Published OnlineFirst April 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2388

Research Article

Poly(ADP-Ribose) Polymerase-1 Inhibitor Treatment Regresses Autochthonous *Brca2/p53*-Mutant Mammary Tumors *In vivo* and Delays Tumor Relapse in Combination with Carboplatin

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Abstract

Germ-line heterozygosity of the *BRCA2* gene in women predisposes to breast and ovarian cancers. Successful therapies targeted specifically at these neoplasms have thus far remained elusive. Recent studies in mice have shown that inhibition of poly(ADP-ribose) polymerase-1 (PARP-1) targets cells lacking *Brca2* and xenografts derived from *BRCA2*-deficient ES cells or Chinese hamster ovary cells. We set out to develop a more relevant preclinical model that will inform and accelerate translation into the clinic. As such, we conditionally deleted *Brca2* and *p53* within murine mammary epithelium and treated the resulting tumors *in situ* with a highly potent PARP-1 inhibitor (AZD2281) alone or in combination with carboplatin. Daily exposure to AZD2281 for 28 days caused significant regression or growth inhibition in 46 of 52 tumors. This response was shown to be specific to tumors lacking both *Brca2* and *p53*. AZD2281/carboplatin combination therapy for 28 days showed no advantage over carboplatin monotherapy. However, if PARP inhibitor treatment was continued, this significantly increased the time to tumor relapse and death in these mice. This preclinical study is the first to show *in vivo* hypersensitivity of spontaneously arising *Brca2*-deficient mammary tumors to PARP-1 inhibition monotherapy or combination therapy. As such, our data add substantial weight to the argument for the use of PARP inhibitors as therapeutic agents against human breast cancers in which BRCA2 is mutated. Moreover, the specificity that we have shown further suggests that PARP inhibitors will be generally effective against tumors caused by dysregulation of components of the homologous recombination pathway. [Cancer Res 2009;69(9):3850–5]

Introduction

BRCA2 acts as a tumor suppressor by interacting with the RAD51 protein during homologous recombination (HR), a process by which DNA is repaired in an error-free manner (1). Deficiency in BRCA2 leads to the employment of error-prone systems of DNA repair, such as nonhomologous end joining, resulting in the types of complex genomic rearrangements that are often the hallmark of tumors caused by such mutations (1). Poly(ADP-ribose) polymerase-1 (PARP) inhibitors have been intensively preclinically tested as potentiators of chemotherapy or radiotherapy (2) and several have recently entered early clinical trials (3). One of the functions of PARP enzymes, particularly PARP-1 and PARP-2, is in the repair of single-stranded DNA breaks (4). Inhibition of this process has been shown to lead to the accumulation of double-stranded breaks, due to the apparent collapse of stalled replication forks, and this opens up a therapeutic window in *BRCA2*-deficient cells because of their inability to repair these lesions in an error-free manner (4). Several studies in cells and xenograft tumor models have shown that *BRCA2* deficiency does indeed sensitize to PARP inhibition (5–7) and also that PARP inhibition sensitizes cells characterized by dysregulation of several other components of the HR pathway (8). These studies have led to the proposal that PARP inhibitors will act as a successful targeted therapy against tumors defective in the HR pathway. However, the response of a relevant preclinical tumor model has yet to be shown.

The aim of this study was to determine the efficacy of a potent PARP-1 inhibitor against *Brca2*-deficient tumors arising naturally in a relevant preclinical mouse model both alone and in combination with an established chemotherapeutic agent. Critically, this model carries important advantages over the xenograft models previously used, in that the tumors arise spontaneously in the mammary gland and are therefore subject to the normal microenvironment and host cell response.

Materials and Methods

Genotyping of mice. PCR conditions for the *blg-cre* transgene and the *Brca2*^{fl}, *p53*^{fl}, and *pten*^{fl} alleles have all been described previously (9–12).

Formulation of AZD2281. AZD2281 (13) was dissolved at 45 mg/mL in DMSO and diluted to 4.5 mg/mL in PBS containing 10% 2-hydroxypropyl-β-cyclodextrin. 10% DMSO in PBS/10% 2-hydroxypropyl-β-cyclodextrin was used as vehicle.

