Resistance to Paclitaxel in a Cisplatin-Resistant Ovarian Cancer Cell Line Is Mediated by P-Glycoprotein

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Abstract
The IGROVCDPP cisplatin-resistant ovarian cancer cell line is also resistant to paclitaxel and models the resistance phenotype of relapsed ovarian cancer patients after first-line platinum/taxane chemotherapy. A TaqMan low-density array (TLDA) was used to characterise the expression of 380 genes associated with chemotherapy resistance in IGROVCDPP cells. Paclitaxel resistance in IGROVCDPP is mediated by gene and protein overexpression of P-glycoprotein and the protein is functionally active. Cisplatin resistance was not reversed by elacridar, confirming that cisplatin is not a P-glycoprotein substrate. Cisplatin resistance in IGROVCDPP is multifactorial and is mediated in part by the glutathione pathway and decreased accumulation of drug. Total cellular glutathione was not increased. However, the enzyme activity of GSR and GGT1 were up-regulated. The cellular localisation of copper transporter CTR1 changed from membrane associated in IGROV-1 to cytoplasmic in IGROVCDPP. This may mediate the previously reported accumulation defect. There was decreased expression of the sodium potassium pump (ATP1A), MRPI and FB which all have been previously associated with platinum accumulation defects in platinum-resistant cell lines. Cellular localisation of MRPI was also altered in IGROVCDPP shifting basolaterally, compared to IGROV-1. BRCA1 was also up-regulated at the gene and protein level. The overexpression of P-glycoprotein in a resistant model developed with cisplatin is unusual. This demonstrates that P-glycoprotein can be up-regulated as a generalised stress response rather than as a specific response to a substrate. Mechanisms characterised in IGROVCDPP cells may be applicable to relapsed ovarian cancer patients treated with frontline platinum/taxane chemotherapy.

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Introduction
The prognosis for women with ovarian cancer is very poor. The majority of patients present with advanced disease and the long-term survival in these patients is 10–30% [1]. Current treatment of ovarian cancer is surgery followed by platinum/taxane combination chemotherapy [1]. The chemotherapeutic drugs cisplatin and paclitaxel are used in the treatment of many solid tumours, including ovarian carcinoma. Cisplatin binds to the DNA strand, hindering both DNA replication and RNA translation and eventually triggering apoptosis. Paclitaxel causes cytotoxicity by binding to and stabilising polymerised microtubules. Due to their differing mechanisms of action, platinum and taxanes are often combined in cancer therapy. Initial responsiveness to chemotherapy in ovarian cancer is high, but up to 80% of patients will eventually relapse and become platinum/taxane resistant.

The IGROVCDPP cisplatin-resistant ovarian cell line is an unusual cisplatin-resistant model, as it is also cross-resistant to paclitaxel. When acquired cisplatin resistance is produced in cell lines, only 17% are also resistant to paclitaxel [2]. 41% of cisplatin drug-resistant models are not resistant to paclitaxel and 28% of cell models become hypersensitive to paclitaxel [2]. This suggests that the majority of cancer patients would benefit from receiving chemotherapy which alternates between cisplatin and paclitaxel, as developing resistance to one drug is less likely to result in resistance to the other. The challenge is how to identify which patients will respond well to alternating therapy between cisplatin and paclitaxel. This is because while the majority of cancer patients may respond well to this treatment strategy, the cross resistant cohort, would respond poorly and need to be treated with alternate therapy.

IGROVCDPP models the resistance phenotype of ovarian cancer patients who have failed standard frontline combination platinum/taxane chemotherapy. Chemotherapeutic drugs which IGROVCDPP is sensitive to may be suitable for the treatment of platinum/taxane resistant ovarian cancer. Studying the IGROVCDPP drug-resistant model will allow us to understand the mechanisms of cross resistance between platinums and taxanes. It is our aim to translate molecular markers of this cross

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resistance phenotypes to the clinical treatment of relapsed drug-resistant ovarian carcinoma.

**Methods**

**Cell Culture and Cytotoxicity Assays**

The human IGROV-1 ovarian cancer cell line and its cisplatin-resistant variant IGROVCDDP were obtained from Prof. Jan Schellens [3,4]. Cells were grown in antibiotic and chemotherapy-free RPMI (Sigma #R8758) with 10% FCS (Lonza, Belgium). Cells were maintained in a humidified atmosphere with 5% CO2 at 37°C, and were mycoplasma-free. To determine cytoxicities cells (1×10^4 cells/well) were plated into flat-bottomed, 96-well plates and allowed to attach overnight. Wells were treated with serial dilutions of drug in a final volume of 200 µL. Drug-free controls were included in each assay. Plates were incubated for a further 5 days and cell viability was determined using an acid phosphatase assay [5].

