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Original Research

Identifying and overcoming a mechanism of resistance to WEE1 kinase inhibitor AZD1775 in high grade serous ovarian cancer cells

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Abstract

Objective: As a result of *TP53* gene mutation high grade serous ovarian cancer (HGSOC) is dependent on the G2 checkpoint for the repair of DNA damage and survival. The key role of WEE1 kinase at this checkpoint makes inhibition of WEE1 kinase in combination with DNA damaging agents an attractive therapeutic strategy for HGSOC. Our aim was to characterise resistance mechanisms to WEE1 inhibitor AZD1775 and identify ways to overcome resistance ready for use in the clinic. **Methods:** AZD1775-resistant HGSOC cell clones were isolated and western blotting, cell cycle analysis, growth assays, RNA-Seq and gene expression analysis were used to characterise resistance mechanisms and investigate a way to overcome resistance. **Results:** A resistance mechanism previously reported in small cell lung cancer did not operate in HGSOC. Instead, resistance resulted from different cell cycle control pathway changes that slow AZD1775-induced cell cycle progression and reduce accumulation of replication associated DNA damage. One major change was reduced levels of CDK1, the substrate for WEE1 kinase inhibition; another was increased levels of PKMYT1, which can also inhibit CDK1. Increased expression of *TGFβ* signalling to slow cell cycle progression occurred in resistant clones. A *TGFβR1* inhibitor overcame resistance in a clone with the highest *TGFβR1* receptor expression. **Conclusions:** Although overexpression of the membrane glycoprotein MDR1 is a common mechanism of drug resistance, it was not involved in our HGSOC cells. Instead AZD1775 resistance resulted from cell cycle control pathway changes that combine to slow AZD1775-induced cell cycle progression and so reduce accumulation of replication-associated DNA damage.

Keywords: ovarian cancer; cell cycle control; WEE1 kinase inhibition; resistance mechanism; DNA repair

1. Introduction

Ovarian cancer is the eighth commonest cause of female cancer mortality worldwide [1]. High grade serous ovarian cancer (HGSOC) is the commonest form and is characterised by *TP53* mutation and genetic instability. There are two major molecular types, homologous recombination repair (HRR) proficient and deficient [2]. Most HGSOC cases are treated by a combination of surgery and adjuvant chemotherapy with carboplatin and a taxane [3]. Patients with HRR-deficient cancers respond well initially, but the majority relapse after first line chemotherapy due to drug resistance. Neoadjuvant chemotherapy prior to surgery is an attractive alternative for some patients that can provide an early indication of chemosensitivity, but there is currently no agreement about which patients are most appropriate for this treatment option [4]. For HRR-deficient HGSOC poly ADP-ribose polymerase (PARP) inhibitors have provided a major advance in treatment options [5], but inhibitor resistance can continue to limit survival [6].

HGSOCs depend on the G2 checkpoint for DNA damage repair because the G1 checkpoint is inactivated by *TP53* mutation. The phosphorylation status of CDK1 controls the G2 checkpoint. DNA damage leads to inhibitory phosphorylation of CDK1 by WEE1 kinase resulting in G2 arrest and so providing time for DNA repair before mitosis [7]. In the absence of G2 arrest cells with damaged DNA enter mitosis prematurely leading to mitotic catastrophe [8–10]. Thus, WEE1 kinase inhibition with AZD1775 together with DNA damaging agents is a particularly attractive therapeutic strategy for HGSOC [11]. Phase I trials have reported target engagement and partial responses in patients with *BRCA*-deficient cancers [12,13]. A series of Phase II studies, involving patients with ovarian and other gynaecological cancers, have reported better progression free survival, or improved response rate with AZD1775 and a DNA damaging agent than with the damaging agent alone, although higher levels of adverse events have also been seen [14–19].



Our aim was to determine AZD1775 resistance mechanisms in HRR-proficient and -deficient HGSOC and find a way to overcome resistance that could be ready to deploy as the inhibitor entered the clinic. CDK1 is phosphorylated on Y15 by WEE1 kinase [20], while a different kinase, PKMYT1 (MYT1), phosphorylates CDK1 at T14 as well as Y15 [20]. We hypothesised that resistance could arise from changes in key DNA damage response and cell cycle control pathways. While our study was in progress resistance to AZD1775 in small cell lung cancer (SCLC) was found to be due to increased expression of AXL and MET receptors activating CHK1 via mTOR and pERK pathways. CHK1 then activated WEE1 kinase and inhibited CDC25 [21]. Resistance could be overcome by targeting AXL or mTOR. We also wished to investigate whether this very different resistance mechanism to the one that we have envisaged operates in HGSOC.

2. Materials and methods

2.1 Cell culture assays

The human HGSOC cell lines ES-2 and OVCAR8, validated by short tandem repeat profiling, were sourced from the American Type Culture Collection. Culture conditions used are described in Supplementary Methods. All experiments were performed on cultures within 10 passages of supply. Clones resistant to WEE1 inhibitor AZD1775 (S1525, Selleckchem, Houston, TX) were isolated by plating cells at 1000 cells/well in 96-well plates in medium containing the selective AZD1775 concentration. Resistant colonies appeared after around 14 days of selection. Single colonies arising in individual wells were picked, expanded and maintained under selection. Selective medium was changed every 3–4 days. Sensitivity of cell lines to AZD1775 was determined by Sulphorhodamine B (SRB) assay [22]. 2000 cells per well were plated in 96-well plates containing a dilution series of AZD1775 (8 wells for each dilution). DMSO, the solvent for AZD1775, was maintained at <0.01% and had no effect on the assay. Each 5-day growth assay was repeated twice. Sensitivity of AZD1775-resistant clones to the TGF β receptor1 inhibitor RepSox (S7223, Selleckchem) was also determined by SRB assay. Dose response curves and IC50 values with 95% confidence intervals were obtained using GraphPad Prism. For cell cycle analysis 50000 cells/well were plated in 6-well plates. Twelve hours after plating cells were treated for 24 or 72 hours with AZD1775 before flow cytometry was carried out as described [23].

2.2 Western blotting

Protein extraction and western blotting were performed as described [24]. Antibodies used are shown in **Supplementary Table 1**.

2.3 RNA-Seq

The first experiment comprised 16 samples (MKG1-16): duplicate cultures of untreated ES-2 parent and ES-2 parent treated for 24 hours with 100 nM AZD1775, triplicate cultures of four resistant clones in their selective concentration of AZD1775. There were two clones resistant to 1000 nM AZD1775 and their progenitors resistant to 250 nM AZD1775. Experiment 2 comprised 12 samples (MKG101-112): triplicate cultures of OVCAR8 parent treated with 100 nM AZD1775 for 24 hours and triplicate cultures of three OVCAR8-derived resistant clones in their selective concentration of 300 nM AZD1775 (see **Supplementary Table 2**). Cells were grown in 25 cm² flasks until they reached 70% confluency and then total RNA was extracted using the RNeasy mini kit (74104, Qiagen Ltd., Manchester, UK). RNA QC and subsequent sequencing were performed at the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh. RNA was quantified and DNA contamination assessed using Qubit RNA HS and dsDNA HS assay kits (Thermo Fisher Scientific) on a Qubit 2.0 Fluorimeter. Samples were checked for RNA integrity with the RNA 6000 Nano Kit (Agilent Technologies UK Ltd., Edinburgh, UK) using an Agilent 2100 Bioanalyser, with RIN numbers for all samples >9.6. 500 ng of each RNA was used to prepare a total RNA library with the TruSeq Stranded Total RNA with Ribo-Zero Gold kit (Illumina, Cambridge, UK). Further details of library preparation, sequencing, bioinformatics analysis and quality control are in Supplementary Methods.

