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**CARBOPLATIN AND TAXOL RESISTANCE DEVELOPS MORE RAPIDLY IN  
FUNCTIONAL BRCA1 COMPARED TO DYSFUNCTIONAL BRCA1 OVARIAN  
CANCER CELLS**

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## **Abstract**

A major risk factor for ovarian cancer is germline mutations of BRCA1/2. It has been found that (80%) of cellular models with acquired platinum or taxane resistance display an inverse resistance relationship, that is collateral sensitivity to the other agent. We used a clinically relevant comparative selection strategy to develop novel chemoresistant cell lines which aim to investigate the mechanisms of resistance that arise from different exposures of carboplatin and taxol on cells having BRCA1 function (UPN251) or dysfunction (OVCAR8). Resistance to carboplatin and taxol developed quicker and more stably in UPN251 (BRCA1-wildtype) compared to OVCAR8 (BRCA1-methylated). Alternating carboplatin and taxol treatment delayed but did not prevent resistance development when compared to single-agent administration. Interestingly, the sequence of drug exposure influenced the resistance mechanism produced. UPN251-6CALT (carboplatin first) and UPN251-6TALT (taxol first) have different profiles of cross resistance. UPN251-6CALT displays significant resistance to  $\text{CuSO}_4$  (2.3-fold,  $p=0.004$ ) while UPN251-6TALT shows significant sensitivity to oxaliplatin (0.6-fold,  $p=0.01$ ). P-glycoprotein is the main mechanism of taxol resistance found in the UPN251 taxane-resistant sublines. UPN251 cells increase cellular glutathione levels (3.0-fold,  $p=0.02$ ) in response to carboplatin treatment. However, increased glutathione is not maintained in the carboplatin-resistant sublines. UPN251-7C and UPN251-6CALT are low-level resistant to  $\text{CuSO}_4$  suggesting alterations in copper metabolism. However, none of the UPN251 sublines have alterations in the protein expression of ATP7A or CTR1. The protein expression of BRCA1 and MRP2 is unchanged in the UPN251 sublines. The UPN251 sublines remain sensitive to parp inhibitors veliparib and CEP8983 suggesting that these agents are candidates for the treatment of platinum/taxane resistant ovarian cancer patients.

**Key Words**

Resistance; comparative; selection strategy; ovarian cancer; BRCA1; carboplatin; taxol

## 1.0 Introduction

Ovarian cancer is the 5th most prevalent cancer amongst European women and is the leading cause of death from a gynaecological malignancy. The majority of patients present with late stage disease and have an approximately 30% 5-year survival rate (*Clarke-Pearson 2009*).

The standard treatment is surgical debulking followed by intravenous platinum-taxane combination chemotherapy (*Ozols 2005; Salzberg et al. 2005*). This treatment often fails and patients relapse with chemoresistant disease.

A strong family history of ovarian or breast cancer, which is often linked to BRCA1/2 germline mutations, is one of the greater risk factors associated with the disease. Deleterious germline mutations are found in 8.6-13.7% of ovarian cancer patients (*Pal et al. 2005; Risch et al. 2001; Rubin et al. 1998*). These mutations cause BRCA1 dysfunction leading to reduced expression of functional BRCA1. A recent study, examining both somatic and germline mutations in ovarian cancer, has revealed that incidence for BRCA1/2 mutations might be even higher at 18.3% (*Hennessy et al. 2010*). A woman with a BRCA1 mutation has a 39-46% chance of developing ovarian cancer (*Hennessy et al. 2009*). BRCA1 function has not been fully elucidated but it has been shown to have roles in a number of cellular processes including DNA damage repair, apoptosis, cell cycle regulation, transcriptional control and ubiquitination (*Kennedy et al. 2002; Murphy et al. 2010*).

A systematic review of the literature by (*Stordal et al. 2007*) revealed that the majority (80%) of cellular models with acquired platinum or taxane resistance displayed an inverse resistance relationship, that is collateral sensitivity to the other agent. A subsequent systematic review by (*Stordal et al. 2009*), revealed that BRCA1 was the mostly likely genetic player in this relationship. Cells with BRCA1 defects have reduced efficiency in repairing DNA adducts

and show increased apoptosis in response to platinum conferring sensitivity (*Foulkes 2006; Xing et al. 2006*). The response to taxanes, in BRCA1 deficient cells is reduced apoptosis conferring resistance (*Lafarge et al. 2001*). The opposite is true for cells with functional BRCA1 (*Quinn et al. 2003; Tassone et al. 2003*).

In this study, chemoresistant ovarian cancer cell lines were developed from established ovarian cancer cell lines using a novel comparative selection strategy. UPN251, which has functional BRCA1 (BRCA1 wild-type due to reversion mutation (*Stordal et al. 2013*)) and OVCAR8, which has dysfunctional BRCA1 leading to reduced BRCA1 expression (due to BRCA1 methylation) were used in order to investigate the development of chemoresistance in relation to BRCA1 status. This study highlights the effects of BRCA1 function and dysfunction on the development of resistance. In particular it focuses on its effects on the inverse resistance relationship between platinum and taxanes and its effect on alternating platinum and taxane doses.

## **2.0 Methods**

### **2.1 Cell culture**

The human ovarian cancer cell lines UPN251 and OVCAR8 were sourced from the MD Anderson Cancer Centre. Cells were grown in RPMI 1640 medium (Sigma R8758-500ML) supplemented with 10% foetal calf serum (Lonza DE14-801F), free of antibiotics. All cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Only cells at log phase of growth were used in experimentation. Cell lines were routinely checked for mycoplasma and were mycoplasma-free. The cell lines are both adherent, grow in a monolayer and are of epithelial serous histotype. UPN251 originated from a patient who had failed first line platinum/taxane chemotherapy and had relapsed after subsequent treatment of 8 rounds of single-agent taxol chemotherapy (personal communication, Hamilton). UPN251 is BRCA1 wild-type due to a secondary reversion mutation (*Stordal et al. 2013*). OVCAR8 was developed from a patient who had undergone treatment with high-dose carboplatin who exhibited progressive ovarian cancer (*Schilder et al. 1990*). OVCAR8 is BRCA1 wild-type but is methylated in the promoter region resulting in reduced gene expression of BRCA1 (*Stordal et al. 2013*). An overview of UPN251 and OVCAR8's BRCA1 and BRCA2 status is given in Table 1. The cell lines were short tandem repeats (STR) fingerprinted in order to confirm identity. Methylation status was examined and confirmed by Myriad Genetics.

### **2.2 Cytotoxicity assays**

Acid phosphatase cytotoxicity assays (*Yang et al. 1996*) were used to determine cytotoxicity as per method used by (*Stordal et al. 2013*). Cells were allowed to attach overnight and then received 5-day exposures to drugs. See supplementary material, Table S1 for list of

chemotherapy drugs used in this study including their molecular weight and conversion of 1unit/ml to unit MW.

### **2.3 Western blotting**

Western blotting was performed as per the method used by (*Stordal et al. 2012*). Primary and secondary antibodies used are listed in supplementary material, Table S2. Drug treated cells received 2µg/ml carboplatin or 15ng/ml taxol for 72 hours.

### **2.4 Total cellular glutathione assay**

Analysis of total cellular levels of glutathione (GSH) was carried out as per the method by (*Stordal et al. 2012*) which was adapted from (*Suzukake et al. 1982*). Plates were read and kinetics measured using the FLUOstar OPTIMA (BMG LABTECH) multifunctional microplate reader (405nm at 30°C).

### **2.5 Cell selection strategy outline**

Cell lines were treated with carboplatin or taxol as per Figure 1. Sublines were named in the format of 'Parental cell line-Round and Treatment'. For example UPN251-4T refers to UPN251 treated with 4 rounds of single-agent taxol treatment'. The round parameter can take the values '1'-'7' and the treatment parameter can be 'C' (single-agent carboplatin), 'CALT' (alternating treatment starting with carboplatin in round 1), 'T' (single-agent taxol) and 'TALT' (alternating treatment starting with taxol in round 1). Treatments were 4-5 weeks apart allowing for all cells to recover before subsequent drugging. For each round of selection:-

Cells were plated into a T25 flask at a cell density of  $2.6 \times 10^4$  cells per flask and drugged on day 2 as per the selection strategy outline (2 and 4µg/ml carboplatin and 60 and 12ng/ml for

UPN251 and OVCAR8 respectively). On day 5 drugged media was removed and replaced with fresh drug-free media. Over subsequent days all T25 flasks were examined for confluence using a novel method to calculate an area fraction output (*Busschots et al. 2015*). Upon reaching confluence, cells were re-seeded into a T75 flask. Leftover cells were used to freeze stocks. Cytotoxicity assays were performed at 1 week intervals for 3 weeks and were compared to the parental lines in order to calculate fold resistance. Once all cells had recovered, the next round of drugging commenced following the same format as above (provided the cells were 4 weeks after drugging, otherwise drugging was delayed until this time).

## **2.6 Statistics**

All experiments were repeated at a minimum in biological triplicate excluding the cell selection strategy. Statistical significance analysis was performed by student's t-test in Microsoft Excel using a two tailed analysis and two samples of equal variance settings.