**TaqMan Low Density Array (TLDA)**

Cells (1.25×10^6 cells/10 cm dish) were plated and allowed to attach and grow for 3 days to reach 70–80% confluence. The cells were washed in 10 mL PBS, centrifuged and the supernatant removed. The cell pellets were stored at −80°C prior to analysis. Total RNA was prepared using a RNeasy Mini Kit (Qiagen, UK). The TLDA array was performed on biological triplicate samples as described in Gillet et al. 2011 [6]. The median expression of each sample was subtracted from all gene expressions for that sample. The data was analysed using BRB ArrayTools, a microarray-data statistical analysis tool [http://linus.nci.nih.gov/BRB-ArrayTools.html] [7]. Genes expressed by less than 50% of the samples were filtered out and a univariate two-sample T-test was performed to determine genes that were significantly different between IGROV-1 and IGROVCDDP based on a p<0.01 cutoff.

**Epirubicin Accumulation Assay**

Cells were plated at a density of 2.5×10^5 in a non-vented T25 flask. The next day the media was removed and the cells were treated with 1 µM epirubicin for 2 hours in the presence or absence of 0.25 µM elacridar, 0.67 µM, 3.33 µM or 33.3 µM cisplatin. Cells were washed with 4 mL of cold PBS and trypsinised. The cells were centrifuged and resuspended in 1 mL of PBS and a cell count performed (9 µL). The remaining cells were centrifuged, supernatant removed and the pellet stored at −20°C prior to analysis. Total epirubicin was then quantified by LC-MS following a liquid-liquid extraction sample preparation, according to the method of Wall et al. 2007 [8].

**Total Cellular Glutathione Assay**

Cells were plated at a density of 1.25×10^6 cells in a 10 cm diameter dish and allowed to attach overnight. The cells were drug treated for 24 hours, then trypsinised and a cell count performed. The cells were washed in 10 mL PBS, centrifuged and the supernatant removed. The cell pellets were stored at −20°C prior to analysis. Total glutathione was determined using a modification of Suzakake et al. [9]. Cell pellets were lysed in 150 µL water and sonicated, 12.5 µL of 30% sulfosalicyclic acid was added and the...
samples were vortexed. After 30 minutes on ice, protein-free supernatants were collected by centrifugation (12,000 g for 5 minutes at 4°C). Glutathione concentration was determined using a reaction mixture containing 20 μL of lysis or standard, 90 μL of triethanolamine buffer, pH 8.0 (0.2 M), 30 μL of NADPH (4 mM) and 20 μL of DTNB (6 mM). After 2 minutes at 30°C, the reaction was started by the addition of 0.5 units of glutathione reductase per well. The plates were read at 405 nm (preheating to 30°C) with kinetic measurement by a plate reader synergy HT, Bio-Tek® (MASON Technology). The rate of change of the kinetic assay was then calculated by KC4 software.

### Glutathione Metabolism

| Gene      | Full Name/Synonyms               | Mean mRNA Fold Change | SD  | P-value |
|-----------|----------------------------------|-----------------------|-----|---------|
| GSR       | Glutathione Reductase            | ↑                     | 1.40| 0.12    |
| GGT1      | Gamma Glutamyl Transpeptidase    | ↑                     | 4.92| 1.75    |

**Table 2.** TLDA IGROV-1 vs IGROVCDDP - Genes of interest by function or pathway.

**Transporters not associated with platinum resistance**

| Gene      | Full Name/Synonyms               | Mean mRNA Fold Change | SD  | P-value |
|-----------|----------------------------------|-----------------------|-----|---------|
| ABCB1     | P-glycoprotein                   | ↑                     | 11.38| 0.45    |
| ABCG2     | BCRP/Breast Cancer Resistance Protein | ↓                  | -2.17| 0.19    |

**DNA Repair**

| Gene      | Full Name/Synonyms               | Mean mRNA Fold Change | SD  | P-value |
|-----------|----------------------------------|-----------------------|-----|---------|
| BRCA1     | Breast Cancer Susceptibility Protein 1 | ↑                   | 2.17| 0.25    |