Treatment of mice. AZD2281 and carboplatin were both injected at 50 mg/kg by single bolus intraperitoneal injection. Bromodeoxyuridine (BrdUrd) labeling reagent (GE Healthcare) was intraperitoneally injected at 30 mg/kg 2 h before animals were culled. Mice were fed standard diet and water *ad libitum* and all experimental procedures were carried out according to current UK Home Office regulations.

Pharmacokinetics/pharmacodynamics of AZD2281 in *Brca2/p53*-mutant mammary tumors. Tumors were removed from mice that were untreated or had received a single intraperitoneal dose of 50 mg/kg AZD2281 either 2 or 24 h previously. Tumor tissue was analyzed for the presence of AZD2281 by mass spectrometry as described previously (7). To determine levels of PARP activity, tumor whole-cell extracts were first

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-08-2388

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analyzed by Western blotting, using the anti-PARP-1 mouse monoclonal antibody 7D3-6 (BD Bioscience), followed by enhanced chemiluminescence detection and quantitative image capture analysis (LAS-3000; Fuji/Raytek). The PARP-1 protein concentration for each extract was determined by two-dimensional densitometry against PARP-1 standards using Advanced Image Data Analyzer imaging software. The equivalent of 20 pg PARP-1 of mouse tumor whole-cell extracts was then activated *ex vivo* by incubating with double-stranded DNA oligos and NAD^+ to stimulate poly(ADP-ribosylation) (PAR formation). PAR formation was then quantified by electrochemiluminescence with a Meso Scale assay, using the anti-PAR mouse monoclonal primary antibody 10H (Serotec), followed by the goat anti-mouse IgG secondary antibody SULFO-TAG (Meso Scale).

Analysis of tumor size. Treatment of palpable tumors was initiated when they reached a size that was measurable accurately by the use of manual spring-loaded calipers. As tumors were treated *in situ*, the starting volumes of tumors varied slightly depending on their position on the body and the ease with which it was possible to start accurate measurement. Tumor volumes were calculated as length × (square of width) / 2 and were taken on the day that treatment started and then usually twice per week henceforth.

Tumor tissue analysis. Tumors were removed from freshly culled mice and tissue was either fixed in formalin and subsequently embedded in paraffin wax for histologic and immunohistochemical analysis, snap-frozen in liquid nitrogen for subsequent protein purification, or stored in RNAlater (Sigma) for subsequent RNA purification using TRIzol reagent (Invitrogen).

Immunohistochemistry. Immunohistochemistry for BrdUrd was done as described previously (14). Cytokeratin 8 was detected using a chicken polyclonal antibody (Abcam; 14053) at 1:800 dilution, vimentin using a rabbit polyclonal antibody (Santa Cruz Biotechnology; C-20) at 1:50 dilution, and Ki-67 using a mouse monoclonal antibody (Vector Laboratories; VP-K452) at 1:20 dilution.

Western blotting. Proteins were extracted from snap-frozen tissue by homogenization in a modified RIPA buffer [50 mmol/L Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA (pH 7.4)] and de-glycosylated using the *N*-Glycosidase F Deglycosylation Kit (Roche). Bcrp-1 was detected using the rat monoclonal antibody bxp-53 (Abcam) at 1:2,000. Equal loading of proteins was assessed using a rat monoclonal antibody against α-tubulin (Abcam) at 1:2,000. Visualization was via enhanced chemiluminescence (GE Healthcare).

Real-time multiplex ligation-dependent probe amplification for multidrug-resistant proteins. Real-time multiplex ligation-dependent probe amplification reactions were done as described elsewhere (15).

Quantitative reverse transcription-PCR. Reactions were done as described previously (16) using established primers for *Brca2* (17) and *p53* (16) expression.

Data analysis. Statistical analyses were done using the nonparametric Mann-Whitney test, except for comparisons of tumor relapse and mouse survival (see Supplementary Tables S1 and S2), which were done using the nonparametric Wilcoxon test. $P < 0.05$ was considered to be statistically significant.