## 3.0 Results

### 3.1 Parental BRCA1 protein expression

The BRCA1 protein expression of OVCAR8 (methylated) and UPN251 (un-methylated) was examined by western blotting. OVCAR8 has  $26\% \pm 7\%$  of the expression of UPN251 ( $p=3.3 \times 10^{-4}$ , Figure 2). This correlates with the BRCA1 methylation status of the cell lines.

### 3.2 Cell selection strategy

The baseline  $IC_{50}$  values of OVCAR8 were carboplatin  $1.3 \pm 0.2 \mu\text{g/ml}$  ( $n=11$ ) and taxol  $1.2 \pm 0.2 \text{ng/ml}$  ( $n=10$ ). The baseline  $IC_{50}$  values of UPN251 were carboplatin  $0.8 \pm 0.1 \mu\text{g/ml}$  ( $n=7$ ) and taxol  $17.9 \pm 5.6 \text{ng/ml}$  ( $n=9$ ). OVCAR8 has a slightly higher baseline  $IC_{50}$  to carboplatin and a much lower baseline  $IC_{50}$  to taxol when compared to UPN251.

#### 3.2.1 Dose optimisation

Doses of drug for carboplatin and taxol used in the selection strategy were selected from the following ranges respectively: - UPN251 ( $0.7\text{-}2 \mu\text{g/ml}$ ,  $10\text{-}100 \text{ng/ml}$ ) and OVCAR8 ( $2.3\text{-}18.5 \mu\text{g/ml}$ ,  $2.3\text{-}14 \text{ng/ml}$ ). Ranges were selected from the results of 3-day cytotoxicity assays on parental cell lines (Table S3) initially encompassing inhibitory concentration (IC) values ranging from 20-80. Clinical relevance was validated by investigating clinical trial publications and using pharmacokinetic studies to translate doses from the clinic into usable doses in the laboratory.

For carboplatin and taxol a dose range of up to  $20 \mu\text{g/ml}$  and  $120 \text{ng/ml}$  respectively was deemed clinically relevant following pharmacokinetic studies for a dose of carboplatin at AUC 5 and taxol at  $175 \text{mg/m}^2$  which are often administered to patients in clinical trials as single agents (*du Bois et al. 1997; Go et al. 1999; ICON3 2002; Joerger et al. 2006;*

*Markman et al. 2010; Mross et al. 2000; Oguri et al. 1988; Ozols et al. 2003; Pfisterer et al. 2006; Rowinsky 1997; Vasey et al. 2004).*

Cells were subjected to 3-day drug exposures and the time taken for cells to recover was recorded and compared to a drug free control. Desired criteria for the selected doses were that cells would display an initially large amount of cell death (>95%) followed by growth to confluence after drug exposure. Carboplatin doses of 4 $\mu$ g/ml and 2 $\mu$ g/ml and taxol doses of 12ng/ml and 60ng/ml were chosen for OVCAR8 and UPN251 respectively. From the recovery plots (Figure 3 (A) and (B)) we can see that recovery from taxol differed from carboplatin. Taxol treated cells saw a sharp decline in cell number over the first number of days followed by a quick return to confluence thereafter. With carboplatin a more prolonged decline and recovery was noted.

### **3.2.2 Recovery**

In general, all cells recovered quicker after drugging as the rounds of selection progressed. Figure 4 (A) shows recovery plots for each cell line grouped per ascending rounds of selection. In round 7 single-agent treatments received twice the usual dose and consequently recovery time increased. UPN251 cells recovered quicker than OVCAR8 cells and in both cell lines it took longer to recover from carboplatin treatments than taxol. Figure 4 (B) shows the sublines that were treated with carboplatin in each round grouped together for comparison purposes. Single-agent carboplatin treatments (solid bars) were compared with alternating treatments (dashed bars) that received carboplatin in each round. There was little difference in recovery between cell lines receiving treatment with the alternating agents compared to cell lines receiving single-agent carboplatin. Round 2 is the only exception to this. Figure 4 (C) shows the same as above but for taxol treatments. In this case alternating treatments always took longer to recover than single-agent treatments when receiving taxol.

### 3.2.3 Fold resistance

The fold resistance of each subline at weekly intervals for 3-weeks in each round of selection for carboplatin is shown in Figure 4 (D) and (E) and for taxol in Figure 4 (F) and (G) for UPN251 and OVCAR8 respectively. By round 6, UPN251-6T treated solely with taxol displayed the highest level of resistance (7-fold,  $p=0.1 \times 10^{-5}$ ). The sublines developed from UPN251 showed higher levels of resistance compared to those developed from OVCAR8. UPN251-6CALT, UPN251-6T and UPN251-6TALT all had significant resistance to taxol (4-8 fold,  $p=0.4 \times 10^{-6}$ - $0.6 \times 10^{-6}$ ) while OVCAR8-6CALT, OVCAR8-6T and OVCAR8-6TALT all had significant resistance to taxol but to a lower extent (1.5-2.5 fold,  $p=0.02$ - $0.2 \times 10^{-6}$ ). All UPN251 sublines after their final round of selection (including UPN251-7T treated only with taxol) had significant resistance to carboplatin (1.6-3.5 fold,  $p=0.3 \times 10^{-3}$ -  $0.5 \times 10^{-6}$ ). Only OVCAR8-7C and OVCAR8-6TALT had significant resistance to carboplatin in the OVCAR8 sublines (1.3-2.6 fold,  $p=0.04$ - $0.3 \times 10^{-3}$ ). But again this was lower than in UPN251 sublines. In as early as the first round of selection UPN251-1C and UPN251-1T was significantly resistant to carboplatin (1.5-fold,  $p=0.3 \times 10^{-2}$ ) and taxol (1.7-fold,  $p=0.8 \times 10^{-2}$ ) respectively. These sublines retained significant resistance with fold resistance increasing from round to round. All UPN251 sublines receiving the opposite selecting agent in round 2 retained some degree of significant resistance to carboplatin, except UPN251-2TALT. However UPN251-2TALT regained a significant level of resistance by round 3.

The cell lines were stable in culture for up to 6 weeks after defrost at which point resistance began to decline (Supplementary material (Figure S1)). Experiments were therefore performed in 6 week blocks.

Figure 5 (A) shows the extent of resistance development after 6 rounds of selection for single-agent treatments in OVCAR8 and UPN251 sublines. This was examined to investigate whether cells with BRCA1 defects (OVCAR8) would develop resistance to platinum slower than taxanes with the opposite being true for cells with functional BRCA1 (UPN251). We found that taxol resistance developed quicker in both models irrespective of BRCA1 status.

Figure 5 (B (i) & (ii)) shows the results of fold resistance after 6 rounds of selection for carboplatin and taxol respectively. This was investigated as we hypothesised that cells receiving alternating treatments of carboplatin and taxol should develop resistance slower or not at all when compared to single-agent treatments. We can see from these graphs that single-agent treatments have higher fold resistance than all of the alternating treatments.

Figure 5 (C (i) & (ii)) shows the results of fold resistance compare the point in time when each subline had received 3 doses of carboplatin or taxol respectively. In this case resistance has developed at the same rate in alternating treatments compared to single agent administration in OVCAR8 and resistance has developed quicker in alternating treatments compared to single agent administration in UPN251.

### **3.3 Mechanisms of drug resistance in UPN251 sublines**

Investigation of drug resistance mechanisms were carried out on UPN251 sublines only. OVCAR8 sublines were not examined any further as they developed only low levels of unstable resistance (Figure 4 (E & G)).

#### **3.3.1 Drug screen**

A drug screen was performed in order to evaluate cross resistance to other drugs and to help elucidate resistance mechanisms that have developed in the cells. 11 drugs and 2 inhibitors were used. Inhibitors include, buthionine sulphoximine (BSO) an inhibitor of glutathione

(GSH) (*Drew et al. 1984*) and elacridar an inhibitor of P-glycoprotein (P-gp) a member of the ATP binding cassette (ABC) transporter family (*Hyafil et al. 1993*). Table 2 gives a summary of all cytotoxicity data collected.

All of the sublines of UPN251 were significantly resistant to carboplatin (fold change=1.5 - 3.2,  $p=0.2 \times 10^{-2}$  -  $0.5 \times 10^{-8}$ ), with single-agent carboplatin developed UPN251-7C being the highest. The addition of 12.5  $\mu\text{g/ml}$  BSO had the effect of lowering  $\text{IC}_{50}$  values across all UPN251 sublines. UPN251 and all sublines (except UPN251-7T) showed significant decreases ( $p=0.0003$ - $0.008$ ). Fold resistance however, stayed at a similar level. Significant cross resistance to cisplatin and copper sulphate ( $\text{CuSO}_4$ ) was also seen in sublines developed with carboplatin treatments. One exception to this is UPN251-6TALT, which is not resistant to  $\text{CuSO}_4$ . Oxaliplatin showed significant cross resistance for sublines developed as single-agent treatments while alternating treatments showed no significant cross resistance. UPN251-6TALT showed collateral sensitivity to oxaliplatin (fold change=0.6,  $p=0.01$ ).