**Western Blots**

Cells (1.25 x 10⁶ cells/10 cm dish) were plated and allowed to attach overnight. The cells were then drug-treated with cisplatin and grown for 3 days. Cells were resuspended in 100 μL lysis buffer (0.01 M Tris/HCl, pH 7.4) and sonicated. 20 μg of protein was diluted in Laemmli sample buffer, boiled for 3 minutes, cooled on ice and loaded onto 12% Tris/glycine gels with a 4% stacking gel. Samples and molecular weight markers were then electrophoresed for 90 minutes at 100 V. The gels were electrotransferred to 0.45 μm nitrocellulose membranes (Biorad) for 90 minutes at 100 V using a wet transfer system (Biorad). The membranes were blocked with 5% non-fat skim milk (Biorad) in PBS for 2 hours, then incubated with the primary antibody prepared in 3% skim milk/0.1% tween/PBS (Table 1) overnight at 4°C [11]. The membranes were washed in 0.3% tween/PBS 5 x 10 minutes and then incubated for 1 hour with a HRP-conjugated secondary antibody (Table 1). Membranes were washed again and exposed to luminol reagent (Santa Cruz) or ECL advanced western blotting reagent (GE Healthcare). Membranes were then exposed to autoradiographic film. β-actin blots were developed using an alkaline phosphatase antibody (Table 1) and Sigma Fast BICP reagent. Densitometry on a minimum of n = 3 biological replicates was performed using Quantity One software (Biorad), using local background correction. Abundance of protein was normalised to ponceau for each sample and then each biological series was normalised to IGROV-1.

**Confocal Microscopy**

Cells (1.5 x 10⁵ cells/well) were plated into 8-well chamber slides and allowed to attach overnight. All washes were with PBS and all incubations were at room temperature unless otherwise specified. The cells were washed twice, and fixed with 4% paraformaldehyde (Sigma) in PBS for 30 minutes at 37°C. The cells were permeabilised with 0.5% Triton-X-100 (Sigma) for 10 minutes
Figure 1. P-gp in IGROV-1 and IGROVCDDP cells. A) Western blot of P-glycoprotein, IGROV-1 (open bars) and IGROVCDDP (grey bars) with and without treatment with 0.67 μM cisplatin for 72 hours (striped bars). Representative image shown. Graph shows quantitation of n = 6 biological repeats normalised to β-actin. * Indicates significant difference from IGROV-1 p < 0.05 student’s t-test. B) Accumulation of epirubicin determined by LC-MS. IGROV-1 (open bars) and IGROVCDDP (shaded bars). Cells were treated with 1 μM epirubicin for 2 hours, 0.25 μM elacridar, 0.67 μM, 3.33 μM or 33.3 μM cisplatin were investigated as modulators of epirubicin accumulation. Graph shows quantitation of n = 3 biological repeats normalised to cell number. * Indicates a significant difference between IGROV-1 and IGROV-CDDP, # Indicates a significant difference on the addition of a modulator (p < 0.05 student’s t-test). C) Cytotoxicity of IGROV-1 and IGROVCDDP to P-glycoprotein and non P-glycoprotein substrates.

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Table 3. Resistance profile of IGROVcDDP to cisplatin and ouabain.

| Drug (Units) | IGROV-1 IC₅₀ Mean ± SD | IGROVCDDP IC₅₀ Mean ± SD | Resistant vs Sensitive Fold, P-value n | IGROV-1 | Ouabain | IGROVCDDP | Ouabain |
|--------------|------------------------|--------------------------|-------------------------------------|---------|---------|----------|---------|
| Platinums    |                        |                          |                                     |         |         |          |         |
| Cisplatin (µM) | 0.14±0.06              | 2.70±1.15                | 18.73                               | >0.001  | 15      |          |         |
| + Ouabain 0.01 nM | 0.07±0.00              | 1.47±0.72                | 21.69                               | 0.027   | 3       | 0.226    | 0.073   |
| Modulator as Single Agent |            |                          |                                     |         |         |          |         |
| Ouabain (nM) | 23.84±9.11             | 3.38±0.59                | 0.14                                | 0.018   | 3       |          |         |

Statistical Analysis

All experiments were performed at minimum in triplicate. Two-sample, two tailed student’s t-tests were used to determine significant differences using p<0.05 as a cut off.

Results

Taxane Resistance in IGROVcDDP is Mediated by P-glycoprotein

The IGROV-1 and IGROVcDDP cells were analysed for 380 genes associated with chemoresistance by TLDA array in order to characterise the mechanisms of platinum and taxane resistance. 145 genes were found to be significantly different between IGROV-1 and IGROVcDDP based on a p<0.01 cutoff. Genes chosen for further analyses were based on the most significant by p-value as well as those pathways previously associated with platinum and taxane resistance (Table 2). All genes listed in table two were validated at the protein level by western blot.