3. Results

3.1 Isolation of clones resistant to AZD1775 from HRR-proficient and HRR-deficient HGSOC cell lines

Two validated HGSOC cell lines [25] were chosen for this study for their ability to form colonies when seeded thinly. Firstly, OVCAR8, with hypermethylation of the *BRCAl* gene promoter [26] and shown to be HRR-deficient by a functional homologous recombination assay [27]. Secondly, ES-2, with an IC50 value in an SRB assay to assess sensitivity to PARP inhibitor olaparib that was >10-fold higher than for OVCAR8 [28] and so was considered to be HRR-proficient. Like other HGSOC cell lines, both ES-2 and OVCAR8 are reported to have *TP53* gene mutations [29]: ES-2, missense mutation in exon7, S241F; OVCAR8, pathogenic RNA splice site mutation and this was confirmed by DNA sequencing (data not shown).

In growth assays ES-2 had an IC50 for AZD1775 of 190 nM (Fig. 1A), while OVCAR8 had an IC50 of 230 nM (Fig. 1B). ES-2 clones resistant to 250 nM AZD1775 (arising at a frequency of 7×10^{-4}) and OVCAR8 clones resistant to 300 nM AZD1775 (frequency 6×10^{-4}) were isolated first. Two ES-2 clones resistant to 250 nM AZD1775 (ES-2 AZ_250:3 and ES-2 AZ_250:7) were then used in a second round of selection to obtain clones resistant to 1000 nM AZD1775 (frequency 3×10^{-4}). See **Supple-**

mentary Fig. 1 for details of clones used in this study and for clone nomenclature. ES-2 clones resistant to 250 nM AZD1775 showed 3-fold increased AZD1775 IC50 values over the ES-2 parent, while their 1000 nM resistant derivatives showed 6 to 11-fold increases (Fig. 1A). OVCAR8 clones resistant to 300 nM AZD1775 showed smaller, ~2-fold IC50 increases compared to OVCAR8 (Fig. 1B).

3.2 Cell cycle profiles of AZD1775-resistant clones are unaffected by AZD1775

Cell cycle profiles for the ES-2 parent and two clones resistant to 1000 nM AZD1775 (ES-2 AZ_1000:7_250:3 and ES-2 AZ_1000:2_250:7), derived from different 250 nM progenitor clones, are shown in Fig. 1C. Cultures were untreated or treated for 24 h with 1000 nM AZD1775. In AZD1775-treated ES-2 cultures the normal sharp distinction between cells in S and G2/M phases was lost. There was an increased frequency of cells in G2/M, reflecting the ability of WEE1 inhibition to drive cells into and then trap them in mitosis leading to a reduced frequency of cells in G1. Profiles of both resistant clones were unaffected by AZD1775. Compared to ES-2 the frequency of S-phase cells in both clones was reduced, likely reflecting the slower growth rate observed for all ES-2 and OVCAR8-derived AZD1775-resistant clones (**Supplementary Table 3**). The two progenitor clones resistant to 250 nM AZD1775 were next treated with 250 nM AZD1775 for 72 h (Fig. 1D). ES-2 cells treated in the same way again showed a highly dysregulated profile, but now with additional populations with subG1 (presumed apoptotic) and >4n DNA content (indicating continued DNA synthesis without cell division). By contrast AZD1775-treated ES-2 AZ_250:1 and ES-2 AZ_250:3 showed normal profiles. Two OVCAR8 clones resistant to 300 nM AZD1775 were also treated for 72 h with 300 nM AZD1775 (**Supplementary Fig. 2**). As expected, AZD1775-treated resistant clones showed normal cell cycle profiles, but the AZD1775 effect on S-phase in the OVCAR8 parent was not as noticeable as on ES-2.

3.3 The mechanism of AZD1775 resistance reported in SCLC does not operate in HGSOc

In SCLC cell lines resistance to AZD1775 was due to increased expression of AXL and MET receptors, leading to increased activation of CHK1 via increased signalling through mTOR and ERK/p90RSK pathways [21]. Western blotting was first carried out to see if the same mechanism of resistance was operating in our HGSOc cell lines (Fig. 2A). Although AXL and MET expression in OVCAR8 clones was slightly increased, none of the ES-2 and OVCAR8 clones showed consistent increased expression of AXL and MET receptors, nor of any markers for activation of their signalling pathways as seen in SCLC (mTOR pS2448, S6 pS240/244, and ERK pT202/Y204). When levels of activated CHK1 pS345 were compared in parents and clones both with and without AZD1775 treatment, it was

clear that the observed increase in CHK1 pS345 was as a result of exposure to AZD1775 rather than as part of the resistance mechanism (Fig. 2E). Activated CHK1 levels were lower in AZD1775-treated resistant ES-2 clones than their parents. We conclude that the mechanism of AZD1775 resistance reported in SCLC is not seen in HGSOc.

3.4 Altered cell cycle control protein levels in AZD1775-resistant HGSOc clones

Sequencing of all the coding exons of the *WEE1* gene from all resistant HGSOc clones found no evidence for mutation, ruling out the most direct resistance mechanism, whereby a *WEE1* mutation would prevent inhibition by AZD1775. Levels of WEE1 and PKMYT1 kinases and their phosphorylation target CDK1 were then determined in resistant HGSOc clones (Fig. 2B). Increased expression of WEE1 kinase above the parental level was found in OVCAR8 clones, but ES-2 clones showed no consistent change. One ES-2 clone, ES-2 AZ_250:3, showed an increase, but the WEE1 kinase level in its derivative clone, ES-2 AZ_1000:2_250:3, was very low. Expression of PKMYT1 was very low in both ES-2 parent and resistant clones. PKMYT1 expression was much higher in OVCAR8 and its resistant clones, with the level in OVCAR8 AZ_300:6 marginally increased over the parent.

The reduction in total CDK1 level seen in some ES-2 clones in Fig. 2B was studied further in a larger group of resistant clones. A reduced level of total CDK1, most noticeable in clones resistant to 1000 nM AZD1775, was seen in most of the ES-2 derived clones tested, but in only a single OVCAR8 clone (Fig. 2C). This suggests that reduced CDK1 levels could be involved in resistance in ES-2, but not in OVCAR8 clones.

To confirm continued WEE1 kinase inhibition by AZD1775 in resistant clones, cell lysates were prepared from ES-2 and two resistant clones grown with and without 250 nM AZD1775 for 24 h (Fig. 2D). Both parent and resistant clones treated with AZD1775 showed a major reduction in phosphorylation of CDK1 at Tyr15, so demonstrating continued target engagement of AZD1775 with WEE1 kinase in resistant clones. The OVCAR8 parent and three resistant clones grown with or without AZD1775 treatment for 48 h also demonstrated clear inhibition by AZD1775 of WEE1 kinase activity (**Supplementary Fig. 3A**).