The UPN251 sublines developed with taxol all have significant taxol resistance (fold change=4.3-9.0,  $p=0.5 \times 10^{-3}$ - $0.2 \times 10^{-7}$ ), with UPN251-7T having the highest fold resistance. UPN251-7C developed with carboplatin was not resistant to taxol. Comparing taxol, with and without 0.25  $\mu\text{g/ml}$  elacridar, across all cell lines reveals significant drops in  $\text{IC}_{50}$  values ( $p=0.1 \times 10^{-3}$  to  $0.4 \times 10^{-7}$ ). An almost identical trend is seen with vinblastine, and olaparib  $\pm$  elacridar which are all P-gp substrates (*Choudhuri et al. 2006; Lawlor et al. 2014*).

Doxorubicin and docetaxel also displayed crossresistance in sublines developed with taxol. A significant degree of collateral sensitivity to docetaxel was observed in UPN251-7C (fold change=0.4,  $p=0.002$ ).

Parp inhibitors veliparib and CEP8983 both showed no significant change in IC<sub>50</sub> when compared to the parental cell lines and could both be candidates for treating platinum/taxane-resistant ovarian cancer.

### **3.3.2 Total cellular glutathione assay**

Using a total cellular glutathione (GSH) assay (Figure 6) no significant difference in GSH levels were seen when UPN251 sublines were treated with carboplatin. However UPN251 parental cells saw a significant 3-fold increase in total cellular GSH levels with the addition of 2µg/ml carboplatin (p=0.02) for a 3-day exposure. Treatment with 12.5µmol BSO for a 3-day exposure gave significantly reduced levels of GSH for UPN251 and its sublines, when compared to treatment free control cells (fold reduction= 9.3-27.8, p=0.5x10<sup>-2</sup>- 0.02). This was the same dose of BSO which was used in our drug screen.

### **3.3.3 Post selection western blots**

P-gp protein expression for UPN251-6CALT and UPN251-7T (control and taxol treated) and UPN251-6TALT (taxol treated) are significantly up-regulated when compared to UPN251 control (Fold Change= 2.3±0.9-7.3±2.8, p=0.04-0.003, Figure 7 (A)). P-gp is significantly decreased in UPN251-7C (carboplatin treated). There are no significant changes in protein expression for both ATP7A (Figure 7 (B)) and CTR1 (Figure 7 (C)) when compared to UPN251 control. MRP2 was not expressed in UPN251 and resistant sublines (supplementary material Figure S2 (A)). There was no change in BRCA1 protein expression between UPN251 parental cells and UPN251-7C and UPN251-7T resistant sublines (supplementary material Figure S2 (C)).

## **4.0 Discussion**

### **4.1 Resistance models**

The mechanism of resistance that develop in a drug-resistance model can differ depending on the method of selection used. The most common methods of selection used to model resistance are increasing continuous administration (*Liu et al. 2010; Smith et al. 2002; Vandier et al. 2000*) and low-dose intermittent incremental inducement (*Godwin et al. 1992; Kars et al. 2006; Teicher et al. 1986*) where cells are exposed sporadically to increasing doses of drug over time. A number of studies have used a pulsed strategy of a 4-hour drug exposure at weekly intervals for 10-12 weeks (*Glynn et al. 2004; Liang et al. 2004; Ying et al. 2012*). Our model has 1 prolonged pulse over 3 days and then recovery in drug free media for 4-5 weeks, which is a more accurate representation of the clinical setting in ovarian cancer, where patients receive drug infusion every 3-4 weeks (*Ozols 2005*). (*Yan et al. 2007*) compare the differences in using the pulse versus intermittent incremental strategy in the same ovarian cancer cell lines. They found great differences in the resistance mechanisms that appeared from both strategies. The consensus was that although the intermittent incremental strategy produced higher levels of fold resistance, the mechanisms evolved using the pulse strategy were closer to the mechanisms seen in the clinic and serves as a more ‘appropriate’ model in studying drug resistance in ovarian cancer. Therefore mechanisms produced from this study have the potential to closely mirror the clinical mechanisms of resistance for ovarian cancer.

### **4.2 Resistance development**

We hypothesised that ovarian cancer cells with BRCA1 defects (OVCAR8) would develop resistance to platinum slower than taxanes with the opposite being true for cells with

functional BRCA1 (UPN251). Figure 5 (A) shows the extent of resistance development after 6 rounds of selection for single-agent treatments. This hypothesis holds true for OVCAR8 sublines, but not for UPN251 sublines, as taxol resistance developed quicker in both models irrespective of BRCA1 status. Possible reasons for this occurrence may be that taxol treated cells recover quicker than carboplatin-treated cells, and therefore resistance can develop faster in taxol-treated cells.

We hypothesised that cells receiving alternating treatments of carboplatin and taxol should develop resistance slower or not at all when compared to single-agent treatments. On first inspection this seems to hold true. Figure 5 (B) shows the results of fold resistance after 6 rounds of selection for taxol and carboplatin. We can see from these graphs that single-agent treatments have higher fold resistance than all of the alternating treatments. Alternatively, however, if we compare the point in time when each subline had received 3 doses of taxol (Figure 5 (C) (i)) or 3 doses of carboplatin (Figure 5 (C) (ii)) during the course of the treatment strategy and compared the extent of resistance development, the opposite conclusion could be reached. In UPN251, despite the fact that alternating treatments received the same amount of taxol or carboplatin over a longer period of time when compared to single-agent treatments (5 or 6 rounds in alternating versus 3 rounds for the single-agent), resistance development was higher in alternating treatments when compared to single-agent treatments. In OVCAR8, alternating and single-agent treatments are at a similar resistance level. This ambiguity in our results may stem from an inability to directly compare the results of the 2 drugs due to their different mechanisms of action and speed of recovery from drug treatment.

An interesting finding from the selection strategy is that cells treated with taxol, having received carboplatin in the previous round, show large increases in taxol resistance, larger than the increase seen when cells were treated with taxol in the previous round (Figure 4 (E)). Cells with carboplatin pre-treatment also take longer to recover compared to cells which have only received taxol as seen from our area fraction data (Figure 4(C)). Carboplatin therefore seems to enhance a cell's capacity to become taxol resistant. Further to this, alternating treatments generally displayed notable jumps in taxol resistance in a round in which they received taxol. This is usually greater than the increase in resistance seen per round from single-agent taxol treatments. This is the opposite of what we would have predicted given the inverse resistance relationship between platinum and taxanes (*Stordal et al. 2009*). We hypothesised that pre-treatment with one agent would sensitise to the other. We saw no evidence of an opposite effect. Taxol pre-treatment did not affect the amount of carboplatin resistance that developed.

In the literature an in vitro study showed that when cisplatin preceded taxol treatment, lessened antitumor activity was seen when compared to taxol before cisplatin (*Kano et al. 1996*). In ovarian cancer cell lines the sequence of cisplatin before taxol reduces taxol induced apoptosis. This was found using DNA fragmentation assays, fluorescence microscopy and flow cytometry (*Judson et al. 1999*). An in vivo mouse study showed that this sequence (cisplatin then taxol) had significant increases in morbidity and mortality associated with it when compared with taxol before cisplatin (*Milross et al. 1995*). In the clinic taxol is given 3 hours before carboplatin in order to circumvent carboplatin's myelosuppressive effects (*Ozols et al. 2003*). Taxol reduces the proportion of bone marrow precursors circulating at the time when carboplatin is given which reduces toxicity when compared to the opposite administration. In non-small cell lung cancer clinical studies, with

chemotherapy naive patients, the sequence of carboplatin then taxol administration in combination treatments, showed no sequence-dependant toxicities or pharmacokinetic interactions. However it is not clear whether the different sequences affected response data (*Giaccone et al. 1995; Huizing et al. 1997*).

Some evidence which supports the inverse resistance relationship hypothesis is that increased P-gp expression was seen in sublines which had taxol treatment during selection while decreased expression was seen in UPN251-7C (carboplatin treated) (Figure 7 (A)). Long term monitoring of UPN251-7C's resistance to taxol showed significant sensitivity to taxol (data not shown). Down regulation of P-gp in this cell line may explain this occurrence. As UPN251-7T is resistant to taxol and has high P-gp expression it may imply that P-gp is involved in the mechanism of the inverse resistance phenotype.

Having received an equivalent cytotoxic drug treatment as UPN251 (BRCA1-wildtype), OVCAR8 (BRCA1-methylated) developed much less resistance to carboplatin or taxol over the same time period. All OVCAR8 sublines were less than 2-fold resistant to carboplatin and less than 2.5-fold resistant to taxol after 6 rounds of selection. This may be due to the cells BRCA1 methylation status. Cells deficient in BRCA1 have reduced efficiency in repairing DNA damage caused by cytotoxic agents. It has been shown that hypermethylation of the BRCA1 promoter region causes increased sensitivity to platinum drugs (*Teodoridis et al. 2005*). Also in two ovarian cancer cell lines decreasing BRCA1 mRNA using inhibition assays correlated to increased sensitivity to platinum (*Quinn et al. 2007*). They also show that patients with low/intermediate levels of BRCA1 mRNA have a significantly improved overall survival following platinum-based chemotherapy compared to patients with high levels of BRCA1 mRNA. (*Zhou et al. 2003*) showed that the ovarian cancer cell line SNU251,

having a mutation in BRCA1 inhibiting its sub-nuclear assembly, increased its sensitivity to taxol. Also UPN251 and OVCAR8 have had different baseline sensitivities to the drugs used as they originated from patients who had had different levels of exposures to carboplatin and taxol which may have affected the development of resistance.

