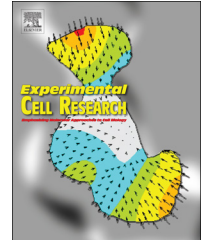


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Research Article

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 CuSO₄ (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 CuSO₄ 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.

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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 [4]. The standard treatment is surgical debulking followed by intravenous platinum-taxane combination chemotherapy ([41,53]). 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 ([43,49,51]). 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% [20]. A woman with a BRCA1 mutation has a 39–46% chance of developing ovarian cancer [19]. 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 ([28,38]).

A systematic review of the literature by [58] 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 [56] 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 [12,66]. The response to taxanes, in BRCA1 deficient cells is reduced apoptosis conferring resistance [29]. The opposite is true for cells with functional BRCA1 [47,61].

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 [59]) 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.

Methods

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% CO₂ 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

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

Cell line	BRCA1		BRCA2
	Mutation status	Methylated	Mutation status
OVCAR8	Wild type	Yes	Wild type
UPN251	Wild type 1199del29 ^a , 1246delA ^b	No	Wild type

Analysis of BRCA1/2 status was carried out by [59].
^a Homozygous deleterious mutation compensated for by another.
^b Reversion mutation.

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 [59]. OVCAR8 was developed from a patient who had undergone treatment with high-dose carboplatin who exhibited progressive ovarian cancer [54]. OVCAR8 is BRCA1 wild-type but is methylated in the promoter region resulting in reduced gene expression of BRCA1 [59]. 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.

Cytotoxicity assays

Acid phosphatase cytotoxicity assays [68] were used to determine cytotoxicity as per method used by [59]. 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 1 unit/ml to unit MW.

Western blotting

Western blotting was performed as per the method used by [57]. Primary and secondary antibodies used are listed in supplementary material, Table S2. Drug treated cells received 2 µg/ml carboplatin or 15 ng/ml taxol for 72 h.

Total cellular glutathione assay

Analysis of total cellular levels of glutathione (GSH) was carried out as per the method by [57] which was adapted from [60]. Plates were read and kinetics measured using the FLUOstar OPTIMA (BMG LABTECH) multifunctional microplate reader (405 nM at 30 °C).

Cell selection strategy outline

Cell lines were treated with carboplatin or taxol as per Fig. 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-

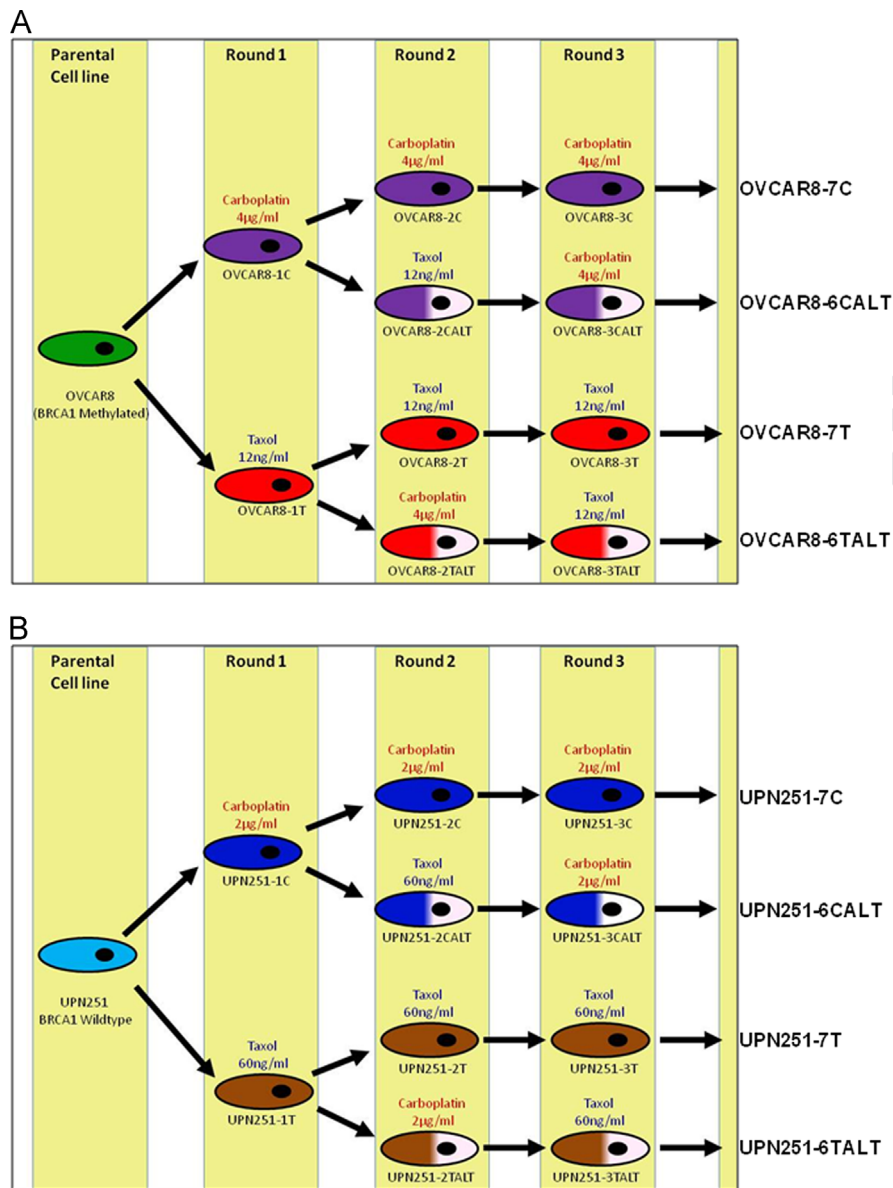


Fig. 1 – (A) and (B).

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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, below steps are followed.

Cells were plated into a T25 flask at a cell density of 2.6×10^4 cells per flask and drugged on day 2 as per the selection strategy outline (2 and 4 $\mu\text{g/ml}$ carboplatin and 60 and 12 ng/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 [2]. 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).

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.

Results

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 ($p = 3.3 \times 10^{-4}$, Fig. 2). This correlates with the BRCA1 methylation status of the cell lines.

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.