Overexpression of the membrane glycoprotein MDR1 is involved in resistance to olaparib and some chemotherapeutic agents [30]. Its possible involvement in AZD1775 resistance was investigated and the result is shown in the bottom panel in Fig. 2E. A cisplatin resistant colorectal cancer cell line overexpressing MDR1, HCT-15, was used as a positive control [31]. Although MDR1 levels showed very minor variation between resistant clones, levels were >10-fold lower than in HCT-15. Continued AZD1775 target engagement, together with failure to observe MDR1 overexpression, both argue strongly against increased expression

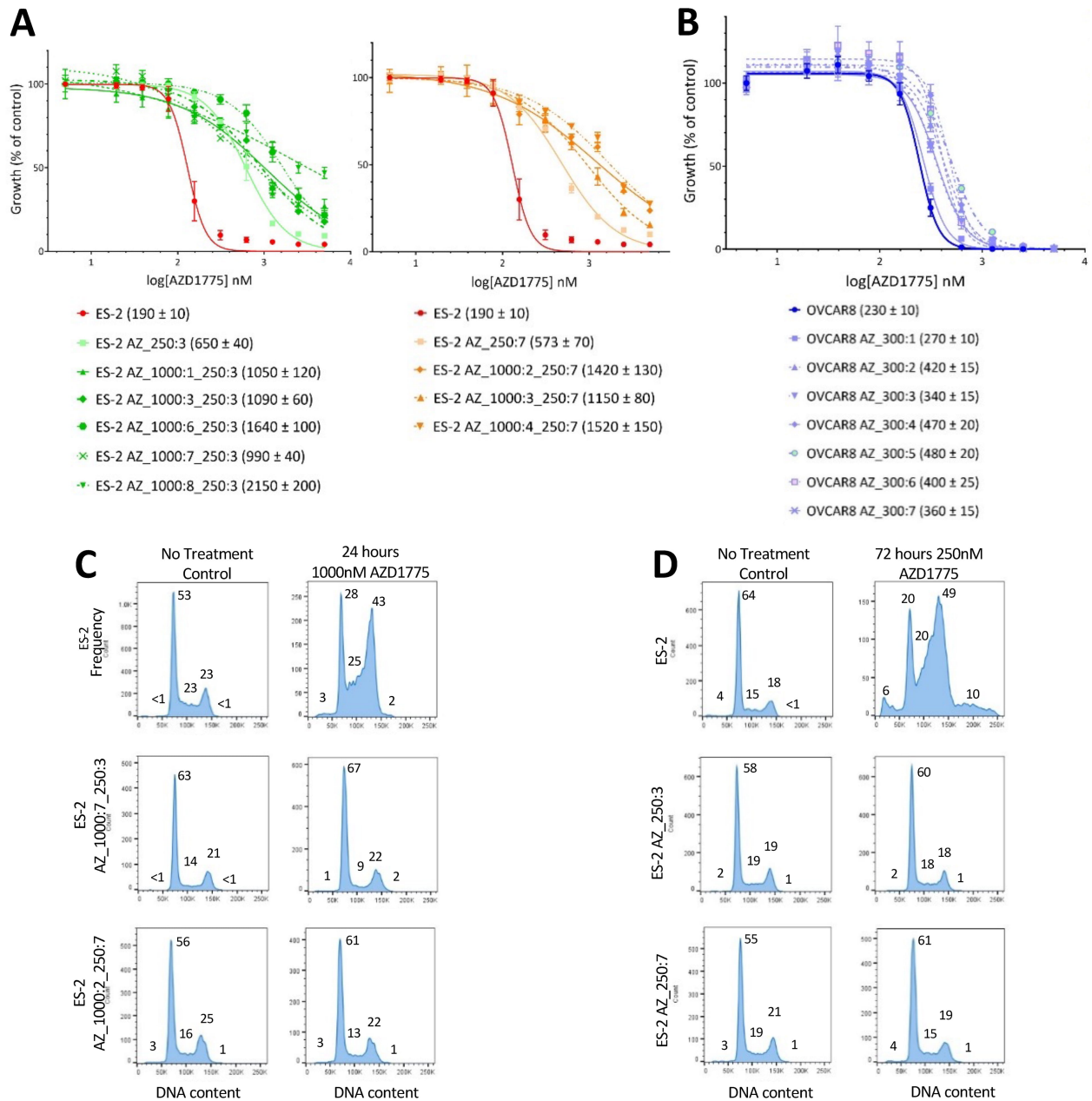


Fig. 1. Growth assay and analysis of cell cycle in resistant clones. (A) Growth curves for two groups of ES-2 clones resistant to 1000 nM AZD1775 and their clonal predecessors resistant to 250 nM. The curve for the ES-2 parent is in red. Left, clonal predecessor ES-2 AZ_250:3, resistant to 250 nM AZD1775 (light green) and five derivatives resistant to 1000 nM (dark green). Right, clonal predecessor ES-2 AZ_250:7, resistant to 250 nM (light orange) and three derivatives resistant to 1000 nM (dark orange). AZD1775 IC50 values (nM ± SEM) for each cell line are shown below the curves. (B) Growth curves for OVCAR8 clones resistant to 300 nM AZD1775. The OVCAR8 parent is shown in blue, clones are shown in light purple. (C) Cell cycle analysis of ES-2 clones resistant to 1000 nM AZD1775. Flow cytometry of untreated ES-2 parent and clones ES-2 AZ_1000:7_250:3 and ES-2 AZ_1000:2_250:7, and cells treated with 1000 nM AZD1775 for 24 hours. x axis, DNA content (intensity of propidium iodide staining); y axis, frequency (count). Across the top of each profile is indicated the percentage of cells with subG1, G1, S, G2/M, and >4n DNA content. Note the aberrant profile for the treated parent ES-2. (D) Cell cycle analysis of ES-2 clones resistant to 250 nM AZD1775. Flow cytometry of untreated parent ES-2 and clones ES-2 AZ_250:3 and ES-2 AZ_250:7, and cells treated with 250 nM AZD1775 for 72 hours. Note the highly dysregulated profile for treated ES-2 parent with discrete subG1 and >4n DNA content populations.