One caveat to our ability to directly compare resistance developed with carboplatin and taxol is that these drugs may not be directly comparable to each other, due to their different rates of recovery after drugging. This was seen in our dose finding experiment for the selection strategy (Figure 3) and in the selection strategy itself (Figure 4). Cells treated with taxol show high initial cell death followed by fast recovery, while carboplatin showed much slower recovery with slight elevations in cell number after drugging, followed by cell death and slow recovery. This difference could be due to the differences in platinum and taxane mechanisms of action. Platinums act mainly by forming nuclear platinum adducts on DNA strands (*Fuertes et al. 2003; Go et al. 1999*), while taxanes act by stabilising microtubules within the cell (*Manfredi et al. 1984; Rao et al. 1995*). Another caveat is that the cell lines used in this study were of different genetic backgrounds having been obtained from different patients. A number of different elements may be at play that has the potential to affect our results. Future studies could be carried out in a BRCA1 mutant cell model and a transfected model where BRCA1 functionality is restored such as UWB1.289 and UWB1.289-BRCA1 (*DelloRusso et al. 2007*).

#### **4.2.1 Mechanisms of taxol resistance**

Taxol resistance cell models are very common and many have been developed for ovarian cancer cell lines (*Duan et al. 1999a; Duan et al. 1999b; Hari et al. 2006; Parekh et al. 1997; Zhang et al. 2012*). Most of these models use different variations on the above mentioned

intermittent incremental inducement and increasing continuous administration strategies. Our work is novel as a pulsed strategy which closely mirrors the clinic has not been used before in ovarian cancer and a model of taxane-resistance has not been previously developed in UPN251 to our knowledge.

Over-expression of P-gp often arises as a mechanism of taxol resistance in cell models. P-gp is the main mechanisms of taxol resistance in our models. Cytotoxicity assays for P-gp substrates taxol, vinblastine and olaparib  $\pm$  elacridar (Table 2) all show highly significant drops in IC<sub>50</sub> when P-gp is blocked with elacridar. Western data showed increased P-gp expression for sublines which had taxol treatment during selection (Figure 7 (A)). Therefore it is likely that taxol is being actively pumped out of the cell by P-gp causing taxol resistance.

#### **4.2.2 Mechanisms of carboplatin resistance**

Carboplatin resistant ovarian cancer cell lines are rare in the literature. This is most likely because a combination of cisplatin and taxol was the standard chemotherapy treatment for advanced ovarian cancer before 2003, until carboplatin and taxol was deemed more favourable due to reduced toxicities associated with carboplatin (*du Bois et al. 2003; Ozols et al. 2003*). A publication by (*Li et al. 2004*) reports on the development of 5 resistance cell models for ovarian cancer cell lines (2 carboplatin, 2 cisplatin and 1 taxol). They found a number of genes which were differentially expressed compared to parental cells across all resistant models. Another study has developed carboplatin resistant sublines from human larynx carcinoma cell line Hep 2 by continuous 5-day exposure of increasing doses of carboplatin. All of the 3 sublines developed had elevated levels of GSH, but only one of these had significant elevations (*Osmak et al. 1995*).

From the results of our GSH assays (Figure 6) we can see that only UPN251 showed a significant increase in total cellular GSH levels in response to carboplatin treatment. The developed UPN251 sublines had no significant increases compared to UPN251 with a carboplatin treatment of 2µg/ml. This suggests that elevated GSH plays a role in the parental cells initial response to carboplatin and that UPN251 resistance sublines utilise other mechanisms. Treatment with a 12.5µM dose of BSO significantly decreases GSH in UPN251 and all sublines. This was the same dose of BSO used in our post selection drug screen with carboplatin (Table 2). Small but significant drops in IC<sub>50</sub> were noted in UPN251 sublines, but no difference in fold change was noted. This indicates that increased total cellular GSH may not be a major mechanism of carboplatin resistance in our developed models.

CuSO<sub>4</sub> (Table 2) had significantly higher IC<sub>50</sub>'s for UPN251-7C and UN251-6CALT. This indicates the possible involvement of copper transporters ATP7A, ATP7B and CTR1 in carboplatin resistance (*Safaei et al. 2005*). However western blots for ATP7A and CTR1 showed little difference in protein expression. These proteins may instead be relocated to different parts of the cell causing a resistance phenotype. An increase of ATP7A and ATP7B in the cellular membrane or a relocation of CTR1 to the golgi apparatus may lead to platinum resistance without a change in protein expression (*Stordal et al. 2012*).

#### **4.2.3 Combined resistance to platinum and taxanes**

Models of taxane-platinum resistance are rare in the literature. One study developed a taxane-platinum resistant model for non-small cell lung cancer by exposing the cells to cycles of taxol and carboplatin, two cytotoxic agents with different mechanisms of action (*Dalvi et al. 2012*). Another study developed a dual carboplatin and docetaxel resistant subline from A2780 ovarian cancer cells which are cross resistant to both agents as well as two singularly

resistant sublines resistant to each agent but not cross resistant to the other. All of the sublines were selected for in parallel (*Armstrong et al. 2012*). Gene profiling revealed that the dual model contains genetic changes not present in the singularly resistant models demonstrating that combined drug resistance may not be a simple combination of changes present in single-agent resistant cell lines but can contain novel changes.

Our model presents this novel aspect of subline development for ovarian cancer where sublines were exposed to alternating sequences of taxol and carboplatin. As a result UPN251-6CALT and UPN251-6TALT show significant cross resistance to both carboplatin and taxol used in their development. They show a carboplatin fold resistance of 1.7 and 2 and a taxol fold resistance of 4.3 and 5.7 respectively (Table 2). This is less than the UPN251 sublines selected with single agents but these have no significant cross resistance to carboplatin or taxol except UPN251-7T which is 1.5 fold resistant to carboplatin.

The cytotoxic agent which our sublines were exposed to first influenced the mechanisms of resistance that arose. UPN251-6CALT and UPN251-6TALT both received 3 rounds of drugging with carboplatin and taxol. The only difference is that UPN251-6CALT received carboplatin in the first round whereas UPN251-6TALT received taxol. As a result UPN251-6CALT displays significant fold resistance to CuSO<sub>4</sub> (2.3 fold, p=0.004) while UPN251-6TALT shows no significant fold resistance. Also UPN251-6TALT shows significant sensitivity to oxaliplatin (p=0.01) while UPN251-6CALT has no significant fold change. This would indicate different resistance mechanisms being selected in these cells depending on initial drug exposure. Also, as neither UPN251-7C nor UPN251-7T had significant sensitivity to oxaliplatin while UPN251-6TALT did, this may indicate a novel mechanism of resistance being generated between this dual carboplatin/taxol resistant model and our singularly non-

cross-resistant models. This evidence is supported by the finding of (*Armstrong et al. 2012*) discussed above.

### **4.3 Treatment options for platinum/taxane resistant ovarian cancers**

The baseline IC<sub>50</sub> values of parp inhibitors CEP8983 and veliparib in OVCAR8 was 2.02±0.3µg/ml (n=6) and 5.8±1.1µg/ml (n=4) respectively and in UPN251 was 1.63±0.1 (n=5) and 17.5±6.8 (n=4) respectively. UPN251 cells were intrinsically more resistant to CEP8983 than OVCAR8 but had similar baseline sensitivity to veliparib. It would be expected that UPN251 cells would be more resistant to both PARP inhibitors as they have functional BRCA1 when compared with OVCAR8 that has non-functional BRCA1.

These two parp inhibitors, CEP8983 and veliparib, were not affected by the multiple mechanisms of resistance that arose in our UPN251 sublines. They did not show any significant resistance development and may be candidates in treating platinum/taxane resistant ovarian cancers. This data and the results of our recent study on a panel of 41 ovarian cancer cell lines (*Stordal et al. 2013*) suggests a broader activity of parp inhibitors in BRCA1 wild-type ovarian cancer. This activity is likely due to a variety of mechanisms causing dysfunction in homologous recombination repair.

## 5.0 Conclusions

The development of taxane resistance was not slower than the development of platinum resistance in cells with functional BRCA1 as was expected per the inverse resistance relationship. Taxol resistance developed quicker in BRCA1-wildtype and BRCA1-methylated cells. Both resistance to carboplatin and taxol developed quicker and more stably in UPN251 (BRCA1-wildtype) compared to OVCAR8 (BRCA1-methylated). Also alternating carboplatin and taxol treatment delays but does not prevent resistance development when compared to single agent administration. This was expected from the inverse resistance relationship. However, interestingly, the sequence of drug exposure influenced the resistance mechanism that developed in resultant sublines. UPN251-6CALT and UPN251-6TALT have different profiles of cross resistance to drugs, one having received carboplatin and one having received taxol in round one of development being their only difference. Finally over expression of P-gp is the dominant mechanism of taxol resistance present in our UPN251 resistant sublines whereas multiple mechanisms of carboplatin resistance are postulated to be present in our cell models.