Dose optimisation

Doses of drug for carboplatin and taxol used in the selection strategy were selected from the following ranges respectively:—UPN251 (0.7–2 $\mu\text{g/ml}$, 10–100 ng/ml) and OVCAR8 (2.3–18.5 $\mu\text{g/ml}$, 2.3–14 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 to 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 ng/ml respectively was deemed clinically relevant following pharmacokinetic studies for a dose of carboplatin at AUC 5 and taxol at 175 mg/m^2 which are often administered to patients in clinical trials as single agents ([8,16,23,24,35,37,39,42,45,50,65]).

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 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ and taxol doses of 12 ng/ml and 60 ng/ml were chosen for OVCAR8 and UPN251, respectively. From the recovery plots (Fig. 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.

Recovery

In general, all cells recovered quicker after drugging as the rounds of selection progressed. Fig. 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. Fig. 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. Fig. 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.

Fold resistance

The fold resistance of each subline at weekly intervals for 3-weeks in each round of selection for carboplatin is shown in Fig. 4(D) and (E) and for taxol in Fig. 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, $p = 0.4 \times 10^{-6}$ – 0.6×10^{-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, $p = 0.02$ – 0.2×10^{-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, $p = 0.3 \times 10^{-3}$ – 0.5×10^{-6}). Only OVCAR8-7C and OVCAR8-6TALT had significant resistance to carboplatin in the OVCAR8 sublines (1.3–2.6 fold, $p = 0.04$ – 0.3×10^{-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

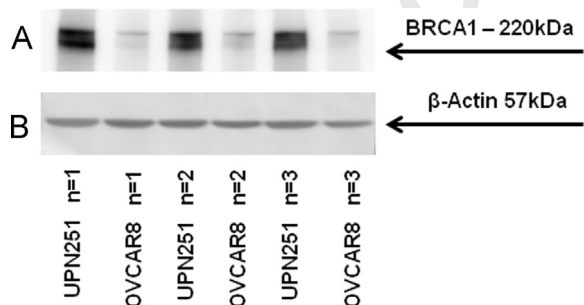


Fig. 2 – (A) and (B).

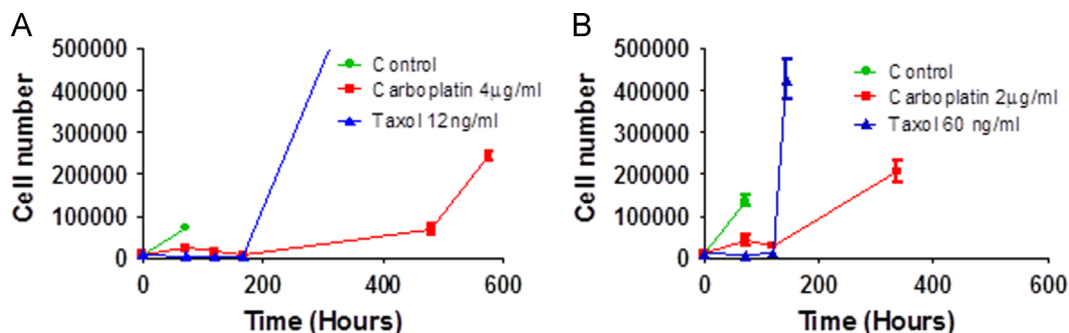


Fig. 3 – (A) and (B).

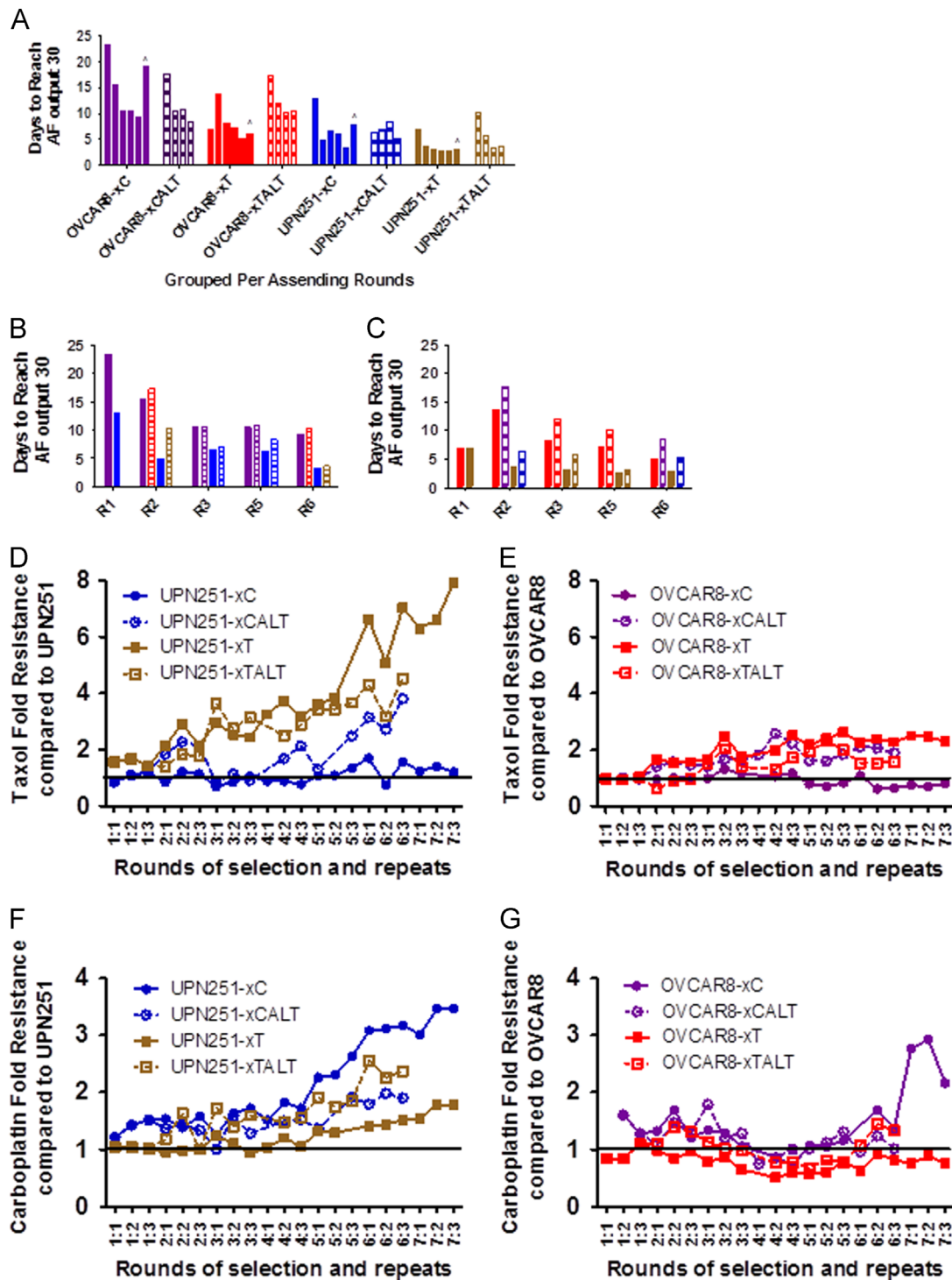


Fig. 4 – (A)–(G).