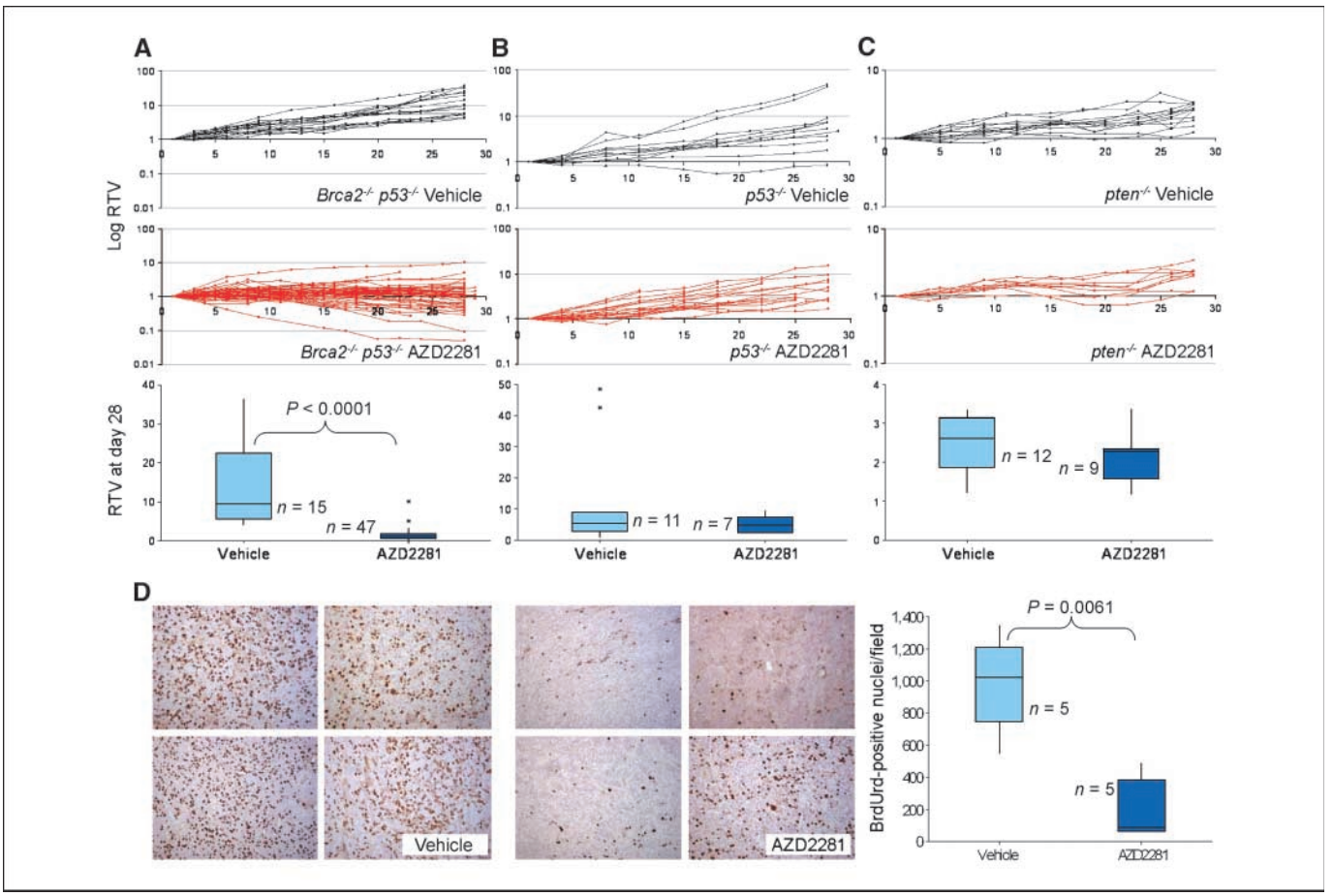

Figure 1. Responses of mammary tumors from mice treated daily with 50 mg/kg AZD2281 or vehicle for 28 d. *A* to *C,* changes in RTV in *Brca2/p53*-deficient (*A*), *p53*-deficient (*B*), or *pten*-deficient (*C*) mammary tumors. *Top* and *middle,* responses to daily vehicle treatment or daily AZD2281 treatment respectively. *X axis,* day of treatment; *Y axis,* log RTV; each line represents the response of an individual tumor. *Bottom,* box plots of RTV at day 28. *D,* representative sections of BrdUrd-stained *Brca2/p53*-deficient tumors treated with either vehicle (*left*) or AZD2281 (*middle*) for 28 d. *Right,* box plot comparing BrdUrd positivity in these sections. *Y axis,* number of positively stained cells per field of view.

