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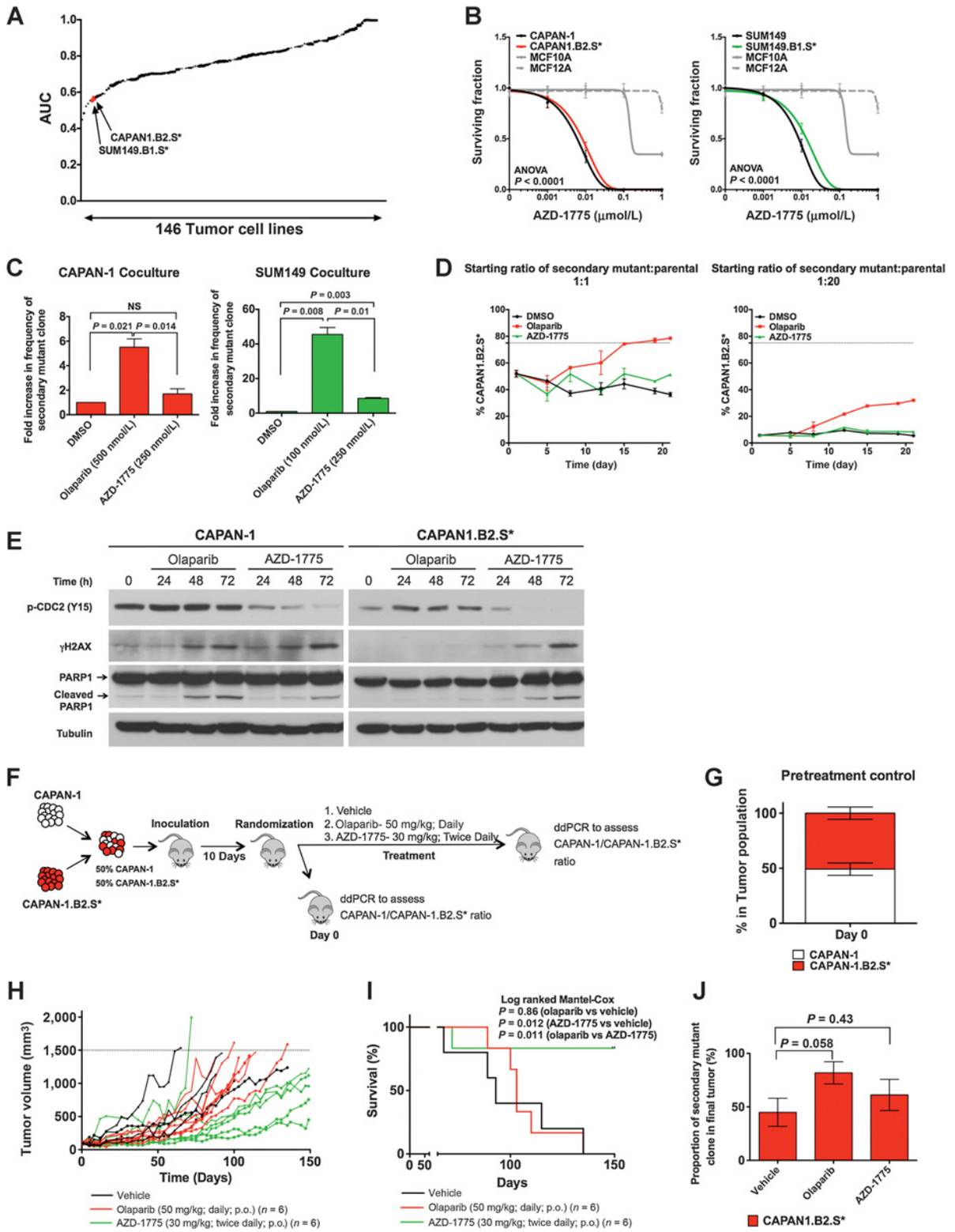

Figure 5.