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 (Fig. S1)). Experiments were therefore performed in 6 week blocks.

Fig. 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.

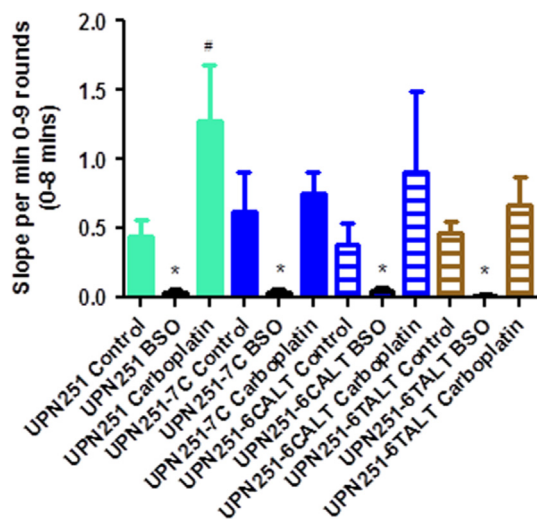


Fig. 5

Fig. 5(B (i) and (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.

Fig. 5(C (i) and (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.

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 (Fig. 4(E and G)).

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. A total of 11 drugs and 2 inhibitors were used. Inhibitors include, buthionine sulphoximine (BSO) an inhibitor of glutathione (GSH) (Drew, Miners [7]) and elacridar an inhibitor of *P*-glycoprotein (*P*-gp) a member of the ATP binding cassette (ABC) transporter family [22]. 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, $p = 0.2 \times 10^{-2}$ – 0.5×10^{-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 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 (CuSO_4)

was also seen in sublines developed with carboplatin treatments. One exception to this is UPN251-6TALT, which is not resistant to 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, $p = 0.5 \times 10^{-3}$ – 0.2×10^{-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 IC_{50} values ($p = 0.1 \times 10^{-3}$ to 0.4×10^{-7}). An almost identical trend is seen with vinblastine, and olaparib ± elacridar which are all *P*-gp substrates ([3,30]). 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_{50} when compared to the parental cell lines and could both be candidates for treating platinum/taxane-resistant ovarian cancer.

Total cellular glutathione assay

Using a total cellular glutathione (GSH) assay (Fig. 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 2 $\mu\text{g/ml}$ carboplatin ($p = 0.02$) for a 3-day exposure. Treatment with 12.5 μmol 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, $p = 0.5 \times 10^{-2}$ – 0.02). This was the same dose of BSO which was used in our drug screen.

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 , Fig. 7(A)). *P*-gp is significantly decreased in UPN251-7C (carboplatin treated). There are no significant changes in protein expression for both ATP7A (Fig. 7(B)) and CTR1 (Fig. 7(C)) when compared to UPN251 control. MRP2 was not expressed in UPN251 and resistant sublines (supplementary material Fig. S2(A)). There was no change in BRCA1 protein expression between UPN251 parental cells and UPN251-7C and UPN251-7T resistant sublines (supplementary material Fig. S2(C)).

Discussion

Resistance models

The mechanism of resistance that develops 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 ([33,55,64]) and low-dose intermittent incremental inducement ([17,27,62]) where cells are