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Results and Discussion

Generation and characterization of *Brca2/p53*-mutant mammary tumors. To generate mammary tumor-bearing mice, we used *cre-lox* technology to conditionally delete genes specifically within mammary epithelium. For this, we used the *blg-cre* transgene, which is known to be specific to mammary epithelial cells (9). Mice carrying the *blg-cre* transgene were crossed with mice carrying the floxed *Brca2* (10) and *p53* (11) alleles to generate *blg-cre*$^{+}$ *Brca2*$^{fl/fl}$ *p53*$^{fl/fl}$ and *blg-cre*$^{+}$ *Brca2*$^{fl/fl}$ *p53*$^{fl/+}$ animals. Animals of these genotypes developed mammary tumors from as early as age 6 months. Quantitative reverse transcription-PCR showed that levels of *Brca2* and *p53* were very low (≤20%) in a panel of 9 tumors (data not shown), indicating that deletion of these two genes was responsible for tumor development. Furthermore, histologic analysis of 34 tumors (Supplementary Fig. S1*A* and *B*) revealed morphologic changes consistent with previous studies of murine tumors characterized by mutation of both *Brca2* and *p53* (11, 18).

To determine if we could deliver inhibitory concentrations of the highly potent PARP-1 inhibitor AZD2281 to *Brca2/p53*-deficient mammary tumors, we simultaneously measured levels of AZD2281 and assayed for PARP-1 activity following treatment. Two hours after a single injection of 50 mg/kg AZD2281, the inhibitor was present in the tumors at high levels and PARP activity was reduced significantly (Supplementary Fig. S1*C*; Mann-Whitney, $P = 0.013$; $n = 6$). By 24 h after dosing, the inhibitor had been cleared from the tumors and PARP activity had returned to pretreatment levels (Supplementary Fig. S1*C*).

AZD2281 regresses spontaneously arising *Brca2/p53*-mutant mammary tumors. To analyze the effect of AZD2281 as a monotherapy, *Brca2/p53*-deficient tumor-bearing mice were treated daily with PARP inhibitor or vehicle. Changes in tumor size were measured biweekly and tumor volumes were calculated and compared with the measured volume on the first day of treatment to give the relative tumor volume (RTV). Eighteen vehicle-treated mice all showed rapid tumor growth throughout the 28-day period of treatment, with 3 mice being sacrificed before day 28 due to deteriorating health associated with growth of the tumor (Fig. 1*A*). In contrast, of 52 tumors arising in mice that were subsequently treated with AZD2281, 46 showed significant responses ranging from cessation of tumor growth to substantial regression (Fig. 1*A*). Of these, 3 were in mice that were culled before the end of the 28-day period of treatment due to deteriorating health. Comparison of RTV at day 28 (Fig. 1*A*) shows a significant response to AZD2281 therapy in comparison with vehicle (Mann-Whitney, $P < 0.0001$; $n = 15$ for vehicle and $n = 47$ for AZD2281). To determine whether AZD2281 showed specific activity against mammary tumors lacking *Brca2*, we also tested the response in tumors caused by deletion of either *p53* or *pten* in mammary epithelium (Fig. 1*B* and *C*). There was no significant difference following vehicle or AZD2281 treatment in either cohort of mice. To assess tumor proliferation following 28 days of treatment, mice from all treatment cohorts were injected with the S-phase labeling reagent BrdUrd 2 h before sacrifice, and tissue sections from these tumors were stained with an antibody against BrdUrd. The results indicate that proliferation was significantly reduced in *Brca2/p53*-deficient tumors when mice had been treated with AZD2281 (Fig. 1*D*; Mann-Whitney, $P = 0.0061$; $n = 5$). However, there was no reduction in tumor proliferation following AZD2281 treatment in either *p53*- or *pten*-deficient tumor-bearing animals (data not shown). These data show conclusively that AZD2281 is highly effective against tumors defective in *Brca2*. Moreover, this specificity strongly argues that PARP inhibition will be effective against other tumors characterized by dysregulation of the HR pathway as suggested previously (8).