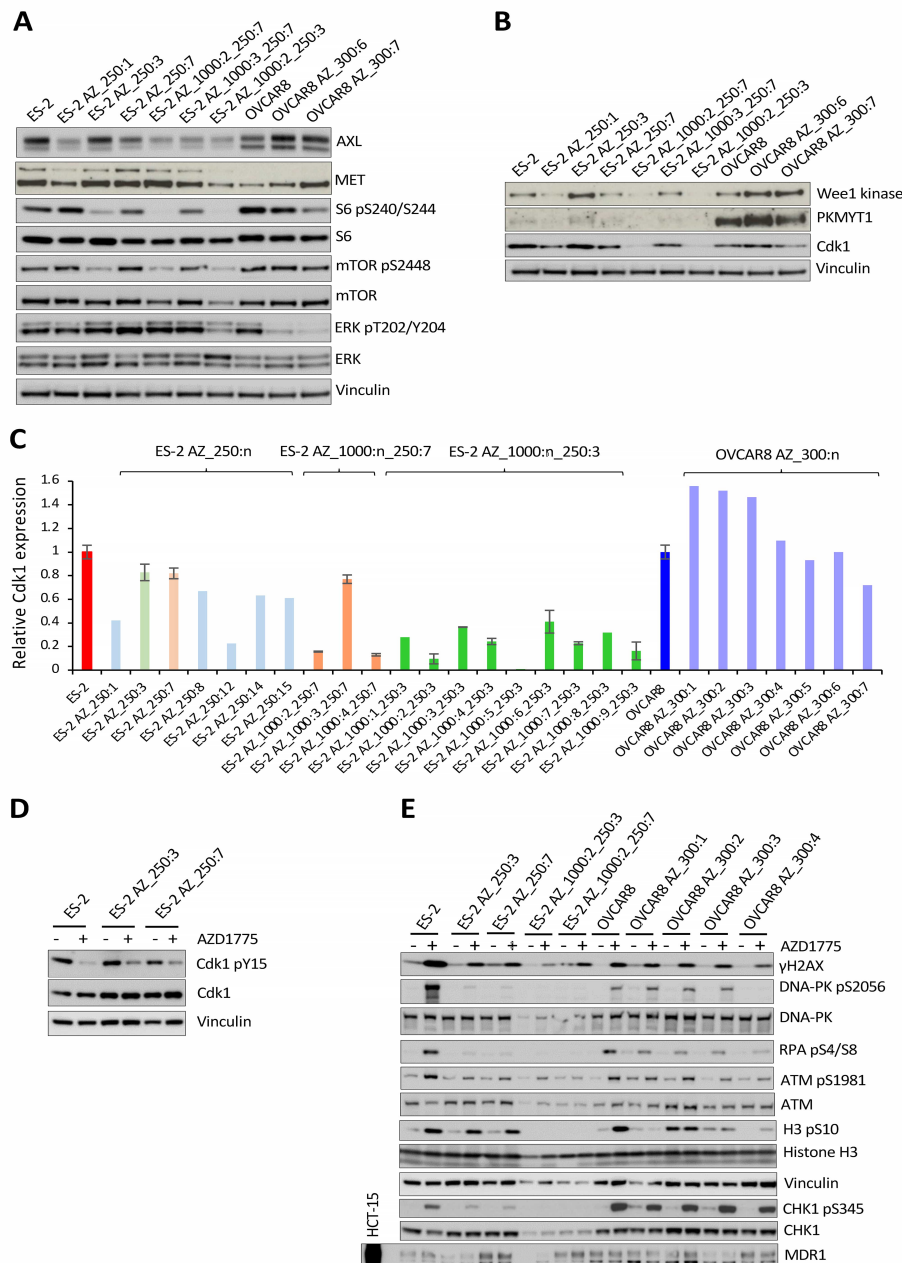


Fig. 2. Investigation by western blotting of the resistance mechanism to AZD1775 in HGSOE clones. (A) Upregulation of AXL or MET signalling is not involved in the resistance mechanism. Western blots of ES-2, OVCAR8 and AZD1775-resistant clones for AXL (138 kDa), MET (140 kDa), S6 pS240/244 (32 kDa), S6, mTOR pS2448 (289 kDa), mTOR, ERK pT202/Y204 (42,44 kDa), ERK and vinculin (115 kDa) as loading control. (B) Changes in levels of WEE1, PKMYT1 and CDK1 in resistant clones. Western blots of WEE1 kinase (95 kDa), PKMYT1 (65 kDa), CDK1 (34 kDa) and vinculin as loading control. (C) CDK1 protein levels are reduced in ES-2 clones resistant to AZD1775. Total CDK1 levels in resistant clones are shown in the histogram. Levels are expressed relative to the respective parent lines and are corrected for loading control differences. Error bars indicate that the values are means from three different determinations. (D) Continued target engagement in AZD1775-resistant ES-2 clones. Western blots of resistant clones and ES-2 parent for CDK1 pY15, total CDK1 and with vinculin for loading control. Lysates were made from cultures grown with (+) or without (-) 250 nM AZD1775 for 24 h. (E) Altered DDR in resistant clones. Western blots of γ H2AX (15 kDa), DNA-PK pS2056 (450 kDa), DNA-PK, RPA pS4/S8 (32 kDa), ATM pS1981 (370 kDa), ATM, Histone H3 pS10 (17 kDa), Histone H3, CHK1 pS345 (54 kDa), CHK1, MDR1 (130-180 kDa) for resistant clones and ES-2 and OVCAR8 parents with vinculin as loading control. Lysates were made from cultures grown in the absence (-) or presence (+) of AZD1775 for 24 h. 250 nM AZD1775 was used for the ES-2 parent, 300 nM for OVCAR8, and the selective AZD1775 concentration for resistant clones. Note increased MDR1 levels in colorectal cancer cell line HCT-15.

of this drug transporter as a mechanism of AZD1775 resistance in our HGSOC cells.

3.5 The DNA damage response is altered in AZD1775-resistant HGSOC clones

In addition to preventing G2 arrest and trapping cells in mitosis, inhibition of WEE1 kinase also disables the S-phase checkpoint resulting in faster passage through S-phase and increased levels of replication-associated DNA damage, such as single- and double-stranded DNA breaks, stalled replication forks, and single-stranded DNA regions [32–34]. The DNA damage response (DDR) between untreated and AZD1775-treated parent and resistant clones was compared by western blotting (Fig. 2E and quantified in **Supplementary Fig. 3B**). As expected, all AZD1775-treated lysates showed increased levels of the DNA damage marker γ H2AX [35] compared to untreated lysates. However, when treated with AZD1775, all resistant ES-2 clones showed reduced γ H2AX levels compared to the ES-2 parent. Clone OVCAR8 AZ_300:1 showed the same reduced γ H2AX response to AZD1775 as the ES-2 clones, while three other OVCAR8 clones showed a similar increased response as OVCAR8. There were also major reductions in levels of activated DNA-PK, which is involved in dsDNA break repair [36], and of activated RPA, a marker for ssDNA regions [37], in all AZD1775-treated ES-2 clones compared to the treated parent. AZD1775-treated OVCAR8 AZ_300:4 showed the same low level of activated DNA-PK as ES-2 clones, while the remaining three OVCAR8 clones showed the same high level of activated DNA-PK as the OVCAR8 parent. Activated RPA levels were slightly lower in three treated OVCAR8 clones than the OVCAR8 parent and marginally increased in OVCAR8 AZ_300:3.

The activated ATM response mirrored the DNA-PK and RPA result, with generally reduced activation levels in treated resistant clones relative to treated parent. The altered DDR to AZD1775 in resistant clones relative to the parent suggests that the ability to reduce the level of DNA damage resulting from exposure to AZD1775 could be an important component in the development to resistance. Altered DDR was more pronounced in resistant ES-2 than OVCAR8 clones and we also observed interclonal heterogeneity in the response.

Levels of a marker for chromosomal condensation and mitosis, H3 pS10 [38], were 8–10-fold elevated in both treated parental lines compared to untreated cultures (Fig. 2E and quantified in **Supplementary Fig. 3B**), reflecting the ability of AZD1775 to prevent G2 arrest resulting in premature entry into mitosis [11]. Whereas, in resistant clones where levels of H3 pS10 could be detected, the level of elevation of H3 pS10 in AZD1775-treated compared to untreated cultures was much lower, indicating that this could also be important for resistance. Although note that failure to see increased H3 pS10 expression on

AZD1775 treatment in OVCAR8 AZ_300:2 was due to unusually high expression in the untreated lysate rather than a smaller increase on treatment.

3.6 Cell cycle and related pathway genes have altered expression in AZD1775-resistant clones

To better understand the transcriptional events associated with drug resistance we carried out RNA sequencing across our cell lines. Because we were most interested in identifying changes in gene expression in resistant clones that allowed them to grow in the continuous presence of AZD1775, while parental cells died on prolonged exposure, we made two separate gene expression comparisons. At the centre were parental cells treated with AZD1775. To one side they were compared against resistant clones treated with AZD1775. To the other side they were compared against untreated parental cells. Gene Set Enrichment Analysis (GSEA, see Supplementary Methods) of differentially expressed genes between parental and resistant clones was carried out. The analysis revealed particular enrichment across a number of altered gene networks, including cell cycle regulation, TGF β signalling which has an important role in cell cycle arrest, and apoptosis. Since our western blotting analysis also indicated that altered cell cycle control protein levels were involved in resistance to AZD1775, we decided to concentrate on a detailed comparison of gene expression differences between parent and resistant clones for these three networks (Fig. 3). For each network a clear change in expression was observed between the ES-2 parent (treated and untreated) and the two treated clones resistant to 1000 nM AZD1775. Many highly expressed genes in the parent showed reduced expression in the clones, and vice versa. This shift was not simply due to global alterations, as evidenced by analysis of a similar sized control set of randomly selected genes. An intermediate expression pattern was seen in the clones resistant to 250 nM AZD1775 from which the 1000 nM clones were derived. A higher concentration of AZD1775 would likely have been required for the difference in expression of cell cycle control genes between untreated and treated ES-2 parent to better reflect the very different cell cycle profiles shown in Fig. 1C. Treated clones OVCAR8 AZ_300:2 and OVCAR8 AZ_300:3 showed a similar altered pattern of cell cycle control gene expression compared to the treated OVCAR8 parent as seen in the comparison between ES-2 clones and the ES-2 parent (**Supplementary Fig. 4**).