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

Drug (Units)	Parent UPN251 IC ₅₀		Carboplatin single agent UPN251-7C IC ₅₀			Alternating carboplatin first UPN251-6CALT IC ₅₀			Taxol single agent UPN251-7T IC ₅₀			Alternatingtaxol first UPN251-6TALT IC ₅₀			
	Mean ±SD	n	Mean ±SD	n	F	Mean ±SD	n	F	Mean ±SD	n	F	Mean ±SD	n	F	
Platinums and heavy metals															
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 (12.5 µg/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 ₄ (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	
Taxanes															
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 (0.14 µg/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	
Parp Inhibitors															
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 (0.14 µg/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 (0.14 µg/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	
Vinca Alkaloids															
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 (0.14 µg/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	
Anthracyclines															
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	
Inhibitors															
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 on the addition of a modulator ($p < 0.05$ Students t -test).
 * 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 ($p < 0.01$ Students t -test).
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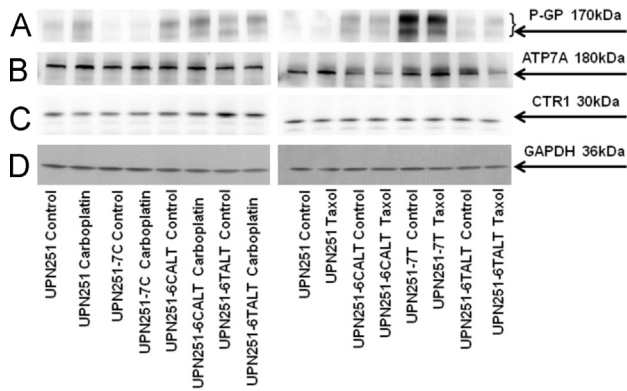
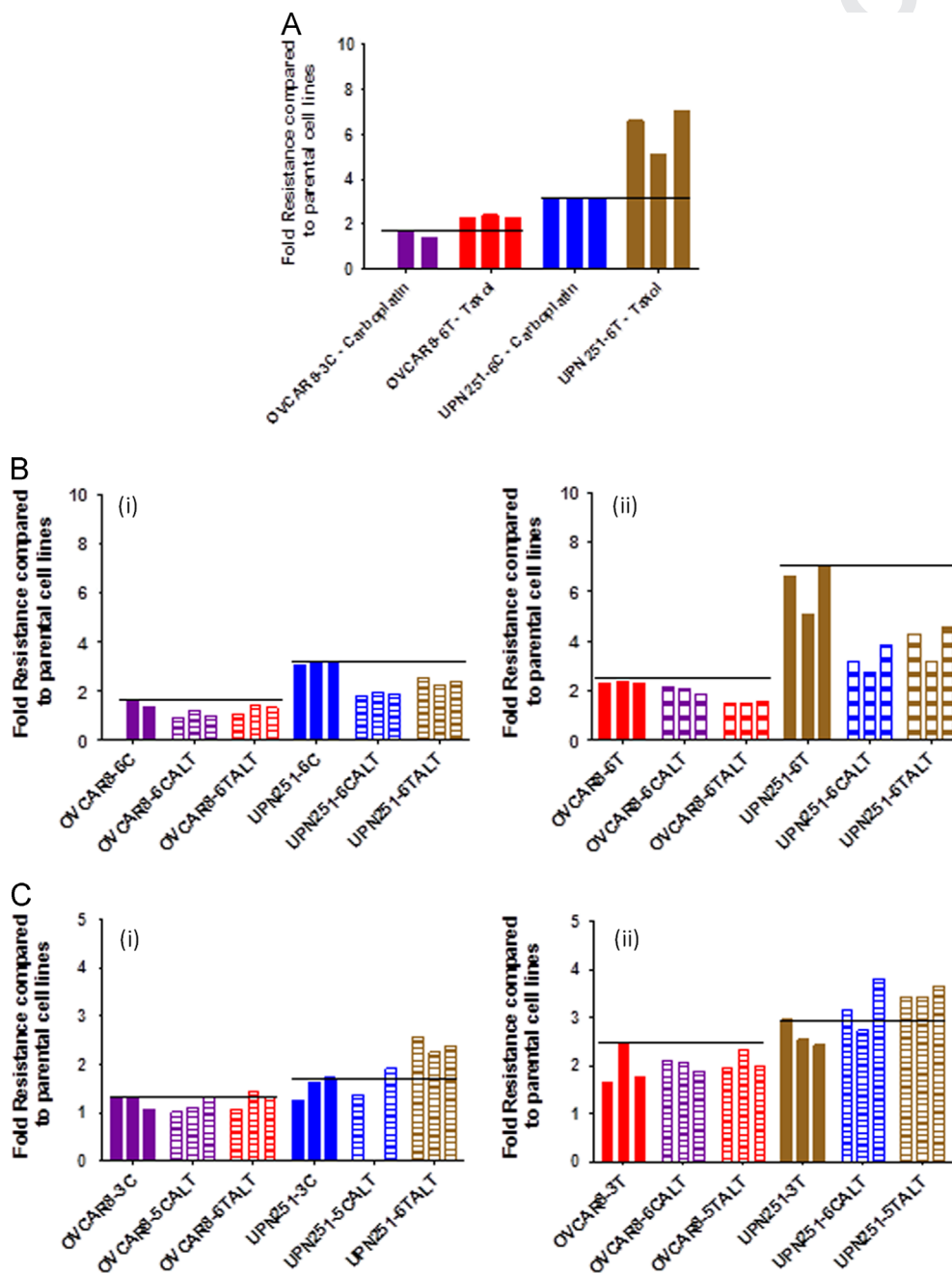


Fig. 6 – (A)–(D).

exposed sporadically to increasing doses of drug over time. A number of studies have used a pulsed strategy of a 4-h drug exposure at weekly intervals for 10–12 weeks ([15,32,69]). 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 [41]. Ref. [67] compares 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.

Resistance development

We hypothesised that ovarian cancer cells with BRCA1 defects (OVCAR8) would develop resistance to platinum drugs slower than taxanes with the opposite being true for cells with functional BRCA1 (UPN251). Fig. 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. Fig. 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 three doses of taxol (Fig. 5(C) (i)) or three doses of carboplatin (Fig. 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 (five or six rounds in alternating versus three 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 two 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 (Fig. 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 (Fig. 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 [26]. 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 [25]. 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 [36]. In the clinic taxol is given 3 h before carboplatin in order to circumvent carboplatin's myelosuppressive effects [42]. 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 ([14,21]).

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) (Fig. 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 ([63]). Also in two ovarian cancer cell lines decreasing BRCA1 mRNA using inhibition assays correlated to increased sensitivity to platinum drugs [46]. 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. Ref. [71] 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 (Fig. 3) and in the selection strategy itself (Fig. 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. Platinum drugs act mainly by forming nuclear

platinum adducts on DNA strands ([13,16]), while taxanes act by stabilising microtubules within the cell ([34,48]). 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 [6].

Mechanisms of taxol resistance

Taxol resistance cell models are very common and many have been developed for ovarian cancer cell lines ([10,11,18,44,70]). 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 ± elacridar (Table 2) all show highly significant drops in IC₅₀ when *P*-gp is blocked with elacridar. Western data showed increased *P*-gp expression for sublines which had taxol treatment during selection (Fig. 7(A)). Therefore it is likely that taxol is being actively pumped out of the cell by *P*-gp causing taxol resistance.

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 ([9,42]). A publication by [31] 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 [40].

From the results of our GSH assays (Fig. 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 2 µg/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 12.5 µM 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 IC₅₀ 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.

CuSO₄ (Table 2) had significantly higher IC₅₀s for UPN251-7C and UN251-6CALT. This indicates the possible involvement of

copper transporters ATP7A, ATP7B and CTR1 in carboplatin resistance (Safaei, Howell [52]). 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 [57].

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 [5]. 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 [1]. 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 CuSO₄ (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 [1] discussed above.

Treatment options for platinum/taxane resistant ovarian cancers

The baseline IC₅₀ values of parp inhibitors CEP8983 and veliparib in OVCAR8 was 2.02 ± 0.3 µg/ml (*n*=6) and 5.8 ± 1.1 µg/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

1181 veliparib. It would be expected that UPN251 cells would be more
1182 resistant to both PARP inhibitors as they have functional BRCA1
1183 when compared with OVCAR8 that has non-functional BRCA1.

1184 These two parp inhibitors, CEP8983 and veliparib, were not
1185 affected by the multiple mechanisms of resistance that arose in
1186 our UPN251 sublines. They did not show any significant resistance
1187 development and may be candidates in treating platinum/taxane
1188 resistant ovarian cancers. This data and the results of our recent
1189 study on a panel of 41 ovarian cancer cell lines [59] suggests a
1190 broader activity of parp inhibitors in BRCA1 wild-type ovarian
1191 cancer. This activity is likely due to a variety of mechanisms
1192 causing dysfunction in homologous recombination repair.

