Collateral Sensitivity to Cisplatin in KB-8-5-11 Drug-resistant Cancer Cells

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Abstract. *Background: KB-8-5-11 cells are a drug-resistant cervical cell model that overexpresses ABCB1 (P-glycoprotein). KB-8-5-11 has become sensitive to non-ABCB1 substrate cisplatin. Understanding the mechanism of collateral sensitivity to cisplatin may lead to biomarker discovery for platinum sensitivity in patients with cancer. Materials and Methods: A Taqman low-density array was used to characterize the expression of 380 genes previously associated with chemoresistance. Identified pathways were further analyzed using cytotoxicity assays, metabolomics and western blots. Results: KB-8-5-11 cells were sensitive to $CuSO_4$ and the glutathione inhibitor buthionine sulphoximine. Expression of ATPase, Cu^{2+} transporting alpha (ATP7A) and ATP7B were decreased at the protein and gene levels respectively in KB-8-5-11. KB-8-5-11 had decreased gene expression of glutathione S-transferase pi 1 (GSTP1), GSTA4 and GSTK1. Cisplatin treatment significantly lowered total cellular glutathione in parental KB-3-1 cells. Glutathione also tended to be lower in KB-8-5-11 cells compared to KB-3-1 cells. Conclusion: KB-8-5-11 cells have alterations in their copper transporters and glutathione metabolism, contributing to their cisplatin-sensitive phenotype.*

The chemotherapeutic drugs cisplatin and paclitaxel are used in the treatment of many solid tumours, including ovarian and cervical cancer. Cisplatin binds to DNA, hindering replication and triggering apoptosis. Paclitaxel causes cytotoxicity by binding to and stabilising polymerised microtubules. Due to their differing mechanisms of action, platinums and taxanes are often combined in cancer therapy.

When acquired paclitaxel resistance is produced in cell lines, only 17% are also resistant to cisplatin (1). A total of 41% of paclitaxel drug-resistant models are not resistant to cisplatin, and 28% of cell models become collaterally sensitive to cisplatin (1). This suggests that the majority of patients with cancer who have failed taxane-based therapy would benefit from receiving cisplatin. The challenge is how to identify which patients are potentially collaterally-sensitive to cisplatin.

The KB-8-5-11 drug-resistant cervical cancer cell line is resistant to paclitaxel and many other chemotherapy agents as it overexpresses the ATP-binding cassette (ABC) transporter P-glycoprotein (ABCB1) (2). We screened KB-8-5-11 for resistance to cisplatin and found that it had become hypersensitive relative to KB-3-1 parental cells. This article will characterize the mechanism of collateral sensitivity to cisplatin in KB-8-5-11 with the aim of developing biomarkers for detecting platinum sensitivity in patients with solid tumours.

Materials and Methods

Cell culture and cytotoxicity assays. KB-3-1 and its colchicine-resistant variant KB-8-5-11 (2) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma #D5671, Dublin, Ireland), containing 1% Pen strep, 2% L-glutamine and 1% Na pyruvate with 10% foetal calf serum (FCS) (Lonza, Verviers, Belgium). KB-8-5-11 cells were routinely grown with colchicine in the media and the drug was removed for three days prior to the start of all experiments. All cell lines were maintained in a humidified atmosphere with 5% CO_2 at 37˚C. All cultures were tested routinely and were *Mycoplasma*-free. To determine the cytotoxicity of chemotherapy drugs, cells were plated into flat-bottomed, 96-well plates at a cell density of 5×10^3 cells/well and allowed to attach overnight. Wells were treated in triplicate with serial dilutions of drug in a final volume of 200 μl and incubated for a further five days. Cell viability was then determined using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (3). Cisplatin was obtained from the St. James' Hospital pharmacy, Dublin, Ireland. Paclitaxel, $CuSO_4$ and BSO were obtained from Sigma, Dublin, Ireland. Elacridar was obtained from

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Table I. *Resistance profile of KB-3-1 and KB-8-5-11 cells.*

Drug (Units)	KB-3-1 IC_{50}	KB-8-5-11 IC_{50}	Resistant *vs.* sensitive			KB-3-1 +/- Elacridar	KB-8-5-11 +/- Elacridar
	Mean±SD	Mean±SD	Fold	*p*-Value	n	*p*-Value	*p*-Value
Cisplatin (μM)	0.56±0.12	0.308±0.044	–1.81	**<0.001**	7		
Paclitaxel (nM)	0.70±0.23	61.70±16.09	88.70	**<0.001**	4		
+ Elacridar 0.25 μM	0.71±0.04	0.93±0.16	1.31	**0.032**	4	0.921	**<0.001**
$CuSO_4$ (μM)	0.658±0.11	0.29±0.071	–2.27	**0.037**	5		
BSO (μM)	35.23±5.64	15.43±4.25	–2.27	**0.008**	3		

IC_{50}, Half maximal inhibitory concentration.