Fig. 4 is adapted from the KEGG cell cycle pathway to show gene expression changes between parent and resistant clones in key parts of the pathway. Changes in gene expression levels for the entire cell cycle control pathway for which expression data were obtained are shown in **Supplementary Figs. 5–7**. Fig. 4A shows the comparison between AZD1775-treated and untreated ES-2 parent. As would be expected for untreated actively cycling cells, increased expression of cyclins (*CCNE* and *CCNA*)

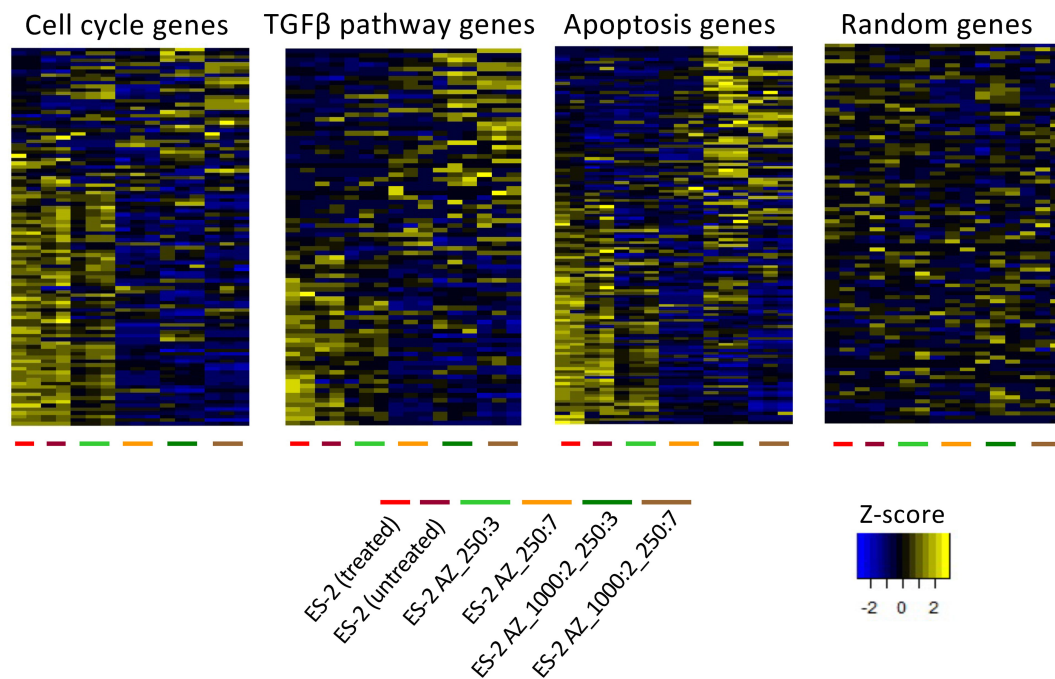


Fig. 3. Altered gene expression patterns for cell cycle and related pathways between resistant ES-2 clones and the AZD1775-sensitive parent. Shown are Z-score heat maps on a linear colour scale for genes in cell cycle control, TGF β and apoptosis pathways in duplicate ES-2 untreated and AZD1775-treated samples and in triplicate samples for treated resistant clones ES-2 AZ_250:3, ES-2 AZ_250:7, ES-2 AZ_1000:2_250:3 and ES-2 AZ_1000:2_250:7. Yellow indicates increased and blue indicates reduced expression. A randomly selected gene set of equivalent size was used as a control. See **Supplementary Table 4** for the gene lists.

and cyclin dependent kinases (*CDK2*) and reduced expression of CDK inhibitors (notably *CDKN1A* and *CDKN2B*) was observed compared to the treated parent. Reduced expression of the TGF β pathway in untreated compared to treated parent was indicated by low expression of *SMADs2-4*, which slow cell cycle progression by inducing expression of *CDKN1A* and *CDKN2B*. Expression of *SKP2*, which encodes an E3 ubiquitin protein ligase mediating degradation of CDKN1A-C, was strongly increased in untreated compared to treated ES-2 parent.

Comparison of treated resistant ES-2 clones with treated ES-2 parent revealed a number of differences (Fig. 4B). There was decreased expression of most cyclin and *CDK* genes in resistant clones. There was also down-regulation of many other genes involved in active cycling, growth and cell division. Unlike the treated parent, the TGF β pathway was activated in both clones, although the mechanism of activation between clones appeared different. Increased expression of *TGF β R1* receptor was found in ES-2 AZ_1000:2_250:3, while ES-2 AZ_1000:2_250:7 has increased *TGF β* ligand expression. Increased *SMAD3* expression was highest in ES-2 AZ_1000:2_250:3. Strongly increased expression in both resistant clones of *CDKN1A* and *CDKN2B* was the other striking change. Expression of both these *CDKN* genes is efficiently induced by *SMAD3/4* [39]. Increased *CDKN* inhibitor expression was consistent

with reduced expression of *CDKs* and cyclins. There was no evidence for the postulated involvement in resistance of increased expression of *WEE1*, *PKMYT1*, or *CDC14A* in the ES-2 clones. Increased expression of *TP53* and reduced expression of *CDC25A-C* indicated some increased expression of DDR pathway genes in the clones (**Supplementary Fig. 6**). The ability of p53 to activate *CDKN1A* and *GAD45* in the DDR is inoperative as a result of *TP53* mutation found in both ES-2 and OVCAR8. However, note that the increased activity through the TGF β pathway fulfils the same function.

Cell cycle control gene expression comparison between treated resistant OVCAR8 clones and treated OVCAR8 parent showed less marked changes and more heterogeneity than seen with ES-2 clones (Fig. 4C). Decreased expression of most *CDK* and cyclin genes was again observed, but there was increased expression of *CCNE1-2* and *CDK6* in OVCAR8 AZ_300:2 and OVCAR8 AZ_300:3. TGF β pathway expression was again elevated with increased expression of *TGF β R1* and *TGF β 2* in OVCAR8 AZ_300:4 and elevated *SMAD3/4* in both OVCAR8 AZ_300:2 and OVCAR8 AZ_300:3. The elevated *CDKN1A* and *CDKN2B* expression observed in the ES-2 clones was not seen in OVCAR8 clones. Unlike the situation in ES-2, all OVCAR8 clones had increased *WEE1* expression and OVCAR8 AZ_300:2 and OVCAR8 AZ_300:3 also showed in-