1193 Conclusions

1194 The development of taxane resistance was not slower than the
1195 development of platinum resistance in cells with functional BRCA1
1196 as was expected per the inverse resistance relationship. Taxol
1197 resistance developed quicker in BRCA1-wildtype and BRCA1-
1198 methylated cells. Both resistance to carboplatin and taxol developed
1199 quicker and more stably in UPN251 (BRCA1-wildtype) compared
1200 to OVCAR8 (BRCA1-methylated). Also alternating carboplatin
1201 and taxol treatment delays but does not prevent resistance
1202 development when compared to single agent administration. This
1203 was expected from the inverse resistance relationship. However,
1204 interestingly, the sequence of drug exposure influenced the resis-
1205 tance mechanism that developed in resultant sublines. UPN251-
1206 6CALT and UPN251-6TALT have different profiles of cross resistance
1207 to drugs, one having received carboplatin and one having received
1208 taxol in round one of development being their only difference.
1209 Finally over expression of P-gp is the dominant mechanism of taxol
1210 resistance present in our UPN251 resistant sublines whereas
1211 multiple mechanisms of carboplatin resistance are postulated to
1212 be present in our cell models.

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1219 Appendix A. Supporting information

1220 Supplementary data associated with this article can be found in
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1222 REFERENCES

- 1223 [1] S.R. Armstrong, R. Narendrula, B. Guo, A.M. Parissenti, K.L.
1224 McCallum, S. Cull, C Lanner, Distinct genetic alterations occur in
1225 ovarian tumor cells selected for combined resistance to carbop-
1226 latin and docetaxel, *J. Ovarian Res.* 5 (1) (2012) (40-40).
1227 [2] Busschots, S., O'Toole, S., O'Leary, J. J., Stordal, B. 2015. Non-
1228 invasive and non-destructive measurements of confluence in
1229 cultured adherent cell lines. *MethodsX*, in press.

- 1230 [3] S. Choudhuri, C.D. Klaassen, Structure, function, expression, 1241
1242 genomic organization, and single nucleotide polymorphisms of
1243 human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux
1244 transporters, *Int. J. Toxicol.* 25 (4) (2006) 231-259. 1244
1245 [4] D.L. Clarke-Pearson, Screening for ovarian cancer, *N. Engl. J. Med.* 1245
1246 361 (2) (2009) 170-177. 1246
1247 [5] M.P. Dalvi, C. Behrens, M. Suraokar, L. Girard, Y. Xie, I. Wistuba, 1247
1248 J.D. Minna, Abstract 821: developing a molecular understanding
1249 of non small cell lung cancer (NSCLC) resistance to platin-taxane
1250 chemotherapy, *Cancer Res.* 72 (8) (2012) (Supplement) 821-821). 1249
1251 [6] C. DelloRusso, P.L. Welch, W. Wang, R.L. Garcia, M.C. King, E.M. 1250
1251 Swisher, Functional characterization of a novel BRCA1-null
1252 ovarian cancer cell line in response to ionizing radiation, *Mol.*
1253 *Cancer Res.* 5 (1) (2007) 35-45. 1253
1254 [7] R. Drew, J.O. Miners, The effects of buthionine sulphoximine 1254
1255 (BSO) on glutathione depletion and xenobiotic biotransforma-
1256 tion, *Biochem. Pharmacol.* 33 (19) (1984) 2989-2994. 1255
1257 [8] A. du Bois, H.J. Luck, K. Buser, H.G. Meerpohl, C. Sessa, U. 1256
1257 Klaassen, H. Meden, H. Bochtler, K. Diergarten, Extended phase II
1258 study of paclitaxel as a 3-h infusion in patients with ovarian
1259 cancer previously treated with platinum, *Eur. J. Cancer* 33 (3) 1258
1260 (1997) 379-384. 1259
1261 [9] A. du Bois, H.J. Luck, W. Meier, H.P. Adams, V. Mobus, S. Costa, 1261
1262 T. Bauknecht, B. Richter, M. Warm, W. Schroder, S. Olbricht, U.
1263 Nitz, C. Jackisch, G. Emons, U. Wagner, W. Kuhn, J. Pfisterer, For 1263
1264 the Arbeitsgemeinschaft Gynakologische Onkologie (AGO) Ovar-
1265 ian Cancer Study Group, A randomized clinical trial of cisplatin/
1266 paclitaxel versus carboplatin/paclitaxel as first-line treatment of 1265
1267 ovarian cancer, *J. Natl. Cancer Inst.* 95 (17) (2003) 1320-1329. 1266
1268 [10] Z. Duan, A.J. Feller, R.T. Penson, B. Chabner, M.V. Seiden, 1267
1268 Discovery of differentially expressed genes associated with
1269 paclitaxel resistance using cDNA array technology: analysis of
1270 interleukin (IL) 6, IL-8, and monocyte chemotactic protein 1 in 1270
1271 the paclitaxel-resistant phenotype, *Clin. Cancer Res.* 5 (11) (1999) 1271
1272 3445-3453. 1272
1273 [11] Z. Duan, A.J. Feller, H.C. Toh, T. Makastorsis, M.V. Seiden, TRAG-3, 1273
1274 a novel gene, isolated from a taxol-resistant ovarian carcinoma 1274
1275 cell line, *Gene* 229 (1-2) (1999) 75-81. 1275
1276 [12] W. Foulkes, BRCA1 and BRCA2: chemosensitivity, treatment 1275
1276 outcomes and prognosis, *Fam. Cancer* 5 (2) (2006) 135-142. 1276
1277 [13] M.A. Fuertes, J. Castilla, C. Alonso, J.M. Perez, Cisplatin biochem- 1277
1278 ical mechanism of action: from cytotoxicity to induction of cell 1278
1279 death through interconnections between apoptotic and necrotic 1279
1280 pathways, *Curr. Med. Chem.* 10 (3) (2003) 257-266. 1280
1281 [14] G. Giaccone, M. Huizing, P.E. Postmus, W.W. ten Bokkel Huinink, 1281
1282 M. Koolen, Z.N. van, J.B. Vermorken, J.H. Beijnen, O. Dalesio, H.M. 1282
1283 Pinedo, Dose-finding and sequencing study of paclitaxel and 1283
1284 carboplatin in non-small cell lung cancer, *Semin. Oncol.* 1284
1285 22 (4 Suppl. 9) (1995) 78-82. 1285
1286 [15] S.A. Glynn, P. Gammell, M. Heenan, R. O'Connor, Y. Liang, 1285
1286 J. Keenan, M. Clynes, A new superinvasive in vitro phenotype 1286
1287 induced by selection of human breast carcinoma cells with the 1287
1288 chemotherapeutic drugs paclitaxel and doxorubicin, *Br. J. Cancer* 1288
1289 91 (10) (2004) 1800-1807. 1289
1290 [16] R.S. Go, A.A. Adjei, Review of the comparative pharmacology and 1290
1291 clinical activity of cisplatin and carboplatin, *J. Clin. Oncol.* 17 (1) 1291
1292 (1999) (409-409). 1291
1293 [17] A.K. Godwin, A. Meister, P.J. O'Dwyer, C.S. Huang, T.C. Hamilton, 1292
1293 M.E. Anderson, High resistance to cisplatin in human ovarian 1293
1294 cancer cell lines is associated with marked increase of glu- 1294
1295 tathione synthesis, *Proc. Natl. Acad. Sci. U.S.A.* 89 (7) (1992) 1295
1296 3070-3074. 1296
1297 [18] M. Hari, F. Loganzo, T. Annable, X. Tan, S. Musto, D.B. Morilla, J.H. 1297
1298 Nettles, J.P. Snyder, L.M. Greenberger, Paclitaxel-resistant cells 1298
1299 have a mutation in the paclitaxel-binding region of Beta-tubulin 1299
1300 (Asp26Glu) and less stable microtubules, *Mol. Cancer Ther.* 5 (2) 1300
1301 (2006) 270-278.