The gene expression of P-glycoprotein (P-gp) is increased in IGROVcDDP (Table 2), and there is a corresponding increase in protein expression (Figure 1A). A 3-day treatment with low dose cisplatin tended to increase P-gp expression in both IGROV-1 and IGROVcDDP but this was not significant (Figure 1A). P-gp was also confirmed to be functionally active in IGROVcDDP with an epirubicin accumulation assay (Figure 1B). The IGROVcDDP cells have significantly lower levels of P-gp substrate epirubicin after a 2-hour exposure compared to IGROV-1. When IGROVcDDP was treated with 0.25 µM of the P-gp inhibitor elacridar, which prevents the action of the drug pump [12] the accumulated mass of epirubicin increased and was significantly higher than that of the parent IGROV-1 cells. The increase above the level of IGROV-1 is an interesting observation, and may be due to the IGROVcDDP cells being so dependent on P-glycoprotein for drug efflux; they suffer more accumulation of drug when it is inhibited.

IGROVcDDP cells were screened for their response to a variety of chemotherapeutics (Figure 1C). IGROVcDDP cells are significantly resistant to non-P-gp substrates cisplatin and carboplatin [13]. IGROVcDDP is also significantly resistant to P-gp substrates [14]; taxanes, paclitaxel and taxotere, the anthracycline epirubicin and vinca alkaloid vinblastine. In contrast, IGROVcDDP is hypersensitive to treatment with non-P-gp substrate 5-FU [15]. IGROVcDDP is resistant to MRPI substrate methotrexate [16] but not resistant to BCRP substrate SN-38 (Figure 1C). Treatment with 0.25 µM elacridar significantly reverses the resistance of the IGROVcDDP cells to all the P-gp substrates, but not the resistance to cisplatin, carboplatin and methotrexate. IGROVcDDP cells are also more sensitive to elacridar treatment than IGROV-1 (Table 3). IGROVcDDP cells have decreased mRNA expression of BCRP (Table 2) and it is not detectable by western blot (data not shown). This suggests that the reversal effects seen with elacridar treatment are specific to P-gp and not BCRP, which elacridar also inhibits.

The impact of co- or pre-treatment with cisplatin on paclitaxel cytotoxicity was investigated and no significant change was observed (data not shown). Similarly, co- or pre-treatment with paclitaxel did not reverse cisplatin resistance (data not shown).

Platinum resistance is associated with an intracellular shift of platinum uptake transporter CTR1 not resistance mediated by MRP2.

MRP2, a transporter which can efflux cisplatin conjugates, had decreased gene expression in IGROVcDDP (Table 2), but was not detectable by western blot in either cell line (data not shown). This suggests that there is no role of the platinum efflux transporter MRP2 in the platinum resistance of IGROVcDDP. Copper transporters can also play a role in platinum uptake (CTR1) and efflux (ATP7A and ATP7B) [17]. A decrease in CTR1 expression or increase in ATP7A or ATP7B could potentially mediate platinum resistance. The IGROVcDDP cells are 2.26 fold resistant to CuSO₄ (Figure 2A) suggesting that copper metabolism may play some role in the mechanism of resistance. There was no significant change in the mRNA expression CTR1, ATP7A and ATP7B on the TLDA array (data not shown). ATP7A protein expression tended to increase in IGROVcDDP in response to cisplatin, but this change is not significant (Figure 2B). However, there was a significant decrease in CTR1 expression, in response to cisplatin drug treatment in the IGROVcDDP cells (Figure 2C). CTR1 is present in the cell membrane in IGROV-1 and shifts intracellularly to the cytoplasm in IGROVcDDP (Figure 2D, E). There is some association of...
Figure 2. Copper Transporters in IGROV-1 and IGROVCDDP Cells. A) Cytotoxicity of IGROV-1 and IGROVCDDP to CuSO₄. B) ATP7A western blot. Open bars are IGROV-1, shaded bars are IGROVCDDP and striped bars indicate treatment with 0.67 μM cisplatin for 72 hours. Representative image shown. Graph shows quantitation of n = 4 biological repeats normalised to β-actin. C) CTR1 western blot. Representative image shown. Graph
CTR1 with the golgi in IGROVCDDP but the staining is consistent throughout the cytoplasm.