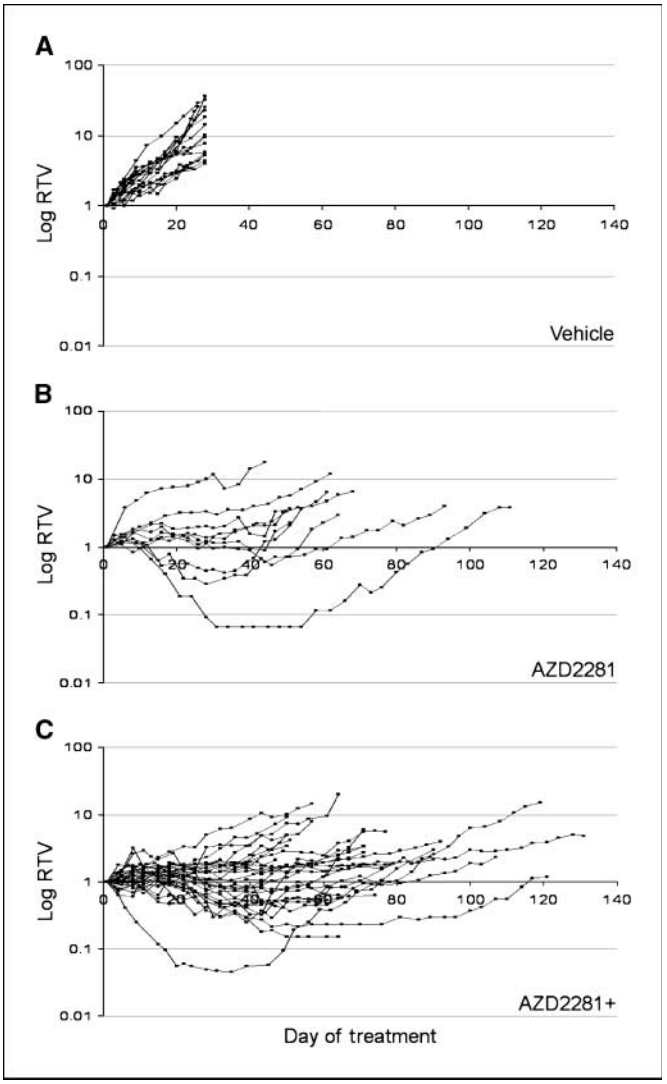

Figure 2. Long-term responses of *Brca2/p53*-deficient tumors from mice treated daily with 50 mg/kg AZD2281. *A,* 28 d of daily vehicle treatment. *B,* 28 d of daily 50 mg/kg AZD2281 treatment. *C,* continuous daily 50 mg/kg AZD2281 treatment. *X axis,* day of treatment; *Y axis,* log RTV; each line represents the response of an individual tumor.

Analysis of the long-term response to AZD2281 treatment. We next wanted to understand the long-term consequences of treatment with AZD2281. Figure 2 shows the responses of tumors from mice that had been treated for 28 days with vehicle (Fig. 2*A*), 28 days with AZD2281 (Fig. 2*B*), or continuously with AZD2281 (Fig. 2*C*). Interestingly, the tumors from either cohort of AZD2281 treatment always relapsed, although this response was heterogeneous in nature, as several tumors showed longer-term responses before relapsing.

Resistance in the clinic is detrimental to a range of therapies, and as such, it is important that we understand the underlying mechanisms. Using a panel of 16 AZD2281-resistant tumors, we firstly performed quantitative reverse transcription-PCR and found

