**Intracellular Platinum Accumulation**

**Effects of Temperature, Ouabain, and Metabolic Inhibitors on Cisplatin Transport.** Fig. 2 shows intracellular platinum accumulation in the 41M/41McisR6 cells (Fig. 2A) and CH1/CH1cisR6 cells (Fig. 2B) immediately after 2-h exposure to various concentrations of cisplatin at either 4 or 37°C. Our previous results have shown that at 37°C, the acquired cisplatin-resistant variant 41McisR6 accumulated 4.8 ± 0.6-fold less platinum when compared to the sensitive 41M cells (8). However, there is a significant reduction (4.0 ± 1.7-fold; P < 0.01) in cisplatin uptake in the parental 41M line at 4°C when compared to incubations at 37°C. No significant differences in platinum accumulation were observed in the 41McisR6 and in the CH1 pair of lines at 4 versus 37°C.

Parent and resistant cells were incubated at 37°C with
various concentrations of cisplatin with or without ouabain (25 μM) for 2 h (Fig. 3). Cells that were treated with cisplatin and ouabain had been preincubated with 25 μM ouabain for 1 h. The concentration of ouabain used conferred a maximum growth inhibitory effect of up to approximately 15%, as assessed by the sulforhodamine B assay (21). The accumulation results obtained with ouabain were similar to those obtained with the temperature data. In the presence of ouabain, platinum uptake was significantly reduced (2.0 ± 0.4-fold, \( P < 0.01 \)) in the parental 41M line only (Fig. 3A). There were no significant differences in uptake in the 41McisR6 (Fig. 3A) and in the CH1 pair of lines (Fig. 3B) in the absence or presence of ouabain.

To prevent the biosynthesis of ATP, 10 mM NaF, and 10 mM NaN₃, which are inhibitors of glycolysis and oxidative phosphorylation, respectively, were used. Fig. 4 shows platinum accumulation in the absence or presence of these metabolic inhibitors immediately after 2-h exposure to cisplatin in 41M/41McisR6 (Fig. 4A) and CH1/CH1cisR6 (Fig. 4B) cell lines. As with ouabain, cells treated with cisplatin and metabolic inhibitors had been preincubated with the metabolic inhibitors for 1 h. In the 41M/41McisR6 cells, platinum accumulation was significantly enhanced (\( P < 0.01 \)), reaching similar levels in both cell lines. This was also observed in the CH1 pair of lines. Across the entire concentration range, platinum uptake was elevated by an average of 2.2- and 6.5-fold in the 41M and 41McisR6 cells, respectively, and 2.0-fold for both CH1 and CH1cisR6 cells. Results from flow cytometry have shown that the integrity of the plasma membrane of the cells was unaltered in the presence of the metabolic inhibitors (Table 1). In one additional experiment, 5 mM 2-deoxyglucose and 10 mM NaN₃ were used; this also resulted in an increase (4-fold) in uptake in the 41M pair of lines.

Effects of Temperature and Ouabain on JM216 Transport. Intracellular platinum levels immediately after 2-h exposure to JM216 at either 4 or 37°C are shown in Fig. 5. We...
equimobar concentration (25 μM), 4lMcisR6 cells in the absence or presence of ouabain. At the across the range of concentrations used, no significant differences in platinum accumulation were observed in the 41M and JM216 transport were determined in the 41M pair of lines. Intracellular cisplatin accumulation in 41M and 4lMcisR6 cells in the absence (0, K) and presence of ouabain (10 μM) were 0.65 ± 0.04 and 0.63 ± 0.06, respectively. In the presence of ouabain, the platinum levels were 0.63 ± 0.1 in the 41M line and 0.73 ± 0.06 in the 4lMcisR6 cells.

In an attempt to determine whether the transport of JM216 across the cell membrane was based on either its lipophilic nature or the fact that platinum(IV) complexes might be more temperature dependent for their accumulation than platinum(II) compounds, intracellular JM118 (one of the major platinum(II) metabolites of JM216) accumulation was measured in the 41M and 41McisR6 cells at 4 and 37°C. Fig. 6 shows that the accumulation of JM118 was significantly reduced (P < 0.01) in both cell lines (3.7 ± 1.9-fold) at 4°C when compared to incubations at 37°C.

**Immunoblot Analysis of Plasma Membrane Components**

Crude plasma membrane proteins of the parental 41M line (Fig. 7, Lane 1) and the three acquired cisplatin-resistant variants 41McisR2, 41McisR4, and 41McisR6 (Fig. 7, Lanes 2–4) were resolved in a gradient (8–16%) SDS-PAGE gel, and subsequently stained with Coomassie blue and silver stain (Fig. 7). An overexpression of a 36,000 protein which correlated with cisplatin resistance was observed. No such differences were observed in the cytosol and whole-cell fractions (data not shown). The phosphorylation status of this protein was determined by immunoblotting with antiphosphotyrosine antibody (RC2OH). Fig. 8 shows that the only significant difference in the phosphorylation status between the 41M and 41McisR6 cells is an increase in tyrosine phosphorylation of the Mr 36,000 protein in the 41McisR6 subline. Moreover, the constitutive level of expression of the Mr 36,000 protein in the CH1 pair of lines and in the two acquired JM216-resistant variants (41M/JM216R and CH1/JM216R) was similar to their respective parental lines (Fig. 9).