Transporters as Biomarkers of the Platinum Accumulation Defect

One of most significant differentially expressed genes in IGROVCDDP was a decrease in expression of the Na$^+/K^+$ pump (ATP1A1) (Table 2), which has previously been associated with platinum accumulation defects [18]. Cisplatin is not transported by ATP1A1 and altered membrane potential may play a role in the passive accumulation of the drug. There was a corresponding decrease in protein expression of ATP1A1 (Figure 3A) and also a sensitivity to treatment with the ATP1A1 inhibitor ouabain [19] (Table 3). However, when 0.01 nM ouabain was co-incubated in a cisplatin cytotoxicity assay rather than reversing the resistance in IGROVCDDP it decreased the IC$_{50}$ of both the IGROV-1 and IGROVCDDP cells equally, the fold resistance between the two cell lines remained constant (Table 3). IGROVCDDP was not resistant to NaCl or KCl as single agents and the addition of 40 mM of these salts did not significantly alter cisplatin cytotoxicity (data not shown).

Previous research has shown decreased expression and an intracellular shift of membrane proteins MRP1 and FBP to be associated with a defect in platinum accumulation in cisplatin-resistant cell lines [20]. Therefore we examined MRP1 and FBP as potential biomarkers of a defect in platinum accumulation in IGROVCDDP. The IGROVCDDP cells have a decrease in mRNA expression of MRP1 (Table 2) as well as a small decrease in MRP1 protein expression in response to cisplatin treatment (Figure 3B). MRP1 distribution was examined by confocal microscopy (Figure 3C,D). Staining in both IGROV-1 and IGROVCDDP cell lines is evident in the cytoplasm and perinuclear region with some accumulations of MRP1 apparent. In IGROV-1 there is more MRP1 above and throughout the blue line on the orthogonal view indicating it is in the apical and middle-section in the cells. A majority of staining in IGROVCDDP is below the blue line it is basolaterally located. FBP is localised mainly adjacent to the nucleus within discrete sub-cellular vesicles; little cytoplasmic staining is evident (Figure 3E,F). There is no change in cellular distribution of FBP between IGROV-1 and IGROVCDDP, but a decrease in expression of FBP in IGROVCDDP is apparent from the confocal images.

Platinum Resistance in IGROVCDDP is Associated with an Increase in Glutathione Recycling not Increased de novo Synthesis

The mRNA expression of glutathione reductase (GSR) and gamma glutamyl transpeptidase (GGT1) were both significantly increased in IGROVCDDP (Table 2). GSR functions to recycle oxidised glutathione within the cell [21,22] and GGT1 recycles glutathione from outside the cell membrane [21,22]. The protein expression of GSR and GGT1 were not increased in the IGROVCDDP cells, GSR was significantly decreased in IGROVCDDP and there was no change in GGT1. (Figure 4A and 4B). However, the enzyme activity of both GSR and GGT1 were significantly increased (Figure 4C and Figure 4D); suggesting that glutathione is being recycled more inside and from outside the cell.

IGROVCDDP cells do not have higher levels of glutathione, and levels are not increased with low-level cisplatin treatment (Figure 4E). The levels of glutathione were also more variable in the IGROVCDDP cells. Treatment with 12.5 μM buthionine sulfoximine (BSO), an inhibitor of γGCS [23] significantly decreased cellular glutathione in both the IGROV-1 and IGROVCDDP cells (data not shown). IGROV-1 and IGROVCDDP also have similar protein expression of γGCS and it is not upregulated in response to low-level cisplatin treatment (Figure 4F). BSO treatment also significantly sensitised both cell lines to cisplatin (Figure 4G). However, the effect was equivalent and the cisplatin fold resistance remained constant (18.76 fold). The IGROVCDDP cells tended to be more sensitive to BSO treatment alone in a cytotoxicity assay, however this was not significant (Figure 4G).

IGROVCDDP has Increased BRCA1 Expression

Increased expression of the DNA repair gene BRCA1 has been previously associated with cisplatin resistance [24]. IGROVCDDP cells have increased mRNA (Table 2) and protein expression of BRCA1 (Figure 5A).

Discussion

P-gp Overexpression is Unusual in a Model of Acquired Cisplatin Resistance

Resistance to paclitaxel in IGROVCDDP cells is mediated by an overexpression of P-gp at the gene (Table 2) and protein level (Figure 1A). P-gp has been shown to be functionally active by cytotoxicity assays (Figure 1C) and epirubicin accumulation assays (Figure 1B). In contrast to other studies [25], short-term cisplatin treatment did not modulate P-gp protein expression, activity or taxane cytotoxicity in IGROVCDDP cells (Figure 1A, 1B, data not shown). It is unusual but not unprecedented to see a model of acquired cisplatin resistance overexpress P-gp (Table 4) [26–30]. This most likely represents a generalised stress response to long-term cisplatin treatment as cisplatin is not a P-gp substrate [13]. P-gp can be up-regulated as part of a response to increased reactive oxygen species (ROS) within a cell [31]. This may be why P-gp expression was induced in IGROVCDDP as ROS are also produced in response to cisplatin [32]. However, as the IGROVCDDP cells are grown without cisplatin in the media, there appears to be either another stimulus favouring the expression of P-gp or P-gp is providing a selective advantage to IGROVCDDP cells.