Santa Cruz Biotechnology, Heidelberg, Germany. Drugs were studied as single agents using the following dose ranges; cisplatin 0.05 μM – 13.3 μM, paclitaxel – 9.37×10^{-14} nM - 937 nM, $CuSO_4$ 1.88 nM – 4.88 μM and BSO 1 μM – 400 μM.

TaqMan low density array (TLDA). The TLDAs were performed as previously described (4).

Metabolomics. Cells were plated at a density of 4.21×10^5 cells per T75 flask and allowed to attach overnight. Cells were treated with 100 ng/ml cisplatin for 72 h, then trypsinised. The cells were then washed in 10 ml phosphate buffered saline (PBS), centrifuged and the supernatant removed. The pellets of cells were stored at –80˚C prior to analysis. The metabolomic profiling was carried out by Metabolon as previously described (5).

Western blots. Cells were grown and treated as described above for metabolomics. The pellets of cells were stored at –20˚C prior to analysis. The western blots were performed as previously described (4). Antibodies were used at a dilution of 1:1,000, unless otherwise indicated. The antibody to ATPase, Cu^{2+} transporting alpha (ATP7A) (rabbit) was a gift from Professor Anthony Monaco (6). The antibodies to β-actin (mouse, A5441), gamma-glutamyltransferase 1 (GGT1, mouse, WH0002678M1), glutathione reductase (GSR, mouse, WH0002936M1), anti-mouse horseradish peroxidase (HRP) (sheep, A6782), anti-rabbit HRP (goat, A4914) and anti-mouse alkaline phosphatase (rabbit, A4312) were obtained from Sigma, Dublin, Ireland. The antibody to solute carrier family 31 (copper transporter), member 1 (SLC31A1, rabbit, NB100-402) was obtained from Novus Biologicals, Cambridge, UK. The antibody to gamma-glutamylcysteine synthetase (GCLC, mouse, ab55435, dilution 1:500) was obtained from Abcam, Cambridge UK. The antibody to ABCB1 (mouse, ALX-801-002-C100, dilution 1:250) antibody was obtained from Alexis Biochemicals, now Enzo Life Sciences, Exeter, UK.

Statistical analysis. All experiments were performed at minimum in biological triplicate. Two-sample, two-tailed Student's *t*-tests were used to determine significant differences using $p\leq0.05$ as a cut off.

Results

KB-8-5-11 cells are collaterally sensitive to cisplatin. KB-8-5-11 cells were resistant to paclitaxel compared to parental KB-3-1 cells (88.70-fold, $p<0.001$, Table I). Paclitaxel resistance in KB-8-5-11 was reversed when cells were treated with 0.25 μM of the ABCB1 inhibitor elacridar (decrease to 1.31-fold, $p<0.001$, Table I). The KB-8-5-11 cells were also sensitive to cisplatin compared to parental KB-3-1 cells (-1.81 fold, $p<0.001$, Table I).

TLDA. Untreated KB-3-1 and KB-8-5-11 cells were analyzed for 380 genes associated with chemoresistance by the TLDA array in order to screen for potential mechanisms of platinum sensitivity. A total of 69 genes were found to be significantly differentially expressed between KB-3-1 and KB-8-5-11 cells, based on a cut-off of $p<0.01$. Genes reported in Table II were selected based on most significant changes as shown by *p*-value, as well as pathways previously associated with platinum sensitivity. Significant differences were observed in ABC transporters, copper transporters and glutathione metabolism.

ABC transporters. The KB-8-5-11 cell line overexpresses the ABC transporter ABCB1 at both the gene (8×10^3 fold, $p<0.001$, Table II) and protein level (6.9 fold, $p<0.001$, Figure 1A). Multidrug resistance-associated protein 2 (ABCC2) gene expression was also increased at a low level (1.8 fold, $p=0.013$, Table II) in the cisplatin-sensitive KB-8-5-11 cells.