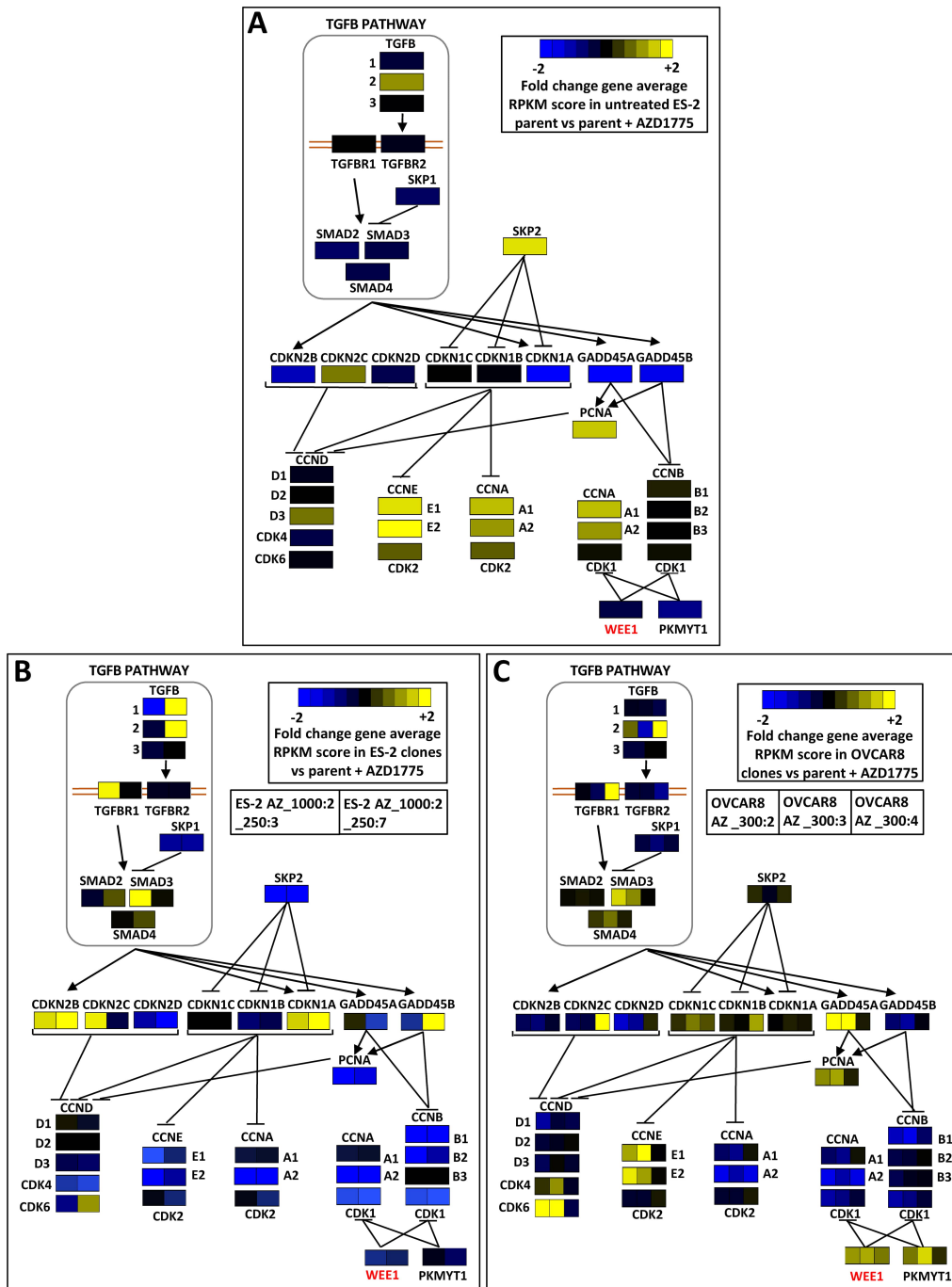


Fig. 4. Gene expression changes in the cell cycle control pathway in resistant clones. Action of a gene at the tail of an arrow increases expression of the gene at the arrowhead, or causes elevated activity of the encoded protein. Lines with bars at the end denote that the gene at the origin of the line decreases expression of the gene at the bar, or decreases activity of the encoded protein. (A) Gene expression changes between untreated and treated ES-2 parent. The fold change in expression of each gene between untreated and treated ES-2 parent (measured as average reads per kilobase per million total reads) is shown on a linear colour scale. Blue indicates reduced expression in untreated ES-2, yellow indicates increased expression. (B) Gene expression changes between two treated ES-2 clones resistant to 1000 nM AZD1775 and treated ES-2 parent. In each gene box, expression in ES-2 AZ_1000:2_250:3 is on the left and ES-2 AZ_1000:2_250:7 is to the right. Blue, reduced expression in ES-2 clone compared to parent; yellow, increased expression in ES-2 clone. (C) Gene expression changes between three treated OVCAR8 clones resistant to 300 nM AZD1775 compared to the treated OVCAR8 parent. For each gene box, expression in OVCAR8 AZ_300:2 is on the left, OVCAR8 AZ_300:3 in the centre and OVCAR8 AZ_300:4 to the right. Blue, reduced expression in the OVCAR8 clone compared to the parent; yellow, increased expression.

creased expression of *PKMYT1*, indicating their possible involvement in resistance. As with ES-2 clones, there was no change in expression of *CDC14A*, *TP53* and *GAD45A*. DDR genes showed increased expression in clones OVCAR8 AZ_300:2 and OVCAR8 AZ_300:3 (Supplementary Fig. 7). DNA replication (*ORC*) and chromosome segregation (*MCM*) genes showed increased expression in resistant OVCAR8 clones, whereas these genes were strongly downregulated in ES-2 clones compared to the treated parent. This, together with increased expression in OVCAR8 clones of genes involved in progression through G1 into S-phase, is hard to reconcile with the longer cell doubling times seen in both ES-2 and OVCAR8 clones (Supplementary Table 3).

3.7 Attempting to overcome resistance

On the basis of our RNA-Seq results, where $TGF\beta$ signalling was upregulated in resistant ES-2 and OVCAR8 clones, we postulated that a targeted inhibitor blockade of this pathway could overcome AZD1775 resistance. RepSox, a $TGF\beta$ receptor1 inhibitor with good specificity and potency [40], was chosen to test our hypothesis. Growth assays were performed on resistant ES-2 and OVCAR8 clones and on parental lines. Resistant OVCAR8 clones (OVCAR8 AZ_300:3 and OVCAR8 AZ_300:4) showed no increased sensitivity to the inhibitor (data not shown). Fig. 5 shows growth assays where ES-2 was compared with resistant clones ES-2 AZ_1000:2_250:3 and ES-2 AZ_1000:2_250:7 in the presence of the inhibitor. IC50 determinations for ES-2 AZ_1000:2_250:3 showed 20-fold increased sensitivity to RepSox compared to both ES-2 parent and ES-2 AZ_1000:2_250:7 and so identified a potential route to overcome AZD1775 resistance in this clone.

4. Discussion

PARP inhibitors have provided a major therapeutic advance for women with HRR-deficient HGSOc [5], although the appearance of resistance can continue to limit survival [6]. The widespread occurrence of *TP53* mutation, leading to reliance on the G2 checkpoint, makes AZD1775 inhibition of *WEE1* kinase together with DNA damaging agents a particularly attractive therapeutic option for all molecular HGSOc subtypes. While *WEE1* kinase inhibition in combination with PARP inhibitors has the additional advantage of inducing both DNA replication stress and nucleotide resource depletion [41]. Our aim was to identify resistance mechanisms to AZD1775 in both HRR-proficient (ES-2) and -deficient (OVCAR8) HGSOc cell lines and then find strategies to overcome resistance that could be deployed in the clinic. The maximum AZD1775 plasma concentration reported in patients with solid refractory tumours is 1650 nM [42]. Thus, the 5-fold increase in IC50 values for AZD1775 that we observed from ~200 nM in ES-2 up to >1000 nM in resistant clones is in the physiologically relevant range to make the difference between cancers respond-