PARPi-resistant, secondary mutant clones and parental tumor cells are sensitive to AZD-1775 *in vitro* and *in vivo*. **A,** Waterfall plot comparing AUC values collated from a 5-day exposure to AZD-1775 from 146 cancer cell lines. **B,** Dose–response survival curves illustrating 6-well clonogenic survival data in CAPAN1, CAPAN1.B2.S*, SUM149, SUM149.B1.S*, MCF-10A, and MCF-12A cells exposed to AZD-1775. **C,** Bar graph illustrating (*Continued on the following page.*)

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marker to enable detection and monitoring of coculture populations via FACS (Supplementary Fig. S5A). We found that in the absence of drug exposure, the DLD1.*BRCA2*$^{WT/WT}$ cells exhibited a selective advantage over DLD1.*BRCA2*$^{-/-}$cells, as previously shown (ref. 29; Supplementary Fig. S5B), and that these cells exhibit more than a 10-fold difference in olaparib sensitivity (Fig. 3A). We then mixed DLD1.*BRCA2*$^{WT/WT}$ cells into DLD1.*BRCA2*$^{-/-}$ cells *in vitro* at starting ratios of 1:1, 1:10, 1:100, and 1:1,000, exposed these cocultures to either olaparib or talazoparib, and monitored the temporal evolution of the population in response to PARPi (Supplementary Fig. S5A). Similar to the CAPAN1 and SUM149 isogenic models, we observed that olaparib and talazoparib both selected for DLD1.*BRCA2*$^{WT/WT}$ cells over DLD1.*BRCA2*$^{-/-}$ cells in a Darwinian fashion (Fig. 3B). For example, both olaparib and talazoparib exposure resulted in a 3-fold increase in DLD1.*BRCA2*$^{WT/WT}$ cells compared with the DMSO-exposed cell population after 13 days of drug exposure (Fig. 3B). In addition, we noticed that the time taken for the DLD1.*BRCA2*$^{WT/WT}$ clone to reach clonal dominance was less in cell populations that had higher starting proportion of DLD1.*BRCA2*$^{WT/WT}$ cells (Fig. 3C–E), as observed in the CAPAN1 coculture model.

Darwinian selection of secondary mutant tumor cells also operates *in vivo*

We also assessed whether a Darwinian process influenced the *in vivo* response to PARPi treatment. To do this, we generated cohorts of mice bearing subcutaneous xenografts consisting of a mixture of CAPAN1 parental and CAPAN1.B2.S* secondary mutant tumor cells (Fig. 4A). We found that inoculating 5×10^6 tumor cells at a 1:1 CAPAN1:CAPAN1.B2.S* ratio reproducibly generated 100 mm^3 xenografts 10 days after innoculation, where each clone was present in equal proportion (Fig. 4B). When tumors reached 100 mm^3, tumor-bearing mice were randomized into the following treatment cohorts to assess the selective pressure of PARPi treatment *in vivo*: (i) olaparib (50 mg/kg) administered once daily, (ii) olaparib (50 mg/kg) administered every other day, (iii) olaparib (50 mg/kg) administered twice a week on days 1 and 4, (iv) drug vehicle administered daily. In addition, sentinel mice were sacrificed prior to treatment so that the CAPAN1:CAPAN1.B2.S* ratio in tumors prior to therapy could be confirmed (Fig. 4A; Supplementary Fig. S6A). We found that 50 mg/kg olaparib treatment, administered daily, every other day, or twice weekly, though well-tolerated, did not decrease tumor growth compared with the vehicle ($P > 0.05$ ANOVA for tumor volume in each olaparib treatment cohort versus vehicle; Supplementary Fig. S6B and S6C). We hypothesized that the absence of overall antitumor efficacy in this particular case might be due to failure to inhibit the PARPi secondary mutant clone in xenografts. To test this, we isolated tumor DNA from olaparib-treated mice (after 28-day treatment) and assessed the relative ratio of parental versus secondary mutant clones by ddPCR. In mice that received drug vehicle alone, the ratio of parental versus secondary mutant clones remained approximately 50% (Supplementary Fig. S6A). However, in mice that received olaparib treatment, the relative frequency of CAPAN1.B2.S* cells increased in response to therapy (Fig. 4C). This increase in CAPAN1.B2.S* frequency, in preference to the parental clone, was dependent upon the periodicity of PARPi administration, for example, daily administration of olaparib caused the greatest increase in CAPAN1.B2.S* enrichment, followed by every other day treatment and then biweekly administration (Fig. 4C and D). This suggested that PARPi administration also selected for secondary mutant tumor cell clones *in vivo* and that the degree of secondary mutant clone selection was related to the extent of selective pressure applied.