Copper transporters. The KB-8-5-11 cells were sensitive to $CuSO_4$ compared to parental KB-3-1 cells (-2.27-fold, $p=0.037$, Table I). *ATP7B* gene expression was decreased in the KB-8-5-11 cells (-1.95-fold, $p=0.03$, Table II). In contrast, there was no change in gene expression of *ATP7A* or *SLC31A1* (Table II). ATP7A protein was significantly decreased in the KB-8-5-11 cells (-1.88-fold, $p=5.6\times10^{-6}$, Figure 1B) while no significant difference was observed for SLC31A1 (Figure 1C).

Glutathione metabolism. BSO is an inhibitor of glutathione synthesis. The KB-8-5-11 cells were sensitive to BSO compared to parental KB-3-1 cells (-2.27-fold $p=0.008$, Table I). Many glutathione-S-transferases (GSTs) showed low-level differences in gene expression between KB-3-1 and KB-8-5-11 cells (Table II). GSTA4, GSTK1 and GSTP1 all showed

Table II. *TLDA gene expression in KB-8-5-11 vs. KB-3-1 cells. Genes of interest by function or pathway.*

Gene	Full Name		Mean fold change	SD	*p*-Value
ABC Transporters					
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	↑↑	8041.65	947.66	**<0.001**
ABCC2	ATP-binding cassette, sub-family G (WHITE), member 2	↑	1.82	0.35	**0.013**
Copper Transporters					
ATP7A	ATPase, Cu^{2+} transporting alpha polypeptide	–	1.27	0.25	0.227
ATP7B	ATPase, Cu++ transporting, beta polypeptide	↓	–1.95	0.03	**0.026**
SLC31A1	Solute carrier family 31 member 1	–	–1.08	0.10	0.604
Glutathione Metabolism					
GSTA4	Glutathione S-transferase alpha 4	↓↓	–2.38	0.04	**0.004**
GSTK1	Glutathione S-transferase kappa 1	↓	–1.52	0.14	**0.024**
GSTM3	Glutathione S-transferase mu 3	↑	1.60	0.14	**0.003**
GSTP1	Glutathione S-transferase pi 1	↓↓	–2.09	0.01	**0.002**
GSTT2	Glutathione S-transferase theta 2	↑	1.48	0.37	**0.006**
GGT1	Gamma-glutamyltransferase 1	–	1.24	0.24	0.167
GSR	Glutathione reductase	–	–1.21	0.01	0.240
GSS	Glutathione synthetase	↑	1.29	0.16	0.038

↑↑, Greater than 2-fold increase; ↓↓, greater than 2-fold decrease; ↑, less than 2-fold increase; ↓, less than 2-fold decrease; -, no significant change in expression.

decreases in expression (-2.38 to -1.52-fold, $p \leq 0.024$, Table II), consistent with cisplatin sensitivity. In contrast, *GSTM3*, *GSTT2* and glutathione synthetase (*GSS*) were increased in gene expression (1.29-1.60 fold, $p \leq 0.038$, Table II). No change was observed in glutathione reductase (GSR) or γ-glutamyl transpeptidase (GGT1) at the gene (Table II) or protein level (Figures 1D and E). No change was observed in the protein expression of γ-glutamyl cysteine synthetase (GCLC) (Figure 1F).

Total cellular glutathione levels were measured by metabolomics. Cisplatin-treated KB-3-1 cells had a lower level of oxidised glutathione (-2.57-fold, $p=0.044$, Figure 2A), reduced glutathione (-1.55-fold, $p=0.007$, Figure 2B) and cysteine-glutathione disulphide (-1.84-fold, $p=0.004$, Figure 2C) compared to control KB-3-1 cells. KB-8-5-11 cells tended to have lower levels of oxidised and reduced glutathione, the decrease observed in cysteine-glutathione disulphide was significant (-1.82-fold, $p=0.045$, Figure 2C). These results suggest that a decrease in glutathione is a part of the initial response to cisplatin treatment in KB-3-1 cells and that this continues to be decreased in KB-8-5-11 cells.