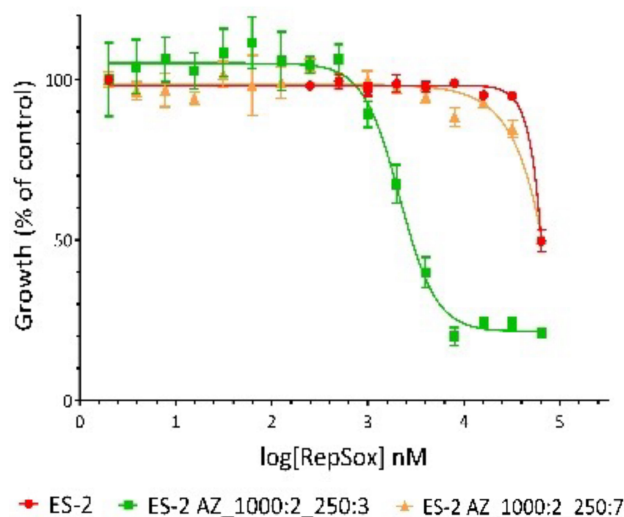


Fig. 5. AZD1775-resistant ES-2 clone with hypersensitivity to a $TGF\beta$ receptor1 inhibitor. Standard 5-day SRB growth assays with two independent resistant ES-2 clones (ES-2 AZ_1000:2_250:3 shown in green, ES-2 AZ_1000:2_250:7 shown in orange) compared to parental ES-2 (shown in red) for $TGF\beta$ receptor inhibitor RepSox. Each growth assay was repeated twice. RepSox IC50 values: ES-2 parent, 60 μ M; ES-2 AZ_1000:2_250:3, 3 ± 0.5 μ M; ES-2 AZ_1000:2_250:7, 60 μ M.

ing or being refractory to AZD1775 therapy. We postulated that resistance could arise through changes in the DDR and cell cycle control pathways (see Fig. 6). During our study a different resistance mechanism was described in SCLC, involving increased expression of AXL and MET receptors signalling down to increase *CHK1* activation and restore G2 arrest [21]. We showed clearly that this mechanism was not operating in HGSOc.

WEE1 gene mutation, preventing inhibitor binding, or increased *WEE1* gene expression could overcome AZD1775 inhibition. Sequencing the *WEE1* gene from resistant clones excluded involvement of coding region mutation in resistance. Western blotting and RNA-Seq for ES-2 clones showed no consistent increase in *WEE1* kinase expression, but an increase at both protein and RNA levels was seen in OVCAR8 clones. Although this could contribute to the development of resistance in OVCAR8, the demonstration of continuing target engagement of *WEE1* kinase by AZD1775 in ES-2 and OVCAR8 clones shows that other changes must also be involved. Continuing target engagement is also consistent with failure to observe increased *MDR1* expression in resistant clones. Resistance to some chemotherapeutics and PARP inhibitors commonly results from overexpression of the *MDR1* drug transporter [30], but we found no evidence that this was responsible for resistance to AZD1775 in our cells.

CDK1 can also be phosphorylated by *PKMYT1* on Y15 as well as T14 [20]. Thus, *PKMYT1* could potentially substitute for AZD1775-inhibited *WEE1* kinase to maintain

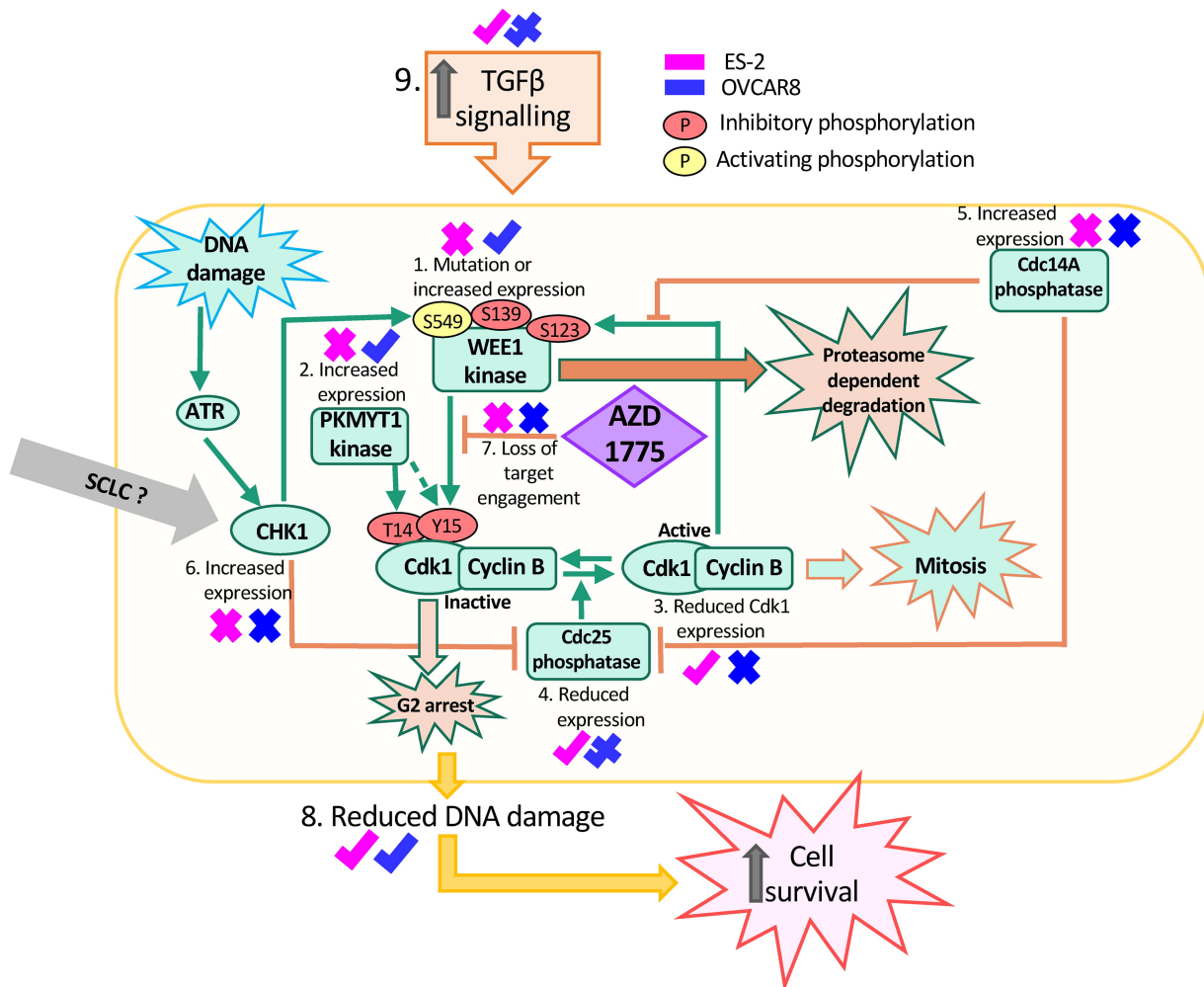


Fig. 6. Mechanisms of resistance to AZD1775 in ES-2 and OVCAR8-derived clones. Schematic showing the cell cycle interactions where we hypothesised that changes could lead to resistance to WEE1 kinase inhibition. Mechanisms 1 to 7 were postulated prior to commencement of the study, mechanisms 8 and 9 were revealed by the study. The figure summarises the results obtained for the different possible mechanisms. Ticks indicate a change that was observed. Crosses denote a possible change that was not seen. Pink ticks and crosses, resistant ES-2 clones; blue ticks and crosses, OVCAR8 clones. Superimposition of a tick and cross denotes a change only found in some resistant clones from that cell line. The crosses underneath postulated mechanism 6, increased expression (activation) of CHK1, indicate that no evidence was found for increased activation of CHK1 as a result of increased signalling from AXL and MET receptors as seen in SCLC.

the ability for G2 arrest. Expression of PKMYT1 was very low in both the ES-2 parent and resistant clones. Western blotting and RNA-Seq showed much higher expression in OVCAR8 with further elevation in most resistant clones, thus supporting the notion that elevated PKMYT1 levels in OVCAR8 clones are involved in resistance.