AZD-1775, a WEE1 kinase inhibitor, targets both parental and secondary *BRCA*-mutant clones *in vitro* and *in vivo*

The coculture model systems described above allowed us to establish that PARPi resistance, when driven by secondary mutations in *BRCA1* or *BRCA2*, can operate along Darwinian principles. We also assessed whether we could identify therapeutic vulnerabilities that would allow targeting of both parental and secondary mutant tumor cell clones as a means to minimize the impact of secondary mutation. We assessed whether small-molecule WEE1 cell-cycle checkpoint kinase inhibitors (WEE1i; ref. 19) might have utility in this regard. We focused on WEE1 inhibitors for a number of reasons. WEE1 prevents premature mitotic entry by phosphorylating and inhibiting cyclin-dependent kinases such as cyclin dependent kinase 1 (CDK1; refs. 31, 32). This activity is particularly critical in tumor cells with p53 pathway defects; p53 defects often cooccur with *BRCA* mutations, and although secondary mutations in *BRCA1/2* drive PARPi resistance, resistant tumors and cell lines remain p53 mutant (13). CAPAN1.B2.S* and SUM149.B1.S* clones retained the p53 mutations present in CAPAN1 and SUM149 parental tumor cell clones (Supplementary Figs. S7 and S8). We also found that in an analysis of *in vitro* sensitivity to the clinical WEE1 kinase inhibitor, AZD-1775, in a panel of tumor cell lines, CAPAN1.B2.S* and SUM149.B1.S* were among the most sensitive of 146 lines profiled (Fig. 5A). We confirmed this AZD-1775 sensitivity in subsequent clonogenic survival experiments and found that, when compared with nontumor breast epithelial cell lines (MCF10A and MCF12A), both CAPAN1 and SUM149-derived secondary mutant tumor cell clones retained profound sensitivity to AZD-1775 seen in parental tumor cells (average 22-fold

(*Continued.*) the increase in secondary mutant clone frequency following 14 days of drug exposure. **D,** Graph showing the frequency of CAPAN1B2*S cells in CAPAN1/CAPAN1B2* cocultures exposed to AZD-1775. Clone frequency was estimated by ddPCR and the time points shown. Error bars represent SEM from three independent measurements. This experiment was conducted alongside the experiment described in Fig. 2D; to allow comparison, the response to olaparib, and DMSO exposure from Fig. 2D is replotted here. **E,** Western blot analysis for CAPAN1 and CAPAN1.B2.S* cells lysates probed for pCDC2(Y15), γ-H2AX (a DNA damage marker), and cleaved PARP1 (a marker of apoptosis). Tubulin was used as a loading control. **F,** Experimental schematic of mixed CAPAN1:CAPAN1.B2.S* xenografts treated with olaparib or AZD-1775. **G,** Bar chart illustrating CAPAN-1 (white) to CAPAN-1.B2.S* (red) clone ratio in in tumour xenografts prior to drug treatment. Values shown from six sentinel animals with mean ± SEM shown. **H,** Tumor volume plotted against length of treatment for individual xenografts comprised of CAPAN1:CAPAN1.B2.S* mixed tumor cells over 150 days ($n = 18$ total, $n = 6$ in each cohort). **I,** Survival curves using maximum tumor size (1,500 mm^3) as a surrogate for survival from the experiment shown in **E**. **J,** Bar chart showing proportion of CAPAN1.B2.S* tumor cells following treatment from the experiment shown in E ($n = 6$, mean ± SEM). *P* values were calculated by Student *t* test.