- [19] B.T. Hennessy, R.L. Coleman, M. Markman, Ovarian cancer, *Lancet* 374 (9698) (2009) 1371–1382. 1361
- [20] B.T.J. Hennessy, K.M. Timms, M.S. Carey, A. Gutin, L.A. Meyer, D.D. Flake, V. Abkevich, J. Potter, D. Pruss, P. Glenn, Y. Li, J. Li, A.M. Gonzalez-Angulo, K.S. McCune, M. Markman, R.R. Broaddus, J.S. Lanchbury, K.H. Lu, G.B. Mills, Somatic mutations in BRCA1 and BRCA2 could expand the number of patients that benefit from poly (ADP Ribose) polymerase inhibitors in ovarian cancer, *J. Clin. Oncol.* 28 (22) (2010) 3570–3576. 1362
- [21] M.T. Huizing, G. Giaccone, L.J. van Warmerdam, H. Rosing, P.J. Bakker, J.B. Vermorken, P.E. Postmus, Z.N. van, M.G. Koolen, W.W. ten Bokkel Huinink, W.J. van der Vijgh, F.J. Bierhorst, A. Lai, O. Dalesio, H.M. Pinedo, C.H. Veenhof, J.H. Beijnen, Pharmacokinetics of paclitaxel and carboplatin in a dose-escalating and dose-sequencing study in patients with non-small-cell lung cancer. The European Cancer Centre, *J. Clin. Oncol.* 15 (1) (1997) 317–329. 1363
- [22] F. Hyafil, C. Vergely, V.P. Du, T. Grand-Perret, In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative, *Cancer Res.* 53 (19) (1993) 4595–4602. 1364
- [23] ICON3, Paclitaxel plus carboplatin versus standard chemotherapy with either single-agent carboplatin or cyclophosphamide, doxorubicin, and cisplatin in women with ovarian cancer: the ICON3 randomised trial, *Lancet* 360 (9332) (2002) 505–515. 1365
- [24] M. Joerger, A.D.R. Huitema, D.H.J.G. van den Bongard, J.H.M. Schellens, J.H. Beijnen, Quantitative effect of gender, age, liver function, and body size on the population pharmacokinetics of paclitaxel in patients with solid tumors, *Clin. Cancer Res.* 12 (7) (2006) 2150–2157. 1366
- [25] P.L. Judson, J.M. Watson, P.A. Gehrig, W.C. Fowler, J.S. Haskill, Cisplatin inhibits paclitaxel-induced apoptosis in cisplatin-resistant ovarian cancer cell lines: possible explanation for failure of combination therapy, *Cancer Res.* 59 (10) (1999) 2425–2432. 1367
- [26] Y. Kano, M. Akutsu, S. Tsunoda, K. Suzuki, Y. Yazawa, In vitro schedule-dependent interaction between paclitaxel and cisplatin in human carcinoma cell lines, *Cancer Chemother. Pharmacol.* 37 (6) (1996) 525–530. 1368
- [27] M.D. Kars, D.I. Ozlem, U. Gunduz, A. Ural, F. Arpacı, J. Molnar, Development of rational in vitro models for drug resistance in breast cancer and modulation of MDR by selected compounds, *Anticancer Res.* 26 (6B) (2006) 4559–4568. 1369
- [28] R.D. Kennedy, J.E. Quinn, P.G. Johnston, D.P. Harkin, BRCA1: mechanisms of inactivation and implications for management of patients, *Lancet* 360 (9338) (2002) 1007–1014. 1370
- [29] S. Lafarge, V. Sylvain, M. Ferrara, Y.J. Bignon, Inhibition of BRCA1 leads to increased chemoresistance to microtubule-interfering agents, an effect that involves the JNK pathway, *Oncogene* 20 (45) (2001) 6597–6606. 1371
- [30] D. Lawlor, P. Martin, S. Busschots, J. Thery, J.J. O’Leary, B.T. Hennessy, B. Stordal, PARP inhibitors as P-glycoprotein substrates, *J. Pharm. Sci.* 103 (6) (2014) 1913–1920. 1372
- [31] L. Li, Y. Luan, G. Wang, B. Tang, D. Li, W. Zhang, X. Li, J. Zhao, H. Ding, E. Reed, Q.Q. Li, Development and characterization of five cell models for chemoresistance studies of human ovarian carcinoma, *Int. J. Mol. Med.* 14 (2) (2004) 257–264. 1373
- [32] Y. Liang, L. O’Driscoll, S. McDonnell, P. Doolan, I. Oglesby, K. Duffy, R. O’Connor, M. Clynes, Enhanced in vitro invasiveness and drug resistance with altered gene expression patterns in a human lung carcinoma cell line after pulse selection with anticancer drugs, *Int. J. Cancer* 111 (4) (2004) 484–493. 1374
- [33] Z. Liu, M. Qiu, Q.L. Tang, M. Liu, N. Lang, F. Bi, Establishment and biological characteristics of oxaliplatin-resistant human colon cancer cell lines, *Chin. J. Cancer* 29 (7) (2010) 661–667. 1375
- [34] J.J. Manfredi, S.B. Horwitz, Taxol: an antimitotic agent with a new mechanism of action, *Pharmacol. Ther.* 25 (1) (1984) 83–125. 1376