Major reductions in total CDK1 protein levels occurred in most ES-2 resistant clones. In the absence of WEE1 kinase inhibition of CDK1, following inhibition of WEE1 itself by AZD1775, reducing total CDK1 levels could result in lower levels of active CDK1 being available to drive cells with damaged DNA into premature mitosis. Our conclusion that reduced total CDK1 levels have an important role in AZD1775 resistance in ES-2 clones is sup-

ported by a report that inhibition of CDK1 increased viability of leukaemic cells treated with AZD1775 and cytarabine [43].

Cell cycle progression is also dependent on CDC25 phosphatase for removal of inhibitory phosphorylations on CDK1 and CDK2 [44]. The CDK1/cyclin B complex itself mediates activating phosphorylation of CDC25C in a positive feedback loop to reinforce the drive into mitosis [45]. Decreased expression of CDC25 phosphatase could keep the CDK1-cyclinB complex inactive and so preserve G2 arrest. CDC25B and C operate in G2/M, while CDC25A functions in S-phase. Our RNA-Seq data showed decreased expression of all three *CDC25* isoforms in ES-2 clones and reduced levels of *CDC25B/C* in OVCAR8 AZ_300:2

and OVCAR8 AZ_300:3. These decreases could also contribute to resistance by reducing the drive for cell cycle progression.

CDC14A removes the CDK1-mediated phosphorylation of WEE1 kinase that marks it for proteasomal degradation [46] and it also inhibits CDC25 [47,48]. Although CDC14A phosphatase expression could potentially help maintain G2 arrest in the presence of AZD1775, we found no evidence for its involvement in resistance.

While this manuscript was in preparation, increased PKMYT1 expression was shown to be responsible for AZD1775 (adavosertib) resistance in breast cancer cells [49]. Based on this result and a previous finding that PKMYT1 knockdown enhanced AZD1775 sensitivity [50], it was suggested that PKMYT1 levels could be a useful biomarker of response to AZD1775. We would caution that we have seen equivalent levels of AZD1775 resistance arising in two HGSOC cell lines with very different levels of PKMYT1 expression and that other changes, such as reducing the level of total CDK1, seem equally effective in generating AZD1775 resistance. Another recent study found that leukemia cell lines were dependent on increased HDAC and MYC expression for AZD1775-resistance [51]. While it would be valuable, given the molecular heterogeneity we observed between independent resistant ES-2 clones, to investigate the resistance mechanism in additional HGSOC cell lines, this was beyond the scope of the current study.

AZD1775 inhibition of WEE1 kinase also inactivates the S-phase checkpoint resulting in faster passage through S-phase and increased replication-associated DNA damage [32–34]. Highly dysregulated cell cycle profiles with abnormal S-phase and continued replication without cell division were observed for AZD1775-treated parental ES-2 cells. While, in marked contrast, resistant ES-2 clones showed normal profiles with reduced numbers of S-phase cells. Increased doubling times for resistant clones support the notion that slowing the cell cycle permits them to overcome the effects of WEE1 kinase inhibition. Further support comes from the result with the mitotic marker phosphorylated Histone H3. Highly elevated levels of this marker were present in both parental lines treated with AZD1775, reflecting its role in blocking G2 arrest leading to premature mitosis. Levels of phosphorylated Histone H3 were much reduced in treated ES-2 and OVCAR8 clones.

According to our model resistant clones should have lower levels of AZD1775-induced replication-associated DNA damage than parental cells. All resistant ES-2 clones had a much-reduced level of γ H2AX, a marker for double-strand DNA breaks, and of activated DNA-PK, needed for repair of ds DNA breaks. Levels of activated RPA, marker for ss DNA regions [36], were also much lower in all ES-2 treated clones and lower in most OVCAR8 treated clones than the treated parents. Further support comes from a report that p21, encoded by *CDKN2A*, limits AZD1775-induced S-phase DNA damage [52]. *CDKN2A* is one of

the genes induced by increased activity of the TGF β pathway in our resistant ES-2 clones. Additional support comes from the finding that DNA damage resulting from silencing of *WEE1* was reduced by treatment of cells with the CDK inhibitor roscovotine [33].

To attempt to overcome resistance we focussed on inhibiting a pathway found to be upregulated in resistant clones on the basis of our RNA-Seq data: TGF β signalling into the cell cycle control pathway to cause cell cycle arrest. A TGF β R1 inhibitor proved effective at controlling the growth of one of the resistant clones. Although components of the TGF β pathway were upregulated in three of the four clones tested, *TGF β R1* receptor expression itself was highest in the responsive clone. Additional studies would be needed to demonstrate the value of the TGF β R1 inhibitor in overcoming AZD1775 resistance. For instance, investigating whether *TGF β R1* overexpression confers AZD1775 resistance to parental ES-2 cells and whether *TGF β R1* knockdown resensitizes the resistant ES-2 clone with the highest level of *TGF β R1* overexpression to AZD1775.

5. Conclusions

We have shown that the mechanism of resistance to WEE1 inhibitor AZD1775 initially reported in small cell lung cancer cell lines [21] does not operate in either of the two main molecular subtypes of HGSOC cell lines. Although overexpression of the membrane glycoprotein MDR1 is a common mechanism of drug resistance in cancer cells, we found no evidence that it was responsible for AZD1775 resistance in our HGSOC cells. Instead AZD1775 resistance in HGSOC cells resulted from changes in the cell cycle control pathway that combine to slow AZD1775-induced cell cycle progression and so reduce accumulation of replication-associated DNA damage. In HRR-proficient ES-2 cells the major change was reduced levels of CDK1, the target for WEE1 inhibition. In HRR-deficient OVCAR8 cells increased levels of another kinase, PKMYT1, that can also inhibit CDK1 appear to have a more important role. It has previously been suggested that PKMYT1 levels could be a useful biomarker of response to AZD1775 [49]. However, we have seen equivalent levels of resistance arising in two HGSOC cell lines with very different levels of PKMYT1 expression and would caution that other changes, such as reducing the level of total CDK1, appear equally effective in generating resistance as high levels of PKMYT1. There was considerable clonal heterogeneity in the cell cycle control gene expression changes seen in resistant cells. We interpret this to reflect the complexity of cell cycle control and the different opportunities that this presents for resistance to arise (see Fig. 6). This clonal heterogeneity could also explain our limited ability to overcome resistance to AZD1775 with an inhibitor targeted against the upregulated TGF β signalling pathway in resistant clones.

Author contributions

DWM and CG designed the research study. MKG and ATW performed the research. MC provided help and advice on laboratory protocols. MJO'C provided help and advice on AZD1775. MKG, JPT and GRG analyzed the data. MKG and DWM wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

MJO'C has ownership interest (including stock and patents) in AstraZeneca. CG discloses research funding from AstraZeneca, Aprea, Nucana, Tesaro, Novartis and BerGenBio; honoraria/consultancy fees from Roche, AstraZeneca, MSD, GSK, Tesaro, Nucana, Clovis, Foundation One, Chugai, Cor2Ed, Takeda and Sierra Oncology; named on issued/pending patents related to predicting treatment response in ovarian cancer outside the scope of the work described here. ATW is a former employee and current shareholder of AstraZeneca. The other authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at <https://www.imrpress.com/journal/EJGO/43/2/10.31083/j.ejgo4302024>.

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