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**Table 1 – Summary of BRCA1/2 and p53 mutation status in UPN251 and OVCAR8 ovarian cancer cells**

	<b>BRCA1</b>		<b>BRCA2</b>
<b>Cell Line</b>	<b>Mutation status</b>	<b>Methylated</b>	<b>Mutation status</b>
OVCAR8	Wild Type	Yes	Wild Type
UPN251	Wild Type 1199del29#, 1246delA*	No	Wild Type

#Homozygous Deleterious mutation compensated for by another \*reversion mutation

Analysis of BRCA1/2 status was carried out by (*Stordal et al. 2013*).

**Table 2 – Resistance profile of UPN251 drug-resistant sublines**

Drug (Units)	Parent		Carboplatin Single Agent			Alternating Carboplatin First			Taxol Single Agent			Alternating Taxol First		
	UPN251 IC <sub>50</sub>		UPN251-7C IC <sub>50</sub>			UPN251-6CALT IC <sub>50</sub>			UPN251-7T IC <sub>50</sub>			UPN251-6TALT IC <sub>50</sub>		
	Mean ± SD	n	Mean ± SD	n	F	Mean ± SD	n	F	Mean ± SD	n	F	Mean ± SD	n	F
<b>Platinums and heavy metals</b>														
Carboplatin (µg/ml)	1.0 ± 0.01	3	3.5 ± 0.07 8***	3	3.3	1.8 ± 0.02 ***	3	1.7	1.6 ± 0.14 **	3	1.5	2.0 ± 0.07 ***	3	2.0
+ BSO (12.5µg/ml)	0.8 ± 0.03 ###	3	2.9 ± 0.16 ##	3	3.4	1.6 ± 0.06 ##	3	1.8	1.3 ± 0.23	3	1.5	1.7 ± 0.07 ##	3	2.1
Cisplatin (µg/ml)	0.15 ± 0.01	3	0.48 ± 0.08 **	3	3.3	0.21 ± 0.02 **	3	1.6	0.15 ± 0.01	3	1.0	0.27 ± 0.04 **	3	1.9
Oxaliplatin (µg/ml)	0.08 ± 0.01	3	0.2 ± 0.03 **	3	2.1	0.08 ± 0.02	3	1.0	0.12 ± 0.003 *	3	1.3	0.04 ± 0.008 *	3	0.6
CuSO <sub>4</sub> (ng/ml)	15.3 ± 1.7	5	28.5 ± 11.4 *	5	1.9	34.7 ± 10.6 **	5	2.3	17.0 ± 2.9	5	1.1	15.0 ± 1.3	5	1.0
<b>Taxanes</b>														
Taxol (ng/ml)	14.9 ± 1.9	3	14.9 ± 1.7	3	1.0	63.6 ± 4.4 ***	3	4.3	133.3 ± 4.7 ***	3	8.9	84.4 ± 11.6 ***	3	5.7
+ Elacridar (0.14µg/ml)	1.8 ± 0.34 ###	3	1.8 ± 0.26 ###	3	1.0	1.9 ± 0.17 ###	3	1.1	1.9 ± 0.17 ###	3	1.0	2.9 ± 0.51 ###	3	1.6
Docetaxel (ng/ml)	4.1 ± 0.3	3	1.5 ± 0.6 **	3	0.4	8.5 ± 1.5 **	3	2.1	20.3 ± 4.0 **	3	4.9	9.0 ± 2.6 *	3	2.2
<b>Parp Inhibitors</b>														
Olaparib (µg/ml)	1.7 ± 0.36	4	3.3 ± 0.85 *	4	2.0	3.9 ± 0.73 **	4	2.3	5.4 ± 0.98 ***	4	3.2	3.7 ± 0.4 ***	4	2.2
+ Elacridar (0.14µg/ml)	1.3 ± 0.14	4	2.5 ± 0.6	4	1.8	1.7 ± 0.36 ##	4	1.2	1.3 ± 0.2 ###	4	1.0	1.4 ± 0.14 ###	4	1.0
Veliparib (µg/ml)	13.1 ± 2.91	3	14.5 ± 0.7	3	1.1	14.6 ± 1.29	3	1.1	10.4 ± 1.58	3	0.8	14.7 ± 1.6	3	1.1
+ Elacridar (0.14µg/ml)	13.7 ± 2.83	3	15.2 ± 1.79	3	1.1	14.0 ± 1.9	3	1.0	10.7 ± 1.81	3	0.8	14.3 ± 1.6	3	1.0
CEP-8983 (µg/ml)	1.4 ± 0.12	3	1.7 ± 0.3	3	1.2	1.3 ± 0.24	3	0.9	1.1 ± 0.25	3	0.8	1.3 ± 0.19	3	0.9
<b>Vinca Alkaloids</b>														
Vinblastine (ng/ml)	9.9 ± 1.1	4	11.9 ± 2.84	4	1.2	29.2 ± 4.54 ***	4	3.0	62.1 ± 3.8 ***	4	6.3	31.2 ± 11.11 **	4	3.2
+ Elacridar (0.14µg/ml)	3.2 ± 0.57 ###	4	5.4 ± 0.65 ##	4	1.7	3.3 ± 0.73 ###	4	1.1	4.6 ± 1.22 ###	4	1.5	3.6 ± 0.73 ##	4	1.1
<b>Anthracyclines</b>														
Doxorubicin (ng/ml)	39.1 ± 10.15	5	46.8 ± 2.96	5	1.2	62.8 ± 3.02 **	5	1.6	117.9 ± 20.2 ***	5	3.0	59.6 ± 6.44 **	5	1.5
<b>Inhibitors</b>														
BSO (µg/ml)	5.1 ± 1.87	4	14.4 ± 2.07 ***	4	2.8	7.6 ± 0.22	4	1.5	7.9 ± 2.8	4	1.5	29.2 ± 11.12**	4	5.7
Elacridar (µg/ml)	2.5 ± 0.4	5	1.5 ± 0.3 **	5	0.6	0.8 ± 0.2 ***	5	0.3	1.3 ± 0.5 **	5	0.5	0.8 ± 0.2 ***	5	0.3

\* Indicates a significant difference between UPN251 parent and UPN251 drug-resistant sublines (p<0.05 students t-test)

\*\* Indicates a significant difference between UPN251 parent and UPN251 drug-resistant sublines (p<0.01 students t-test)

\*\*\* Indicates a significant difference between UPN251 parent and UPN251 drug-resistant sublines ( $p < 0.001$  students t-test)