Many models of acquired drug resistance will have overexpression of an transporter which effluxes the drug that was used to develop the model. Colchicine, a P-gp substrate [33] selected for P-gp overexpression in KB-8-5-11 cells [34] and epirubicin, a MRP1 substrate [35] induced MRP1 expression in CCRF-CEM/ E1000 [36]. However, methotrexate, fluorouracil, chlorambucil, cisplatin, and hydroxyurea have all been shown to transiently induce the expression of P-gp in K562 leukaemia cells when these drugs are not P-gp substrates [15]. It is then up to natural selection if the cells that transiently express P-gp have any other survival advantage and become part of the drug-resistant cell line. In some cisplatin-resistant models which overexpress P-gp, the P-gp has no survival advantage as is not functionally active (SNU-601/Gis10 - Table 4) [29]. Within cisplatin-resistant P-gp overexpressing cell
lines there can also be heterogeneity; in SKOV3/CIS P-gp positive and negative populations were maintained after treatment with cisplatin, indicating that P-gp has no survival advantage for cisplatin treatment [27]. However, P-gp can have anti-apoptotic effects distinct from those associated with transport of cytotoxic drugs, and some may be mediated through efflux of pro-apoptotic glucosylceramide [37,38]. It is also possible that some xenobiotic present in the FCS used to culture the cells could assist in maintaining the P-gp expression in IGROVCDPP.

IGROVCDPP is the only cisplatin-resistant model developed from IGROV-1 known to overexpress P-gp, and consequently have a platinum/taxane-resistant phenotype. Cisplatin-resistant models IGROV-1/Pt0.5 and IGROV-1/Pt1 [39] have the inverse platinum/taxane-resistant phenotype. Other cisplatin-resistant IGROV-1 models have been developed (IGROV-R10, IGROV-1/CP) but they do not appear to have been examined for resistance to P-gp substrates or P-gp expression. However, P-gp has not been identified as differentially expressed by genomic or proteomic profiling [40–44].

**Platinum Resistance is Multifactorial**

Platinum resistance in the IGROVCDPP cells is multifactorial and involves the glutathione pathway and decreased accumulation of drug. This could result either from a complex regulatory pathway which controls many different mechanisms for conferring resistance to cisplatin, or could reflect the fact that the cells were selected in multiple steps and could therefore have accumulated different mechanisms at each step.

IGROVCDPP cells are low-level resistant to CuSO₄ suggesting a role of copper transport in platinum resistance (Figure 2A). The expression of uptake transporter CTR1 is reduced in IGROVCDPP in response to cisplatin drug treatment (Figure 2C), which may contribute to the decrease in platinum accumulation previously reported [3]. CTR1 also shifts from being membrane associated in IGROV-1 to cytoplasmic staining in IGROVCDPP (Figure 2D). The loss of a cisplatin uptake transporter from the cell membrane in IGROVCDPP is likely to be the cause of decreased cellular accumulation of platinum [3]. These results suggest that CTR1 needs to be examined for cellular localisation by immuno-histochemistry (IHC), rather than by RT-PCR or Western blot to be useful as a biomarker of decreased accumulation of cisplatin. However, high levels of CTR1 as measured by RT-PCR and IHC have both been shown to be prognostic of sensitivity to platinum chemotherapy in ovarian cancer [35]. It has been shown with other biomarkers of platinum resistance such as ERCC1 that mRNA expression can be prognostic even if mRNA expression does not directly correlate with the functional role of the protein [46]. ERCC1 is a DNA repair protein and the measurement of gene and protein expression does not strictly correlate with DNA repair activity. It could be similar with CTR1, gene and protein expression being prognostic independent of predicting protein function. By also examining protein localisation the sensitivity and specificity of CTR1 as a biomarker may be improved.

Our results show that while total cellular glutathione is not increased in IGROVCDPP (Figure 4E), the way glutathione is recycled in the cell is enhanced. Increased enzyme activity of GSR (Figure 4C) indicates oxidised glutathione is being recycled more efficiently to its reduced form. Increased enzyme activity of GGT1 (Figure 4D) indicates that GSH is being recovered from outside the cell and the precursor amino acids transported to be available for synthesis of new glutathione inside the cell.