**DISCUSSION**

This study has aimed at identifying the precise mechanism(s) by which cisplatin enters cells in two pairs (parent and acquired cisplatin variants) of human ovarian carcinoma cell lines with distinct mechanisms of resistance to cisplatin: 41McisR6, resistance predominantly through reduced drug accumulation (8), and CH1cisR6, resistance through effects mediated at the DNA level (15). Previous data have shown no

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin (25 μM)</th>
<th>Cisplatin (25 μM) + metabolic inhibitors (10 μM NaF, and 10 μM NaN₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>41M</td>
<td>91.4</td>
<td>81.8</td>
</tr>
<tr>
<td>41McisR6</td>
<td>98.5</td>
<td>91.2</td>
</tr>
<tr>
<td>CH1</td>
<td>100</td>
<td>99.4</td>
</tr>
<tr>
<td>CH1cisR6</td>
<td>100</td>
<td>94.0</td>
</tr>
</tbody>
</table>

Values are the means from two experiments. Cells were treated with 25 μM cisplatin in the absence of metabolic inhibitors for 2 h. Cells were treated with metabolic inhibitors for 1 h prior to the 2-h incubation with cisplatin and metabolic inhibitors.

![Graph](https://example.com/graph.png)

*Fig. 4* Intracellular cisplatin accumulation in 41M (A, ● and ○) and 41McisR6 (A, ▲ and △), and CH1 (B, ■ and □) and CH1cisR6 (B, ● and ○) cells in the absence (○, △, □, ○) or presence (●, ▲, ■, ●) of metabolic inhibitors (10 μM NaF and 10 μM NaN₃). Bars ± SD of triplicate determinations in two experiments. SD was less than the symbol size where not indicated.
involvement of intracellular GSH or metallothioneins in the mechanism of resistance in these cell lines (8, 15). Hence, the 41M and 41McisR6 cells may provide an excellent model system in understanding how cisplatin is transported across the plasma membrane. Alterations in the transmembrane transport of cisplatin are discussed here as a change in the ability of the resistant cells to accumulate intracellular drug relative to that of the parental cell lines.

Recent studies have suggested that cisplatin enters the cell partly by passive diffusion and partly by facilitated transport through a gated channel (9). This article has attempted to address the issue of cisplatin transport in these cell lines by examining the effects of temperature, ouabain, and metabolic inhibitors on drug uptake. Results have shown that at 4°C, there was a marked reduction in cisplatin accumulation in the parental 41M line only, when compared to incubations at 37°C. No differences in uptake were observed in the resistant 41McisR6 cells and in the CH1 pair of lines at either 4 or 37°C. This change in drug accumulation under hypothermic conditions (4°C) in the 41M line may be as a result of differences in membrane fluidity (e.g., through differences in lipid composition) and thereby permeability, differential effects on the underlying cytoskeleton, changes in active drug transport, or altered cellular metabolism (24, 25). Although Mann et al. (26) have found only small differences in membrane lipid composition and no significant differences in membrane fluidity in another
pair of human ovarian cell lines (2008), and there is no further evidence to show that the membrane fluidity is changed in resistant cells, this does not preclude membrane fluidity changes being responsible for the effects observed in the 41M pair of cell lines. To address this possibility, we are currently determining cholesterol levels in these cell lines. However, the temperature data do indicate that the activation energy ($E_a$) for transport is different between 41M and 41McisR6 cells.

Studies have reported that cisplatin accumulation in the 2008 pair of lines was significantly reduced (by 50%) when ouabain (an inhibitor of Na$^+$/K$^+$-ATPase) was added (27). This corresponded with the findings of Ohmori et al. (28). In this study, we have also shown that cisplatin uptake was markedly reduced in the presence of ouabain, but as with the temperature data, only in the parental 41M line. There were no changes in platinum accumulation in the other cell lines in the absence or presence of ouabain. In addition, the effects of metabolic inhibitors (10 mM NaF and 10 mM NaN$_3$) on cisplatin transport were examined in all of the cell lines, and resulted in increased uptake in all lines. In contrast, Andrews et al. (7) have reported a reduction (20–30%) in platinum accumulation in the 2008 pair of cell lines in the presence of 10 mM NaF and 0.25 mM dinitrophenol (inhibitor of oxidative phosphorylation). In this study, we have also reported that 5 mM 2-deoxyglucose and 10 mM NaN$_3$ resulted in an increase in cisplatin uptake in the 41M pair of lines. Moreover, using flow cytometry, we have shown that the integrity of the plasma membrane was unaltered in the presence of NaF and NaN$_3$ (Table 1). Prolonged exposure to metabolic inhibitors can produce many effects that could mask any true effect on an active transport process (e.g., altered cytoskeletal structure or phosphorylation status), and, thus, at present, it is unclear as to the mechanisms responsible for these contrasting observations.