# Indicates a significant difference on the addition of a modulator ( $p < 0.05$  students t-test)

# # Indicates a significant difference on the addition of a modulator ( $p < 0.01$  students t-test)

# # # Indicates a significant difference on the addition of a modulator ( $p < 0.001$  students t-test)

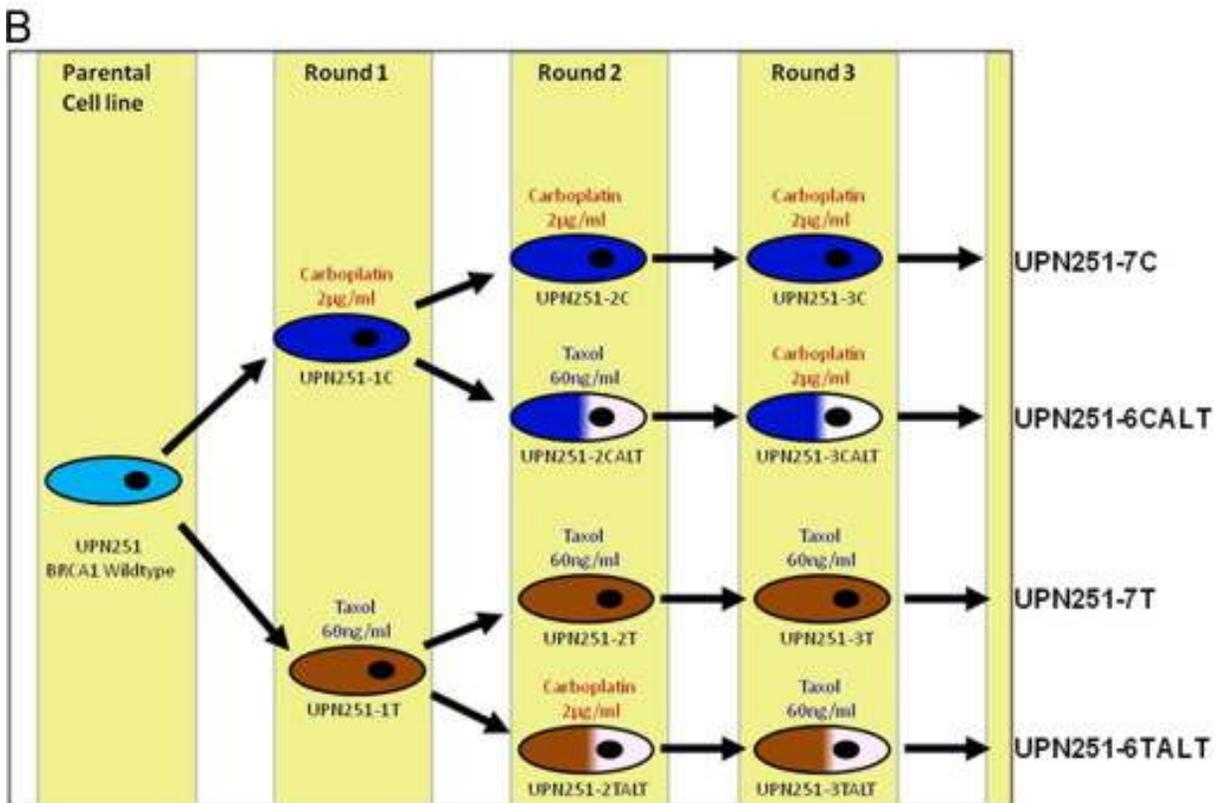
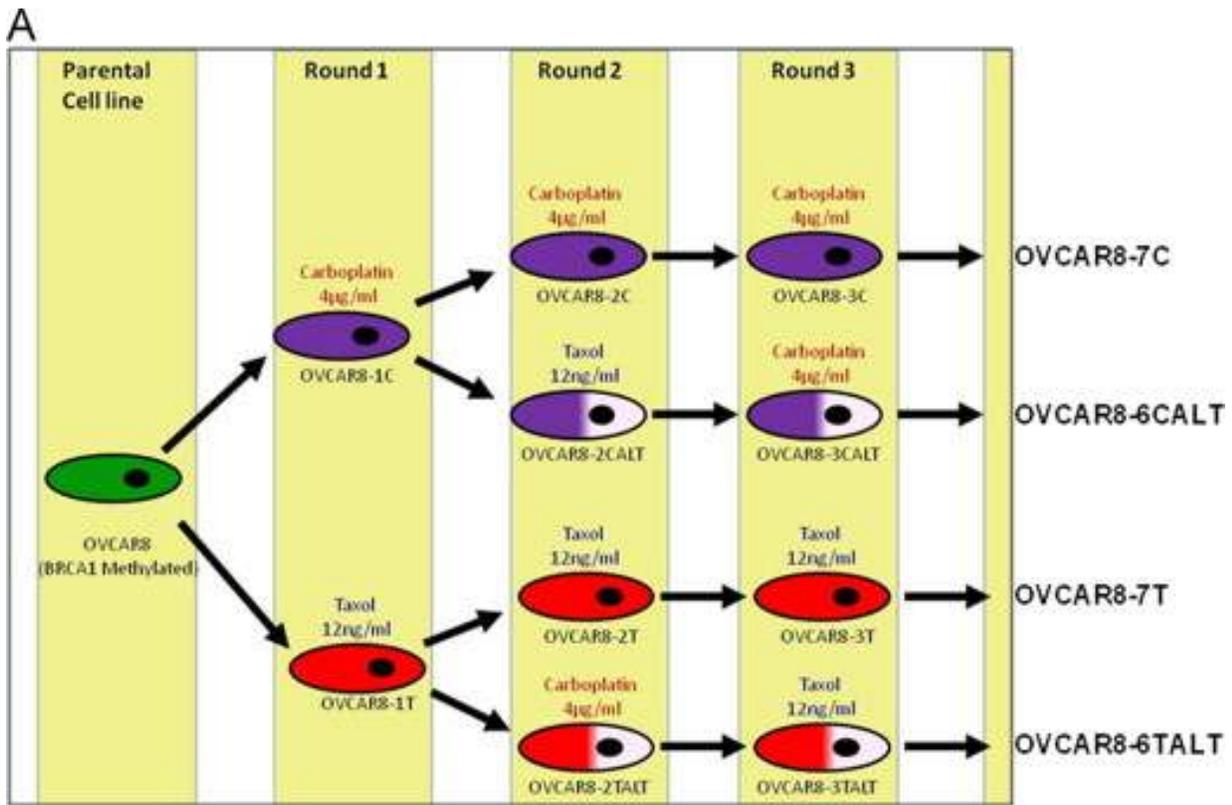


Fig. 1. OVCAR8 and UPN251 treatment strategy for the development of platinum and taxane resistant cell lines. In each case arrows represent progression to the next round with a drug treatment of either single-agent taxol or carboplatin as indicated. Resultant cells are shown from round 1-3 with the drug treatment they received (either carboplatin or taxol) above cells and cell name given below cells. Rounds 4-7 progressed in the same pattern as shown. (A) OVCAR8 parental cells on far left with resultant sublines derived from them to the right. (B) UPN251 parental cells on far left with resultant sublines derived from them to the right.

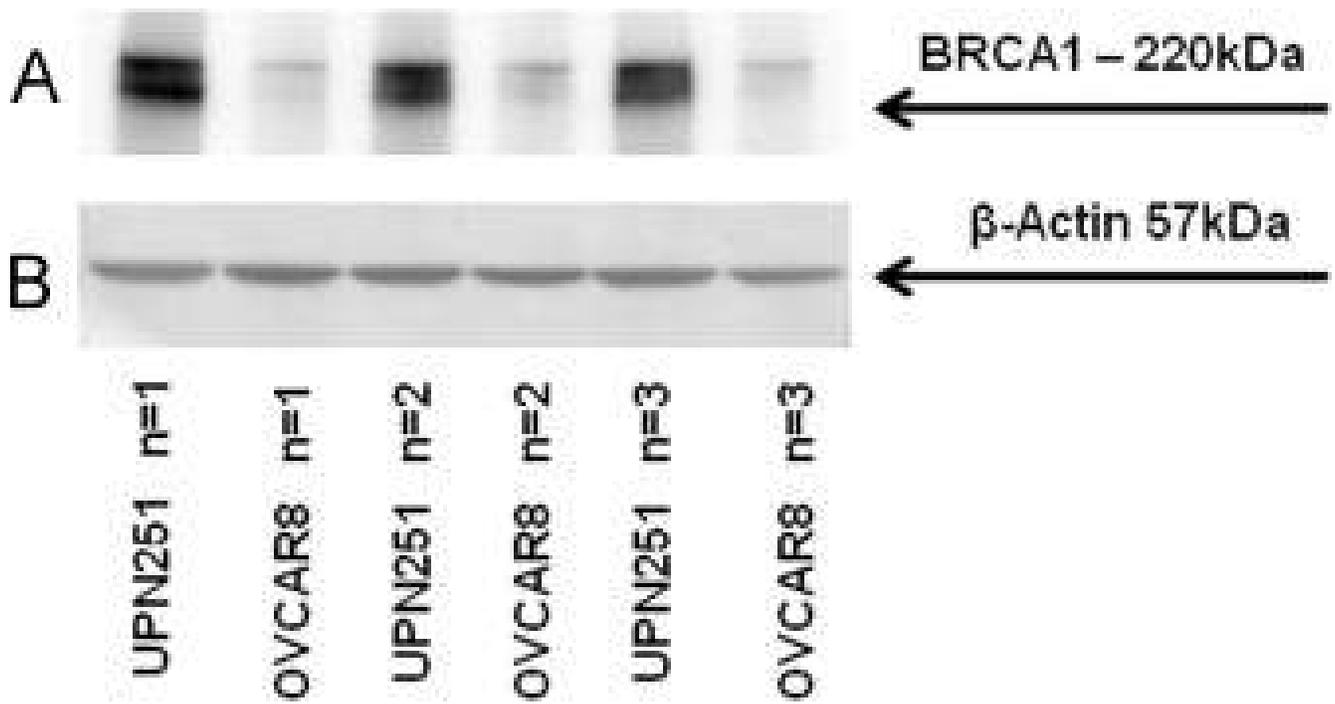


Fig. 2. Protein expression status of BRCA1 in UPN251 and OVCAR8 by western blot. Densitometry on n=3 biological replicates was carried out using Quantity One software (Biorad). Abundance of protein in arbitrary units was normalized to  $\beta$ -actin loading control for each sample and then each biological series was normalized to UPN251 control expression. (A) BRCA1 220kDa (B)  $\beta$ -actin 57 kDa.

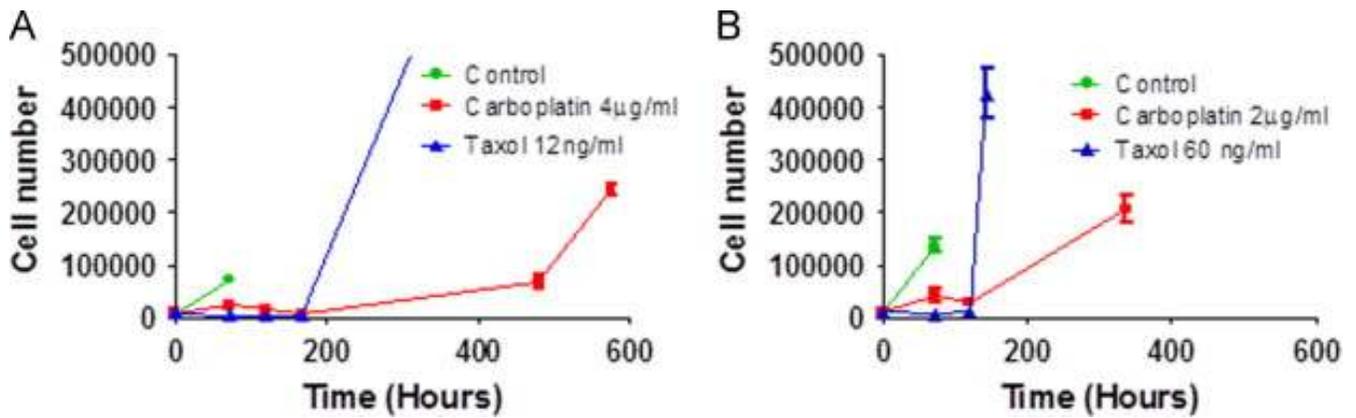


Fig. 3. Recovery plots for dose finding evaluation in cell selection strategy optimisation. Each graph is a representative of 1 of at least 3 biological repeats and shows cell number graphed over time (Hours). (A) Selected dose for taxol on OVCAR8. (B) Selected dose for carboplatin on OVCAR8. (C) Selected dose for taxol on UPN251. (D) Selected dose for carboplatin on UPN251.