**Biomarkers of Decreased Platinum Accumulation**

Platinum accumulation defects mediated by decreased expression of ATP1A1 have been shown in H4-II-E/CDDP cisplatin resistant rat hepatoma cells [18]. The activity of ATP1A1 was previously associated with the mechanism of decreased cisplatin accumulation in IGROVCDPP as co-treatment with the inhibitor ouabain at a dose of 0.5 μg/mL reversed the decrease in accumulation [3]. Our cytotoxicity assays showed no reversal of platinum resistance when ouabain was added as an inhibitor (Table 3). The difference in results between studies may be the difference between a short-term high-dose ouabain treatment for an accumulation assay and a longer-term low-dose ouabain treatment in a cytotoxicity assay. IGROVCDPP cells are more sensitive to ouabain as a single agent (Table 3), consistent with the decrease in ATP1A1 protein (Figure 3A).

A mechanism has previously been described in cisplatin-resistant cell lines with platinum accumulation defects (KB-CP20 and 7404-CP20) in which surface expression of transporters is reduced, and some are overexpressed within cytoplasmic vesicles [47]. Protein expression of MR1 is reduced in KB-CP20 and 7404-CP20 [48] and the protein is localised with the golgi rather than the cell membrane [20]. This is similar to what is observed in IGROVCDPP, decreased mRNA expression of MR1 (Table 2), and decreased expression of MR1 protein in response to cisplatin drug treatment (Figure 3B) and altered localisation within the cell (Figure 3C,D). KB-CP20, 7404-CP20 and IGROVCDPP cells are all resistant to the MR1 substrate methotrexate due to the pump no longer being present on the cell surface, despite a decrease in protein expression [49]. Decreased expression and cytoplasmic localisation of FBP has also been associated with platinum accumulation defects in KB-CP20 and 7404-CP20 [49]. In IGROVCDPP the localisation of FBP does not change but the expression is decreased (Figure 4E,F). FBP is localised intracellularly in discrete vesicles near the nucleus rather than membrane associated. The altered localisation of MR1 and FBP in IGROVCDPP is not extreme as what is seen in KB-CP20 and 7404-CP20 cells [20]. It is clear that to use a shift in MR1 or FBP localisation as a biomarker of platinum accumulation defects the proteins must be strongly associated with the cell membrane in the parent cell line, in IGROV-1 this is not the case. Despite this caveat, a shift in MR1 localisation appears to be more useful as a biomarker of a platinum accumulation defect than gene or protein expression. It has been shown that MR1 gene [50] and protein expression [51] is not predictive platinum resistance in clinical ovarian samples, consistent with the results of this study. The localisation of MR1 has not yet been examined in clinical samples.
Figure 4. Glutathione pathway in IGROV-1 and IGROVCDDP cells. Open bars are IGROV-1, shaded bars are IGROVCDDP and striped bars indicate treatment with 0.67 μM cisplatin. A) Total intracellular glutathione. Graph shows n = 3 biological repeats normalised to cell number. B) γGCS western blot. Representative image shown. Graph shows quantitation of n = 4 biological repeats normalised to β-actin. C) GSR western blot. Representative image shown. Graph shows quantitation of n = 3 biological repeats normalised to β-actin. D) GSR enzyme assay. Graph shows n = 4 biological repeats normalised to cell number. E) GGT1 western blot. Representative image shown. Graph shows quantitation of n = 3 biological repeats normalised to β-actin. F) GGT1 enzyme assay. Graph shows n = 4 biological repeats normalised to cell number. * Indicates significant difference from IGROV-1 p<0.05 student’s t-test. # Indicates significant difference from IGROVCDDP on the addition of cisplatin. G) Modulation of cisplatin cytotoxicity of IGROV-1 and IGROVCDDP with BSO.
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Potential Biomarkers of Platinum/taxane Cross Resistance Versus Inverse Resistance

The IGROVCDDP cells have increased mRNA (Table 2) and protein expression of BRCA1 (Figure 5A), which may contribute to platinum resistance through increased DNA repair. This result is particularly interesting as previously we have associated an increase in BRCA1 with the inverse resistance phenotype; platinum resistance and taxane sensitivity [24]. While an increase in BRCA1 may mediate taxane sensitivity in some models [52,53] if there is an overriding mechanism of taxane resistance (such as P-gp) this effect is cancelled out. Therefore BRCA1 expression cannot be used as a molecular marker for platinum/taxane resistance status without also examining P-gp.

IGROV-1/Pt0.5 and IGROV-1/Pt1, platinum-resistant and taxane-sensitive cells, have increased cellular GSH and decreased GGT1 enzyme activity [39] which is the reverse pattern to that seen in the IGROVCDDP platinum/taxane-resistant cells. Further research is needed to determine if GGT1 activity could be used as a biomarker which could predict whether a cisplatin-resistant cell line is resistant or sensitive to paclitaxel.