- [35] M. Markman, J. Moon, S. Wilczynski, A.M. Lopez, J. Rowland, D.P. Michelin, V.J. Lanzotti, G.L. Anderson, D.S. Alberts, Single agent carboplatin versus carboplatin plus pegylated liposomal doxorubicin in recurrent ovarian cancer: final survival results of a SWOG (S0200) phase 3 randomized trial, *Gynecol. Oncol.* 116 (3) (2010) 323–325. 1377
- [36] C.G. Milross, L.J. Peters, N.R. Hunter, K.A. Mason, L. Milas, Sequence-dependent antitumor activity of paclitaxel (taxol) and cisplatin in vivo, *Int. J. Cancer* 62 (5) (1995) 599–604. 1378
- [37] K. Mross, N. Hollander, B. Hauns, M. Schumacher, H. Maier-Lenz, The pharmacokinetics of a 1-h paclitaxel infusion, *Cancer Chemother. Pharmacol.* 45 (6) (2000) 463–470. 1379
- [38] C.G. Murphy, M.E. Moynahan, BRCA gene structure and function in tumor suppression: a repair-centric perspective, *Cancer J. (Sudbury, Mass.)* 16 (1) (2010) 39–47. 1380
- [39] S. Oguri, T. Sakakibara, H. Mase, T. Shimizu, K. Ishikawa, K. Kimura, R.D. Smyth, Clinical pharmacokinetics of carboplatin, *J. Clin. Pharmacol.* 28 (3) (1988) 208–215. 1381
- [40] M. Osmak, L. Bizjak, B. Jernej, S. Kapitanovi-ć, Characterization of carboplatin-resistant sublines derived from human larynx carcinoma cells, *Mutat. Res. Lett.* 347 (3–4) (1995) 141–150. 1382
- [41] R.F. Ozols, Treatment goals in ovarian cancer, *Int. J. Gynecol. Cancer* 15 (2005) 3–11. 1383
- [42] R.F. Ozols, B.N. Bundy, B.E. Greer, J.M. Fowler, D. Clarke-Pearson, R.A. Burger, R.S. Mannel, K. DeGeest, E.M. Hartenbach, R. Baergen, Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a gynecologic oncology group study, *J. Clin. Oncol.* 21 (17) (2003) 3194–3200. 1384
- [43] T. Pal, J. Permeth-Wey, J.A. Betts, J.P. Krischer, J. Fiorica, H. Arango, J. LaPolla, M. Hoffman, M.A. Martino, K. Wakeley, G. Wilbanks, S. Nicosia, A. Cantor, R. Sutphen, BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases, *Cancer* 104 (12) (2005) 2807–2816. 1385
- [44] H. Parekh, K. Wiesen, H. Simpkins, Acquisition of taxol resistance via P-glycoprotein- and non-P-glycoprotein-mediated mechanisms in human ovarian carcinoma cells, *Biochem. Pharmacol.* 53 (4) (1997) 461–470. 1386
- [45] J. Pfisterer, M. Plante, I. Vergote, A. du Bois, H. Hirte, A.J. Lacave, U. Wagner, A. Stahle, G. Stuart, R. Kimmig, S. Olbricht, T. Le, J. Emerich, W. Kuhn, J. Bentley, C. Jackisch, H.J. Luck, J. Rochon, A. H. Zimmermann, E. Eisenhauer, Gemcitabine plus carboplatin compared with carboplatin in patients with platinum-sensitive recurrent ovarian cancer: an intergroup trial of the AGO-OVAR, the NCIC CTG, and the EORTC GCG, *J. Clin. Oncol.* 24 (29) (2006) 4699–4707. 1387
- [46] J.E. Quinn, C.R. James, G.E. Stewart, J.M. Mulligan, P. White, G.K.F. Chang, P.B. Mullan, P.G. Johnston, R.H. Wilson, D.P. Harkin, BRCA1 mRNA expression levels predict for overall survival in ovarian cancer after chemotherapy, *Clin. Cancer Res.* 13 (24) (2007) 7413–7420. 1388
- [47] J.E. Quinn, R.D. Kennedy, P.B. Mullan, P.M. Gilmore, M. Carty, P.G. Johnston, D.P. Harkin, BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis, *Cancer Res.* 63 (19) (2003) 6221–6228. 1389
- [48] S. Rao, G.A. Orr, A.G. Chaudhary, D.G.I. Kingston, S.B. Horwitz, Characterization of the taxol binding site on the microtubule, *J. Biol. Chem.* 270 (35) (1995) 20235–20238. 1390
- [49] H.A. Risch, J.R. McLaughlin, D.E.C. Cole, B. Rosen, L. Bradley, E. Kwan, E. Jack, D.J. Vesprini, G. Kuperstein, J.L.A. Abrahamson, I. Fan, B. Wong, S.A. Narod, Prevalence and penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with ovarian cancer, *Am. J. Hum. Genet.* 68 (3) (2001) 700–710. 1391
- [50] E.K. Rowinsky, The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents, *Annu. Rev. Med.* 48 (1997) 353–374. 1392
- [51] S.C. Rubin, M.A. Blackwood, C. Bandera, K. Behbakht, I. Benjamin, T.R. Rebbeck, J. Boyd, BRCA1, BRCA2, and hereditary nonpolyposis colorectal cancer gene mutations in an unselected ovarian cancer 1393