Discussion

ABCC2 overexpression has been previously associated with cisplatin resistance in head and neck cancer cell lines, *via* a mechanism involving export of glutathione-platinum conjugates (7). In contrast, ABCC2-deficient rats have been shown to be hypersensitive to cisplatin (8). Low-level *ABCC2* gene expression in a cisplatin-sensitive model such as KB-8-5-11 is therefore unusual (Table II). Colchicine has been shown to be a low-affinity substrate for ABCC2 in the small intestine (9), while another study has shown that colchicine can induce ABCC2 expression in serum, in an animal model (10). Therefore, it is possible that the use of colchicine to maintain the paclitaxel-resistant phenotype in the KB-8-5-11 cells may induce *ABCC2* gene expression (Table II) and that this is unrelated to the observed cisplatin sensitivity. However, it should be noted that although colchicine was used to maintain the resistant phenotype it was removed from the media for the KB-8-5-11 cells three days prior to the start of all experiments.

KB-8-5-11 cells were collaterally sensitive to the glutathione inhibitor BSO (Table I), suggesting a shared mechanism with cisplatin. Glutathione is important in maintaining the redox potential of cells and also inactivates xenobiotics such as cisplatin (11). Consequently, glutathione has been shown to be positively-correlated with cisplatin resistance in a panel of ovarian cancer cell lines (11). The KB-3-1 cells decrease their levels of glutathione in response to cisplatin treatment and this trend is maintained in KB-8-5-11 cells (Figure 2). This suggests that decreased glutathione metabolism is in part mediating the cisplatin-sensitive phenotype of KB-8-5-11 cells. Glutathione depletion has been previously shown to induce ABCB1 expression in endothelial cells of the blood-brain barrier in rats (12). While the ABCB1 overexpression in KB-8-5-11 cells is stable due to DNA amplification, this amplification was preceded in the cell selection process from KB-3-1 by overexpression of the gene (2). Glutathione depletion in response to xenobiotics may be a first response of KB-3-1 cells, such as the one observed with low-level cisplatin treatment in

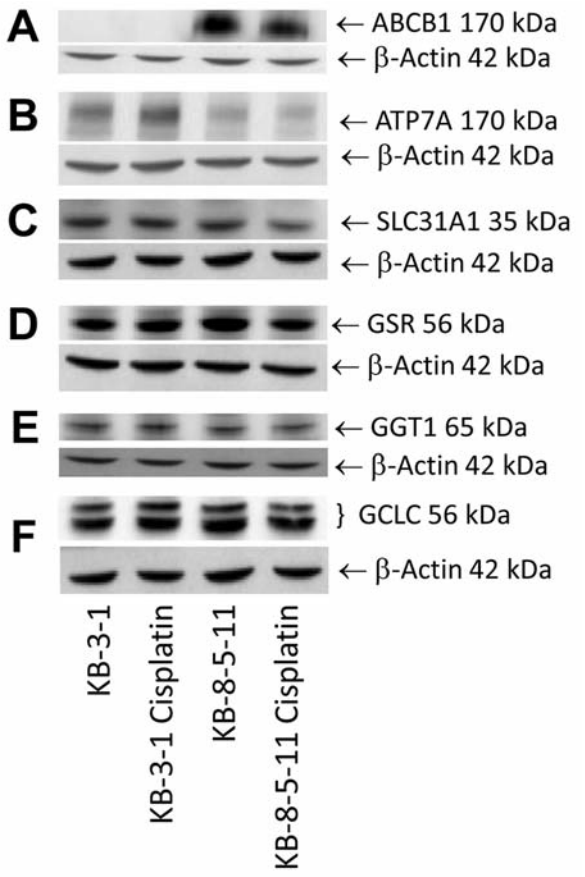

Figure 1. *Western blots of proteins from KB-3-1 and KB-8-5-11 cells in response to cisplatin treatment. KB-3-1 and KB-8-5-11 cells were treated for 72 h with 100 ng/ml of cisplatin and compared to a drug-free control. Western blots are shown for (A) ABCB1, (B) ATP7A, (C) SLC31A1, (D) GSR, (E) GGT1 and (F) GCLC. Representative images of four biological replicates are shown.*