Our previous results have shown that platinum accumulation was not saturable (up to 500 $\mu$M) in all cell lines, which suggests that cisplatin does not enter cells through an active mechanism (8). However, the temperature and ouabain data suggest that, in the parental 41M line, cisplatin may be transported into cells by both passive diffusion and either active transport or facilitated diffusion. In the acquired cisplatin-resistant variant 41McisR6, cisplatin appears to enter cells mainly by passive diffusion. If cisplatin transport is operating by both mechanisms, one would therefore predict that a more lipophilic drug would not be as dependent on facilitated transport for entry, and hence drug accumulation would increase in the resistant cells. JM216 is a highly lipophilic platinum(IV) complex (aqueous solubility of cisplatin and JM216 are 1 183 $\mu$g/ml and 170 $\mu$g/ml, respectively) and has been shown to circumvent the problem of resistance in the 41McisR6 variant by increasing platinum uptake (8). Furthermore, Melish and Kelland (19) have shown that acquired resistance to JM216 in the 41M/JM216R subline was not mediated through reduced accumulation. Our results have reported no differences in JM216 uptake in the 41M and 41McisR6 cells in the absence or presence of ouabain. At 4°C, JM216 uptake was significantly reduced in the 41M and CH1 pairs of lines when compared to incubations at 37°C. Preliminary results have shown that this reduction in uptake at 4°C was not due to altered cellular metabolism of the drug (data not shown). The platinum(II) complex MJ118 possesses similar aqueous solubility to JM216 (the solubility of MJ118 in water is 216 $\mu$g/ml; Ref. 8), and has been shown to be the major metabolite of JM216 in man (29). It would seem that the transport of JM216 is mainly via passive diffusion as a result of its lipophilicity, since the reduction in JM118 accumulation...
closely paralleled the fold reduction observed with JM216 at 4°C. Recently, several studies have found evidence for protein-mediated anticancer drug transport. The main mechanism by which the membrane P-170 glycoprotein-mediated multidrug resistance occurs is through enhanced drug efflux. However, many studies have reported that reduced platinum influx rather than enhanced efflux is a consistent feature of acquired cisplatin resistance (7, 30). This was also observed with the 41M/41McisR6 cells (8). In addition, we have previously shown no overexpression of the P-170 glycoprotein in the 41M/41McisR6 and CH1/CH1cisR6 cell lines (31). This study has revealed a novel, putative plasma membrane protein of Mr 36,000 that is overexpressed in the resistant 41McisR variants, and the level of expression of this protein may relate to the observed reduction of active transport of cisplatin in this subline. Kawai et al. (10) have also shown that the levels of resistance in a series of cisplatin-resistant murine lymphoma cell lines (R1.1 lines), which exhibited reduced drug accumulation, correlated with increased levels of a Mr 200,000 membrane glycoprotein. Other studies however, have reported specific plasma membrane proteins that were reduced in cisplatin-resistant lines exhibiting reduced drug uptake (11-14). It is therefore very difficult to ascertain the role (if any) of this Mr 36,000 protein in the cisplatin-resistant cell line (41McisR6) that exhibited reduced drug uptake as the major mechanism of cisplatin resistance. Further analysis has shown that this Mr 36,000 protein has increased levels of tyrosine phosphorylation in the resistant 41McisR6 cells when compared to the sensitive 41M line. However, preliminary Western blot analysis has shown that this Mr 36,000 protein does not belong to the family of calcium- and phospholipid-binding proteins known as annexin I or II proteins, which are also Mr 36,000 phosphorylated proteins (32) (antibodies to annexin I and II were kindly provided by Dr. Mark Crompton, Institute of Cancer Research, Sutton, Surrey, United Kingdom; data not shown).

Previous studies have reported that the resistance in the CH1cisR6 and in the two acquired JM216-resistant variants 41M/JM216R and CH1/JM216R was not mediated through reduced drug accumulation (8, 19). We have shown that the level of expression of the Mr 36,000 protein in these cell lines was similar to that of their respective parental lines. These results suggest that the Mr 36,000 protein may play a significant role in cisplatin transport in resistant cells with an accumulation defect. However, it must be noted that treatment of cells with cisplatin in vitro results in multiple changes in levels of various protein, including heat shock and cytoskeletal proteins [e.g., human β-tubulin (13), type I keratin (14)].

An understanding on how cisplatin is transported across the plasma membrane may shed some light into why decreased accumulation of cisplatin is a common factor in the in vivo and in vitro acquisition of resistance to this antitumor agent. At present, our working hypothesis is that in the sensitive 41M line, cisplatin enters cells by both passive diffusion and active/facilitated transport, whereas in the resistant 41McisR6 variant (where resistance is predominantly through reduced uptake), the transport of cisplatin is by passive diffusion only. The transport mechanism of JM216 (and JM118) is predominantly through passive diffusion due to the highly lipophilic nature of the drugs.

The overexpression of the putative membrane protein (Mr 36,000) observed in the acquired cisplatin-resistant 41McisR6 line may play a significant role in the transport of cisplatin in resistant cells exhibiting reduced drug uptake. Our future studies will focus on the identification and role of this Mr 36,000 protein by polyclonal antibody production, which may hopefully lead to better strategies for the eradication of resistant tumors.

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