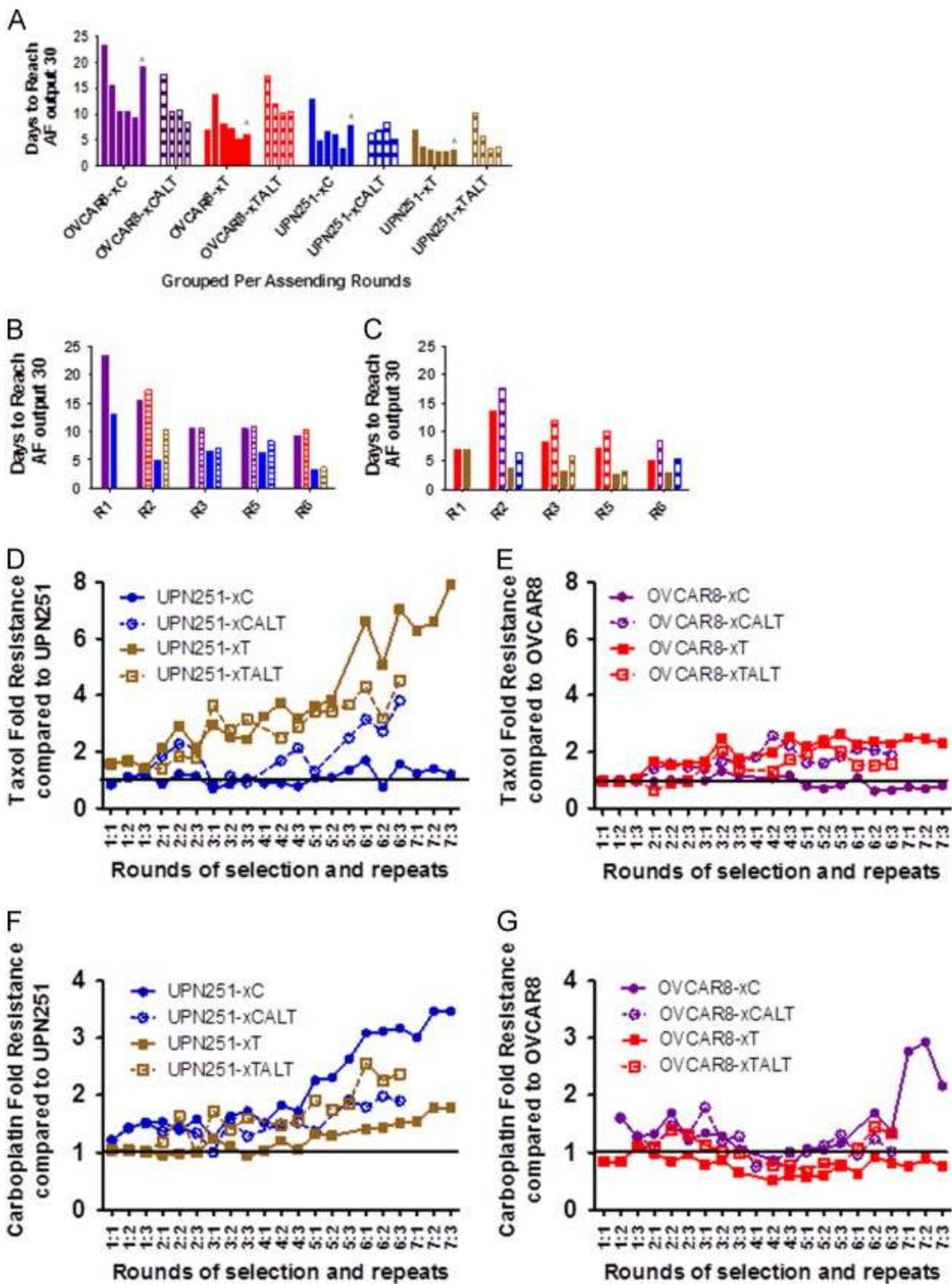


Fig. 4. Selection strategy recovery and fold resistance to carboplatin and taxol when compared to parental cell lines. Recovery is indicated by days to reach AF output 30 shown on y-axis. In bar graphs solid colour indicates a subline which received single agent carboplatin or taxol during every round of development and dashed bars indicate sublines which received alternating treatments of single agent carboplatin or taxol during development. (A) Recovery plots for each cell line (x in cell line name denotes that it represents all rounds of selection) grouped per ascending round of selection (1-7) Round 4 has been excluded from the summary due to technical error. (B) Recovery of carboplatin treated cells in each round. (C) Recovery of taxol treated cells in each round. Fold resistance to taxol ((D) UPN251 sublines (E) OVCAR8 sublines) and carboplatin ((F) UPN251 sublines (G) OVCAR8 sublines) is given from rounds 1-7. The x-axis gives a time progression for 3 weekly cytotoxicity assay in seven rounds of selection ((round number):(assay number)). The y-axis indicates fold resistance compared to parental cells. The horizontal black bar indicates a fold resistance ratio of 1 (No difference to parent cells). ^Received double dose of drug.