Decreased expression of FBP was also seen in IGROV1/Pt 0.5 and IGROV1/Pt 1. However, decreased FBP appeared to be an effect if cisplatin resistance rather than a cause of it as transfection with FBP cDNA did not cause cisplatin sensitivity [54]. The IGROVCDDP, IGROV1/Pt0.5 and IGROV1/Pt1 cells all have decreased expression of FBP, therefore the expression of FBP may be a useful biomarker of platinum resistance but cannot be used to differentiate between taxane sensitivity and resistance.

**Figure 5. BRCA1 in IGROV-1 and IGROVCDDP cells.** Open bars are IGROV-1, shaded bars are IGROVCDDP and striped bars indicate treatment with 0.67 μM cisplatin for 72 hours. A) BRCA1 western blot. Representative image shown. Graph shows quantitation of n = 4 biological repeats normalised to β-actin. * Indicates significant difference from IGROV-1 p < 0.05 student’s t-test.

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**Table 4. Models of acquired cisplatin resistance which express P-glycoprotein.**

| Parent Cell Line | Cancer | Cisplatin IC50 | Resistant Cell Line | Cisplatin IC50 | Cisplatin Fold Resistance | P-glycoprotein Activity | Reference |
|------------------|--------|----------------|---------------------|----------------|--------------------------|-------------------------|----------|
| Colon LoVo      |       | 1.30 mg/ml     | C20.0               | 832±0.12 μg/ml | 6.4                      | Yes                     | [26]     |
| Ovarian SKOV3   |       | 1.19±0.03 mM   | SKOV3/CIS          | 442±0.3 μM    | 3.71                     | Yes                     | [27]     |
| Ovarian SKOV3/10|       | 0.68±0.06 μM   | SKOV3/DDP          | 992±0.34 μM   | 15.64                    | No                      | [28]     |
| Gastric SNU-601  |       | 0.19 μg/ml     | SNU-601/G10        | 92 μg/ml      | 44.42                    | ND                      | [30]     |
| Lymphoma (Rat)  |       | 900 nM         | WS                 | >100 μg/ml    | >226                     | ND                      | [30]     |
|                  |        |                |                     | 78.8          |                         |                         |          |

ND – Not Determined.

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Potential Treatment Strategies for Platinum/taxane Cross Resistant Ovarian Cancer

The only chemotherapy drug that IGROV CDDP was more sensitive to than IGROV-1 was 5-FU (Table 3). This suggests that 5-FU may be a suitable treatment for platinum/taxane resistant ovarian cancer. The changes in folate metabolism, as indicated by decreased expression of FBP (Figure 3E,F) may mediate this sensitivity to 5-FU. Several phase II clinical trials have examined capecitabine, a pro-drug of 5-FU, in platinum-resistant ovarian cancer. Platinum resistance was defined as progressive disease during or within 6 months of platinum treatment; patients in these studies had also received taxanes and therefore are most likely taxane resistant. The response rate of this population to capecitabine was poor 2.8–8.5% [55,56]. This is similar to the response seen with single-agent oxalplatin 7.6% [37] and is worse than retreatment with paclitaxel 35.3% [2]. Collateral sensitivity response seen with single-agent oxaliplatin 7.6% [37] and is worse than retreatment with paclitaxel 35.3% [2]. Collateral sensitivity is worse than retreatment with paclitaxel 35.3% [2]. Collateral sensitivity is worse than retreatment with paclitaxel 35.3% [2]. Collateral sensitivity is worse than retreatment with paclitaxel 35.3% [2]. Collateral sensitivity is worse than retreatment with paclitaxel 35.3% [2]. Collateral sensitivity is worse than retreatment with paclitaxel 35.3% [2].

Conclusions

P-gp overexpression is rare in a model of acquired cisplatin resistance. In the IGROV CDDP cells P-gp causes taxane resistance and overrides any potential taxane sensitivity mediated by increased MDR1 expression. Platinum resistance is multifactorial and is mediated by an increase in glutathione recycling and decreased accumulation of drug. The IGROV CDDP cells were sensitive to 5-FU and this class of chemotherapeutics warrants further preclinical research to determine if they are useful for the treatment of platinum/taxane resistant ovarian cancer.

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Author Contributions

Conceived and designed the experiments: BS SR MG. Performed the experiments: BS MH VM SR JPG. Analyzed the data: BS MH VM SR JPG MG. Contributed reagents/materials/analysis tools: JJO MC. Wrote the paper: BS.

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