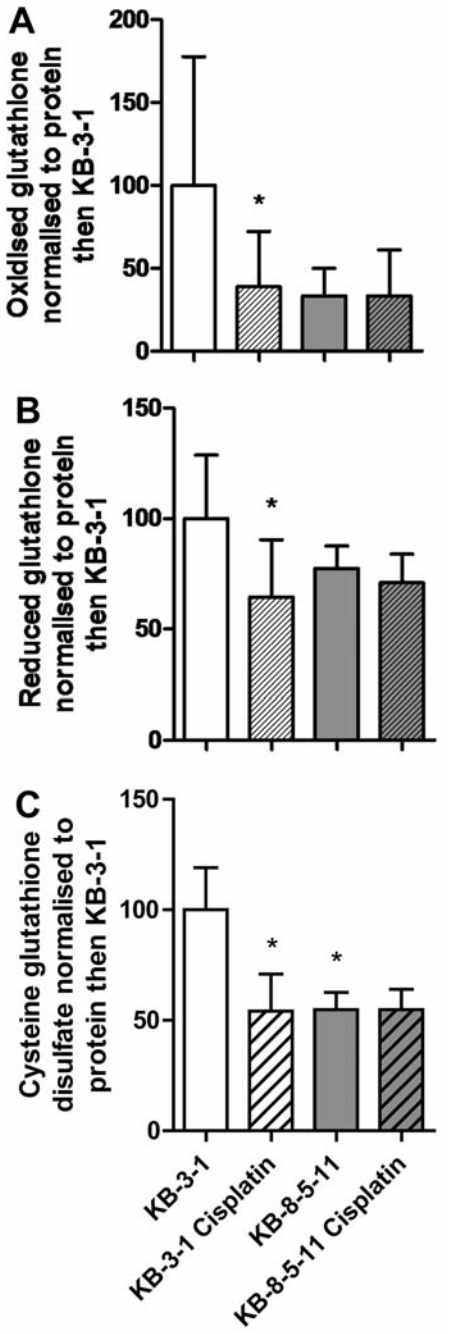

Figure 2. *Total cellular glutathione in KB-3-1 and KB-8-5-11 cells in response to cisplatin treatment. KB-3-1 and KB-8-5-11 cells were treated for 72 h with 100 ng/ml of cisplatin and compared to a drug-free control. Data are shown for (A) oxidised glutathione, (B) reduced glutathione and (C) cysteine glutathione disulfide. Graphs show means and standard deviation of five biological replicates *Indicates a significant difference compared to untreated KB-3-1 cells, $p<0.05$ Student's t-test.*

this study (Figure 2). Glutathione depletion may, therefore, have played a role in establishing the ABCB1 phenotype in KB-8-5-11 cells in response to colchicine treatment.

KB-8-5-11 cells were collaterally sensitive to $CuSO_4$ (Table I), suggesting a shared mechanism with cisplatin. Overexpression of the Cu^{2+} efflux transporter ATP7A has been associated with cisplatin resistance in ovarian cancer cells (13). Conversely, the KB-8-5-11 cells have a decrease in ATP7A protein expression (Figure 1B) and *ATP7B* gene expression (Table II). Cellular glutathione depletion has been shown to impair the copper transport function of ATP7A/B and increase the intracellular Cu^{2+} in CHO/K1 cells (14). Cellular reduced glutathione is required for the reduction of the ATP7A/B CXXC copper binding motifs by glutaredoxin, which then allows copper binding (14). Cisplatin has been shown to react with the same copper-binding motifs in ATP7B and ATP7A is likely to react in a similar fashion based on sequence homology (15). Glutathione depletion (Figure 2) and the lower protein expression of ATP7A (Figure 1B) may therefore interact to mediate the KB-8-5-11 cells sensitivity to Cu^{2+} and consequently to cisplatin.

ATP7A may be useful as a biomarker of cisplatin sensitivity. In ovarian cancer patients treated with platinum-based regimens, higher ATP7A expression, observed by immunohistochemistry, was associated with poorer survival times (16). Determining total cellular glutathione is technically challenging in clinical specimens, requiring for a large amount of fresh tumour tissue. This approach is, therefore, not very suitable for use as a clinical biomarker. Decreases in three of the GSTs (GSTA4, GSTK1 and GSTP1) in KB-8-5-11 cells were consistent with the platinum-sensitive phenotype. Inversely, increased gene expression of *GSTP1, GSTA4* and *GSTK1* have all been associated with the development of cisplatin resistance in a panel of cancer cell lines (17). GSTs have been studied extensively in clinical cancer samples, including investigation of polymorphisms that may increase cancer risk and affect response to chemotherapy. Increased expression of GSTP1 by immunohistochemistry is indicative of a poor prognosis in ovarian cancer patients treated with cisplatin (18).

Conclusion

KB-8-5-11 cells have a multi-factorial mechanism of collateral sensitivity to cisplatin, involving alterations in copper transporters and glutathione metabolism.

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