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difference in AUC, $P < 0.0001$ vs. MCF10A or MCF12A, ANOVA; Fig. 5B). We confirmed these observations using coculture systems and found that at SF_{50} concentrations (concentration required to inhibit 50% of cells) of either olaparib or AZD-1775, olaparib exposure increased the relative frequency of the secondary mutant clones, but AZD-1775 did not (Fig. 5C). This observation was confirmed when we used ddPCR to monitor the frequency of the secondary mutant clone over time in cocultures exposed to AZD-1775 (Fig. 5D). We also observed that parental and secondary mutant SUM149 and CAPAN1 clones were sensitive to additional small-molecule cell-cycle checkpoint inhibitors including PF-477736, a CHK1 inhibitor (33, 34), and VX-970, an ATR inhibitor (35) when compared with nontumor epithelial cells (Supplementary Fig. S9A and S9B). This suggested that even when partial BRCA1 or BRCA2 protein function was restored by secondary mutation, vulnerability to small-molecule inhibitors that target cell-cycle checkpoints still existed. These effects did not appear to represent a relatively nonspecific sensitivity to cytotoxic agents in the parental and secondary mutant tumor cells, as these did not display an overtly distinct level of sensitivity to paclitaxel, capecitabine, or gemcitabine when compared with MCF10 or MCF12A cells (Supplementary Fig. S9C–S9F).

Previous studies have shown that WEE1 inhibitors cause tumor cell cytotoxicity by reducing the extent of CDC2 phosphorylation at Y15 (36). We found that in both CAPAN1 and CAPAN1.B2.S* cells, AZD-1775 exposure caused a decrease in CDC2 Y15 phosphorylation, an effect that was enhanced with prolonged drug exposure (Fig. 5E). We noted that AZD-1775 exposure caused an increase in H2AX phosphorylation (γH2AX), a biomarker of DNA damage, in both CAPAN1 and secondary mutant CAPAN1.B2.S* cells (Fig. 5E). This increase in γH2AX was commensurate with an increase in PARP cleavage, a measure of apoptosis (Fig. 5E). Using FACS profiling, we found that AZD-1775 exposure had a very similar effect on cell-cycle fractions in both CAPAN1 and CAPAN1.B2.S* cells, both of which demonstrated a profound reduction in the fraction of cells in active S-phase, with a commensurate increase in the proportion of cells in nonreplicating S phase (Supplementary Fig. S10). In CAPAN1 cells, AZD-1775 exposure caused a reduction in the active S-phase fraction from 25.9% to 3.4% (with a 3.9%–52.1% increase in nonreplicating S-phase), while CAPAN1.B2.S* cells showed a reduction in active S-phase from 27.8% to 4.2% (with a 3.3%–51.4% increase in nonreplicating S-phase). These observations were reminiscent of those seen in H3K36me3-deficient cells, where WEE1 inhibition also caused a severe reduction in the active S-phase fraction (37). This suggested that WEE1 inhibition targeted CAPAN1 cells in S-phase, regardless of whether *BRCA2* was dysfunctional (as in CAPAN1) or somewhat reconstituted by the presence of a secondary *BRCA2* mutation (as in CAPAN1.B2.S*).

To investigate whether WEE1 inhibitor sensitivity in PARPi-sensitive and resistant clones also operated *in vivo*, we assessed the effect of AZD-1775 treatment on mice bearing mixed CAPAN1/CAPAN1.B2.S* xenografts (each clone present at a 1:1 ratio, Fig. 5F). Mice with established tumors were treated with either AZD-1775, olaparib, or drug vehicle. Sentinel mice sacrificed prior to treatment showed the CAPAN1:CAPAN1.B2.S* ratio in tumors prior to therapy was 1:1 (Fig. 5G). We used the time taken for tumors to reach 1,500 mm^3 as a surrogate measure of survival (Fig. 5H) and found that while olaparib treatment had minimal benefit ($P = 0.86$, log-rank Mantel–Cox test compared with vehicle), AZD-1775 treatment led to a significant survival benefit ($P = 0.011$, log-rank Mantel–Cox test compared with olaparib; Fig. 5I). Consistent with these observations, ddPCR analysis of tumors at the end of treatment showed that olaparib therapy caused a relative enrichment in the frequency of the secondary mutant clone ($P = 0.058$ compared with vehicle, Student *t* test) while AZD-1775 did not ($P = 0.43$, compared with vehicle, Student *t* test; Fig. 5J).