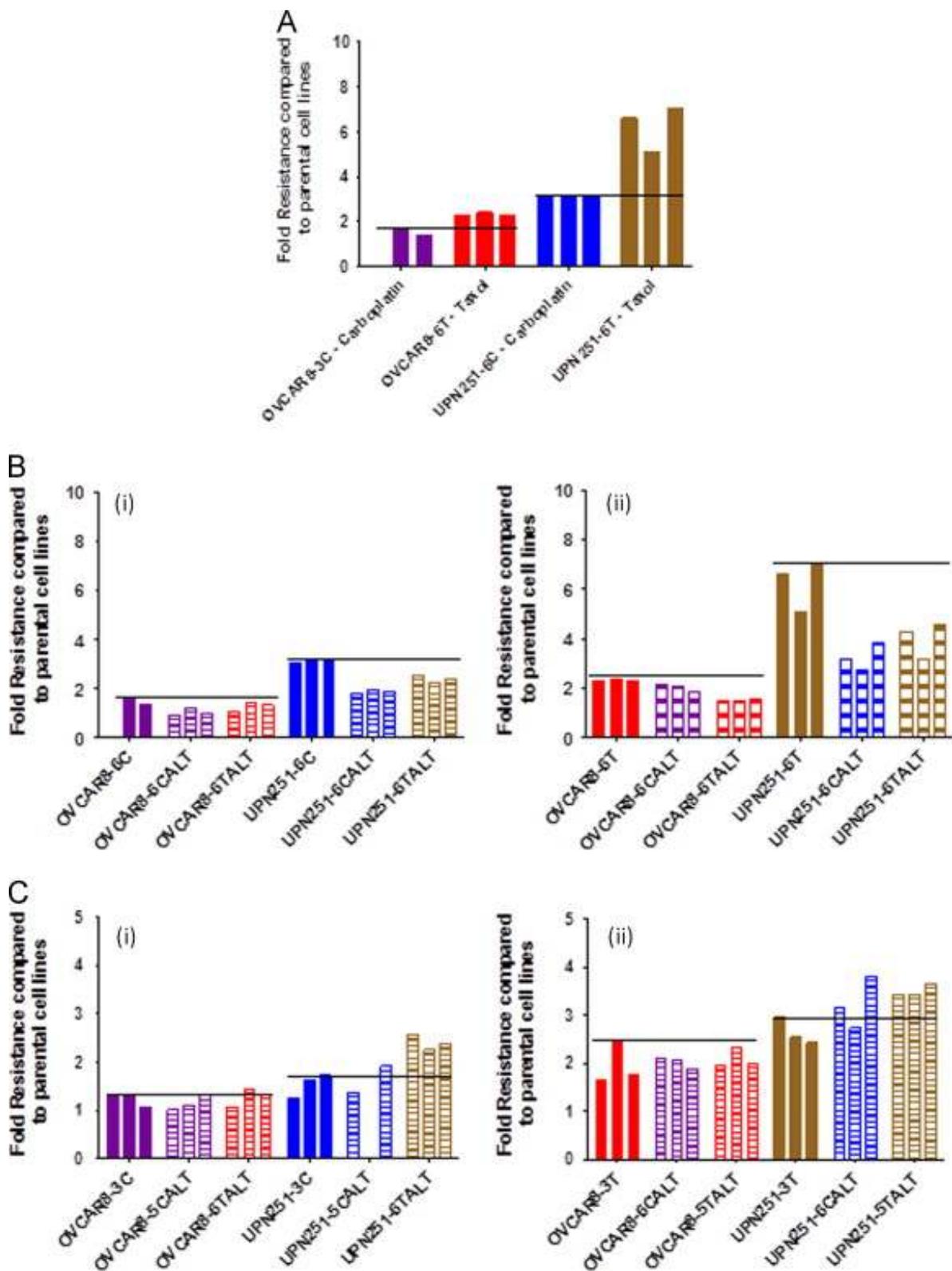


Fig. 5. Selection strategy fold resistance outcomes - Investigation of hypotheses. In each graph the y-axis shows fold resistance compared to parental cell lines. The x-axis shows resistant sublines. In Graph (A) it also indicates the drug used in cytotoxicity assay represented in the graph. Grouped bars represent cytotoxicity assays for 3 weeks following recovery. In (A) horizontal lines are used to show relative differences in carboplatin and taxol resistance development, while in (B) & (C) they show relative differences in carboplatin or taxol single-agent resistance development compared to alternating treatments. (A) Development of resistance to single-agent taxol and carboplatin for both OVCAR8 and UPN251. (B) Fold resistance to (i) carboplatin and (ii) taxol when compared to parental cell lines in round 6 of selection. (C) Fold resistance to (i) carboplatin and (ii) taxol when compared to parental cell lines when each cell line had received 3 doses of either carboplatin or taxol.

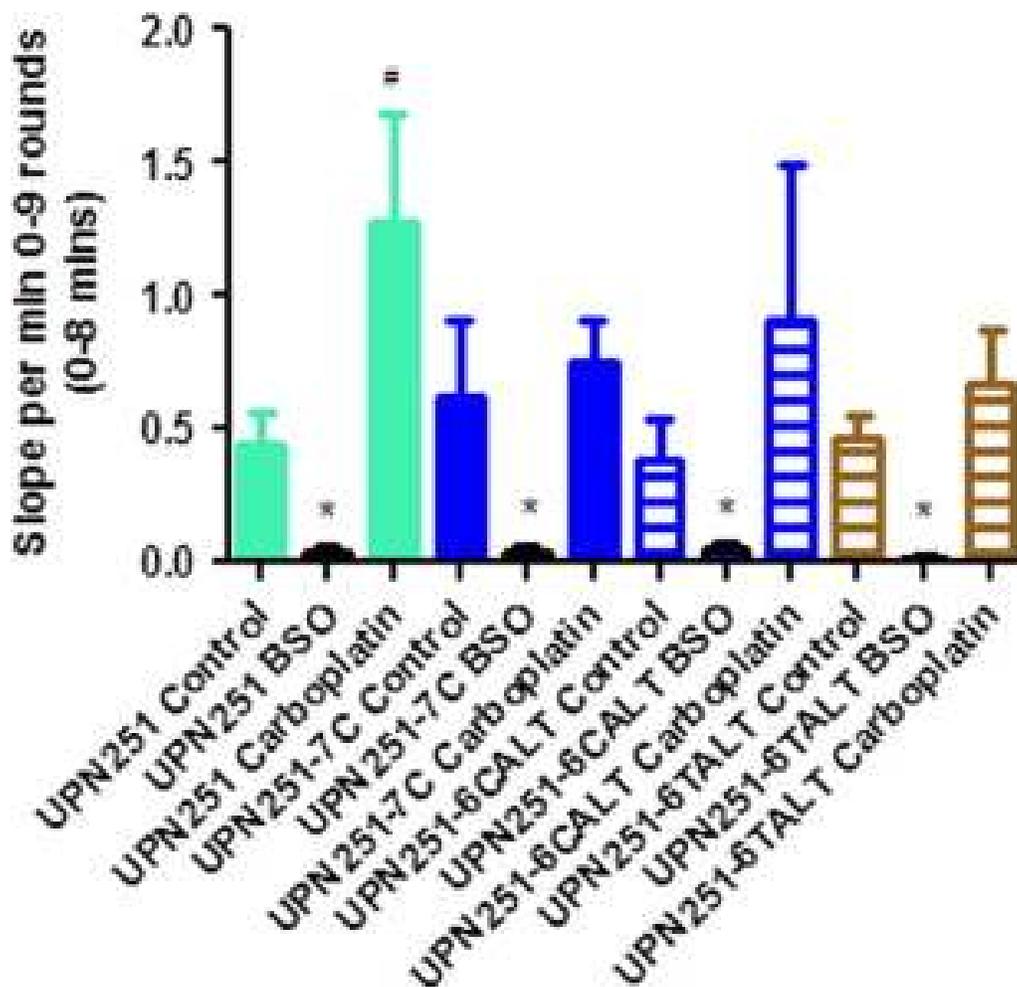


Fig. 6. Total cellular glutathione assay. Parental cells UPN251 and resistant sublines are shown on x-axis as control, BSO or carboplatin treated. The y-axis gives slope per minute of 0-9 rounds of the assay run over 8mins. 3-day exposures to drug was used in this experiment. \* Indicates a significant difference between control and BSO treatment ( $P < 0.05$ , Student *t*-test) # Indicates a significant difference between control and carboplatin treatment ( $P < 0.05$ , Student *t*-test).

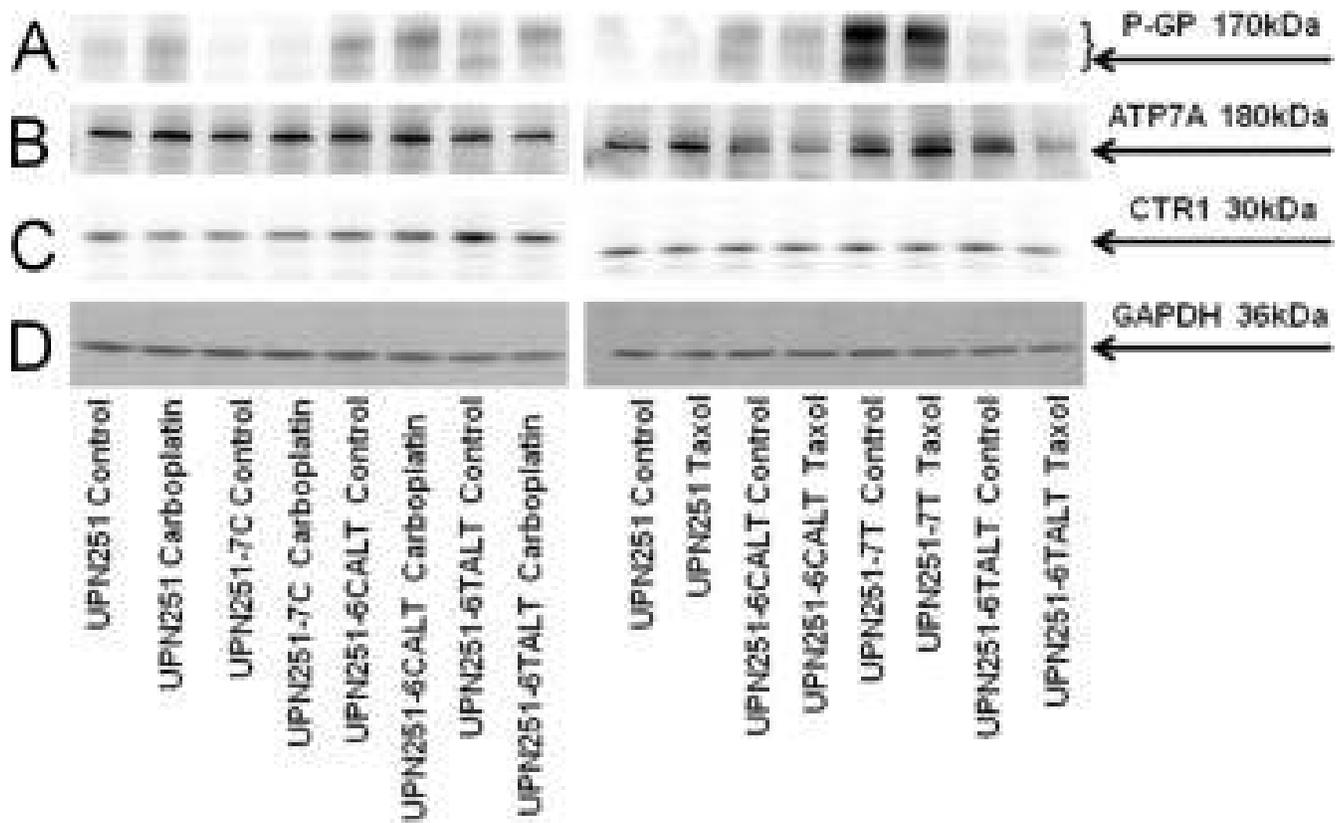


Fig. 7. Protein expression by western blotting for UPN251 and sublines treated with carboplatin and taxol. All protein samples were normalised to GAPDH and then UPN251 control. Drug treated cells were exposed to 2 $\mu$ g/ml carboplatin or 15ng/ml taxol for 72hours before and compared to drug free controls. (A) P-glycoprotein (B) ATP7A (C) CTR1 (D) GAPDH loading control. Representative images of at least three biological replicates are shown.