- 1421 population: relationship to family history and implications for
1422 genetic testing, *Am. J. Obstet. Gynecol.* 178 (4) (1998) 670–677.
- 1423 [52] R. Safaei, S.B. Howell, Copper transporters regulate the cellular
1424 pharmacology and sensitivity to Pt drugs, *Crit. Rev. Oncol.*
1425 *Hematol.* 53 (1) (2005) 13–23.
- 1426 [53] M. Salzberg, B. Thurlimann, H. Bonnefois, D. Fink, C. Rochlitz, M.
1427 R. von, H. Senn, Current concepts of treatment strategies in
1428 advanced or recurrent ovarian cancer, *Oncology* 68 (4-6) (2005)
1429 293–298.
- 1430 [54] R.J. Schilder, L. Hall, A. Monks, L.M. Handel, A.J. Fornace Jr., R.F.
1431 Ozols, A.T. Fojo, T.C. Hamilton, Metallothionein gene expression
1432 and resistance to cisplatin in human ovarian cancer, *Int. J. Cancer*
1433 45 (3) (1990) 416–422.
- 1434 [55] V. Smith, M.G. Rowlands, E. Barrie, P. Workman, L.R. Kelland,
1435 Establishment and characterization of acquired resistance to the
1436 farnesyl protein transferase inhibitor R115777 in a human colon
1437 cancer cell line, *Clin. Cancer Res.* 8 (6) (2002) 2002–2009.
- 1438 [56] B. Stordal, R. Davey, A systematic review of genes involved in the
1439 inverse resistance relationship between cisplatin and paclitaxel
1440 chemotherapy: role of BRCA1, *Curr. Cancer Drug Targets* 9 (3)
1441 (2009) 354–365.
- 1442 [57] B. Stordal, M. Hamon, V. McEneaney, S. Roche, J.P. Gillet, J.J.
1443 O’Leary, M. Gottesman, M. Clynes, Resistance to paclitaxel in a
1444 cisplatin-resistant ovarian cancer cell line is mediated by P-
1445 Glycoprotein, *PLoS One* 7 (7) (2012) (e40717-e40717).
- 1446 [58] B. Stordal, N. Pavlakis, R. Davey, A systematic review of platinum
1447 and taxane resistance from bench to clinic: an inverse relation-
1448 ship, *Cancer Treat. Rev.* 33 (8) (2007) 688–703.
- 1449 [59] B. Stordal, K. Timms, A. Farrelly, D. Gallagher, S. Busschots, M. I.
1450 Renaud, J. Thery, D. Williams, J. Potter, T. Tran, G. Korpany, M.
1451 Cremona, M. Carey, J. Li, Y. Li, O. Aslan, J.J. O’Leary, G.B. Mills, B.T.
1452 Hennessy, BRCA1/2 mutation analysis in 41 ovarian cell lines
1453 reveals only one functionally deleterious BRCA1 mutation, *Mol.*
1454 *Oncol.* 7 (3) (2013) 567–579.
- 1455 [60] K. Suzukake, B.J. Petro, D.T. Vistica, Reduction in glutathione
1456 content of L-PAM resistant L1210 Cells confers drug sensitivity,
1457 *Biochem. Pharmacol.* 31 (1) (1982) 121–124.
- 1458 [61] P. Tassone, P. Tagliaferri, A. Perricelli, S. Blotta, B. Quaresima, M.L.
1459 Martelli, A. Goel, V. Barbieri, F. Costanzo, C.R. Boland, S. Venuta,
1460 BRCA1 expression modulates chemosensitivity of BRCA1-
1461 defective HCC1937 human breast cancer cells, *Br. J. Cancer* 88 (8)
1462 (2003) 1285–1291.
- 1463 [62] B.A. Teicher, C.A. Cucchi, J.B. Lee, J.L. Flatow, A. Rosowsky, E. Frei,
1464 Alkylating agents: in vitro studies of cross-resistance patterns in
1465 human cell lines, *Cancer Res.* 46 (9) (1986) 4379–4383.
- 1466 [63] J.M. Teodoridis, J. Hall, S. Marsh, H.D. Kannall, C. Smyth, J. Curto,
1467 N. Siddiqui, H. Gabra, H.L. McLeod, G. Strathdee, R. Brown, CpG
1468 island methylation of DNA damage response genes in advanced
1469 ovarian cancer, *Cancer Res.* 65 (19) (2005) 8961–8967.
- 1470 [64] D. Vandier, V. Calvez, L. Massade, A. Gouyette, L. Mickley, T. Fojo,
1471 O. Rixe, Transactivation of the metallothionein promoter in
1472 cisplatin-resistant cancer cells: a specific gene therapy strategy,
1473 *J. Natl. Cancer Inst.* 92 (8) (2000) 642–647.
- 1474 [65] P.A. Vasey, G.C. Jayson, A. Gordon, H. Gabra, R. Coleman, R.
1475 Atkinson, D. Parkin, J. Paul, A. Hay, S.B. Kaye On behalf of the
1476 Scottish Gynaecological Cancer Trials Group Phase III randomized
1477 trial of docetaxel-carboplatin versus paclitaxel-carboplatin as
1478 first-line chemotherapy for ovarian carcinoma, *J. Natl. Cancer*
1479 *Inst.* 96 (22) (2004) 1682–1691.
- 1480 [66] D. Xing, S. Orsulic, A mouse model for the molecular character-
1481 ization of BRCA1-associated ovarian carcinoma, *Cancer Res.* 66
1482 (18) (2006) 8949–8953.
- 1483 [67] X.D. Yan, M. Li, Y. Yuan, N. Mao, L.Y. Pan, Biological comparison of
1484 ovarian cancer resistant cell lines to cisplatin and taxol by two
1485 different administrations, *Oncol. Rep.* 17 (5) (2007) 1163–1169.
- 1486 [68] T.T. Yang, P. Sinai, S.R. Kain, An acid phosphatase assay for
1487 quantifying the growth of adherent and nonadherent cells, *Anal.*
1488 *Biochem.* 241 (1) (1996) 103–108.
- 1489 [69] W. Ying, S. Wang, J. Shi, Y. Sun, ER–/ER+ breast cancer cell lines
1490 exhibited different resistance to paclitaxel through pulse selec-
1491 tion, *Med. Oncol.* 29 (2) (2012) 495–502.
- 1492 [70] J. Zhang, J. Zhao, W. Zhang, G. Liu, D. Yin, J. Li, S. Zhang, H. Li,
1493 Establishment of paclitaxel-resistant cell line and the underlying
1494 mechanism on drug resistance, *Int. J. Gynecol. Cancer* 22 (9)
1495 (2012) 1450–1456.
- 1496 [71] C. Zhou, J.L. Smith, J. Liu, Role of BRCA1 in cellular resistance to
1497 paclitaxel and ionizing radiation in an ovarian cancer cell line
1498 carrying a defective BRCA1, *Oncogene* 22 (16) (2003) 2396–2404.