Discussion

In this study, we used CRISPR-generated *BRCA1* or *BRCA2* secondary mutant daughter clones alongside isogenic parental cell lines to demonstrate that PARPi exposure selects for secondary mutant clones in a Darwinian manner, both *in vitro* and *in vivo*. We found that the extent of selection for secondary mutant clones was influenced by the frequency of drug administration. In mice bearing tumors comprised of an equal proportion of *BRCA2*-mutant and *BRCA2* secondary mutant tumor cells, olaparib had minimal effects on tumor growth, but did preferentially select for the secondary mutant daughter clone over the parental tumor cell. It would be reasonable to infer that high frequencies of secondary mutant cells hinder the therapeutic effectiveness of PARP inhibitors. We also found that a WEE1 inhibitor, AZD-1775, had a greater therapeutic effect on mixed parental/secondary mutant tumors than olaparib. This example suggests that therapeutic vulnerabilities might still exist in tumors that have a high frequency of secondary mutant clones. Our data also suggest that secondary mutant and parental tumor cells also show sensitivity to other cell cycle/DNA damage repair inhibitors, including CHK1 and ATR inhibitors (Supplementary Fig. S9). It seems possible that while secondary *BRCA1* or *BRCA2* gene mutations restore some HR function, these are unlikely to reverse the complex set of genomic rearrangements, aneuploidy, and p53 mutations found in *BRCA1* or *BRCA2* mutant tumors prior to treatment (38). We hypothesize that these latter characteristics sensitize tumor cells to drugs such as WEE1 inhibitors, perhaps explaining why AZD-1775 targets both parental and secondary mutant clones. This hypothesis remains to be tested, but the observation that secondary mutant tumor cells are sensitive to AZD-1775 raises the possibility that therapeutic vulnerabilities still exist in PARPi-resistant tumors.

In clinical studies, the MTD for single-agent AZD-1775 was identified as 225 mg twice per day orally over 2.5 days per week for 2 weeks per 21-day cycle, a dosing regimen sufficient to elicit a number of antitumor responses (39). In our *in vivo* studies (Fig. 5) we used 30 mg/kg AZD-1775 twice-daily treatments for the entire duration of the study (150 days). This treatment approach was well tolerated in mice and based on prior mouse-based experiments using this WEE1 inhibitor (40). Nevertheless, it is possible that a similar constant dosing approach may not be well-tolerated in humans. Subsequent work might assess the potential of using intermittent WEE1 inhibitor dosing schedules to assess whether these also elicit a survival benefit in experiments similar to those shown in Fig. 5.

One implication of this work is that the detection of secondary *BRCA1* or *BRCA2* mutations in patients could be important in influencing the choice of therapy. At present, secondary mutations in *BRCA1* or *BRCA2* can be detected by Sanger DNA sequencing (14–16) or by targeted DNA capture and deep sequencing (13). Circulating tumor DNA and circulating tumor cells might also

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display some of the secondary *BRCA1* or *BRCA2* mutations found in solid tumors. Detecting secondary mutations in such liquid biopsies might allow the early emergence of secondary mutations to be identified as a biomarker predicting the eventual clinical manifestation of PARPi resistance. One avenue we will now explore is to utilize the *in vivo* system we have described here to assess this possibility. A key quality of the model systems described here is that they allow the construction of cocultures and xenografts where the frequency and identity of secondary mutants is known. This will hopefully facilitate experiments that aim to examine further principles that govern clonal evolution and influence drug resistance in *BRCA1*- or *BRCA2*-mutant cancers. Alongside these models, we also note that the first PDX with PARPi resistance-causing mutations have been recently described (41). These provide another system in which to assess how the clonal structure of tumors evolve in response to therapy. The combined use of engineered systems, such as that described here, alongside PDX systems will be critical in establishing what factors determine the response to treatment, and importantly, what therapeutic approaches could be taken to minimize the impact of secondary *BRCA1/2* gene mutations.

Disclosure of Potential Conflicts of Interest

N.C. Turner reports receiving commercial research support from AstraZeneca and is a consultant/advisory board member for AstraZeneca. A. Ashworth provided expert testimony for AstraZeneca. C.J. Lord has received speakers bureau honoraria from AstraZeneca and Vertex and is a consultant/advisory board member for Vertex and Sun Pharma. A. Ashworth and C.J. Lord are named inventors on patents describing the use of PARP inhibitors and stand to gain from their use as part of the ICR "Rewards to Inventors" scheme. No potential conflicts of interest were disclosed by the other authors.

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Molecular Cancer Therapeutics

Modeling Therapy Resistance in *BRCA1/2*-Mutant Cancers

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