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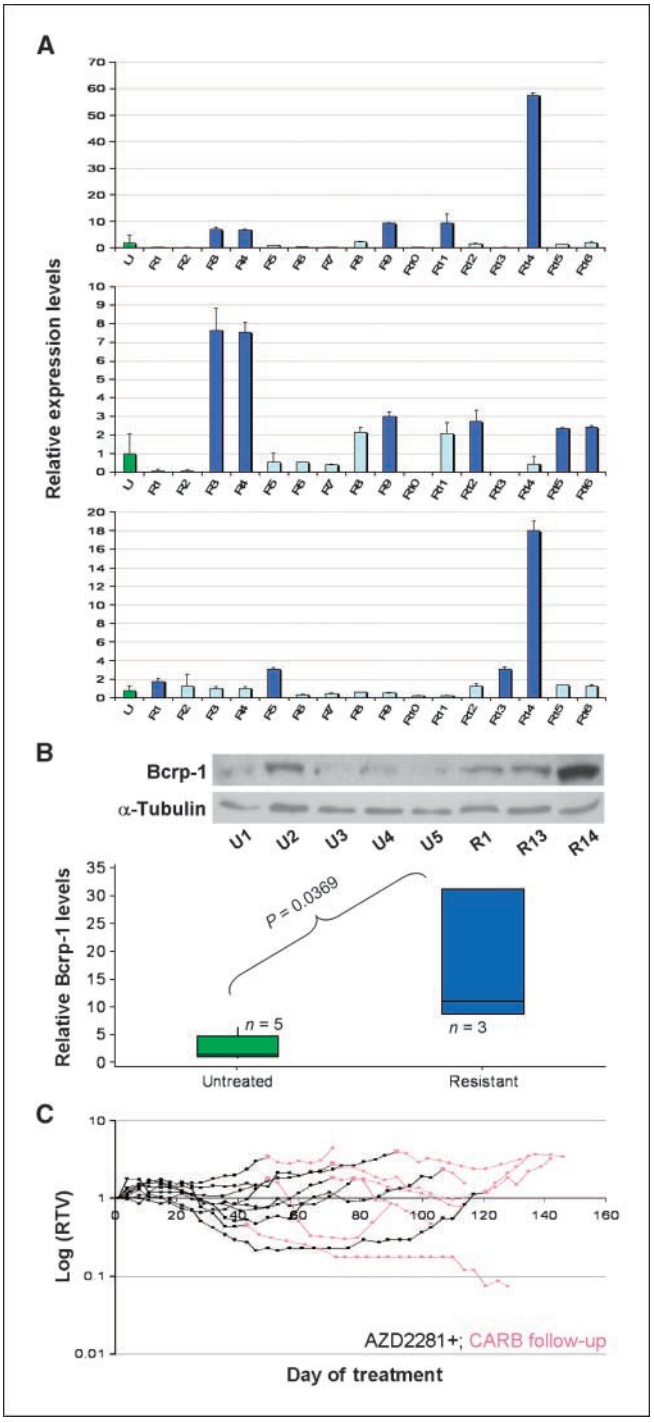

Figure 3. Analysis of AZD2281-resistant *Brca2/p53*-mutant mammary tumors. *A,* real-time multiplex ligation-dependent probe amplification of MDR proteins Mdr1a (*top*), Mdr1b (*middle*), and Bcrp-1 (*bottom*). *Green columns, U,* mean of 16 untreated control tumors; *blue columns, R1* to *R16,* individual resistant tumors. Dark blue shading shows that the level of expression in the resistant tumor is at least 2-fold greater than in the untreated tumors. *Y axis,* average expression levels compared with the combined expression of the three control markers ActB, Hprt1, and Hsp90. *B,* Western blots of Bcrp-1 and α-tubulin on untreated tumors (*U1-U5*) and AZD2281-resistant tumors (*R1, R13*, and *R14*; *top*). *Bottom,* box plot of bcrp-1 levels in the five untreated and three resistant tumors. Values on Y axis are relative and were calculated by measuring band intensities of the Western blots for bcrp-1 and α-tubulin. *C,* treatment of AZD2281-resistant tumors with carboplatin. *Black lines,* a period of daily 50 mg/kg AZD2281 treatment; *pink lines,* a switch to a period of weekly 50 mg/kg carboplatin treatment. *X axis,* day of treatment; *Y axis,* log RTV; each line represents the response of an individual tumor.

that levels of *Brca2* and *p53* were as low as those seen in untreated tumors (data not shown). This allowed us to rule out the possibility that resistance to AZD2281 was caused by a secondary mutation within the *Brca2* gene in these tumors as recently reported in two independent studies (19, 20). Such a genetic event would be very unlikely in our model system anyway, due to the nature of the large deletion, which conditionally inactivates the *Brca2* gene. To test another potential mechanism of resistance to AZD2281, we analyzed the same tumors in terms of their multidrug-resistant (MDR) expression profiles using real-time multiplex ligation-dependent probe amplification (15, 16). In 11 of 16 tumors analyzed, it was noted that at least a 2-fold up-regulation of one or more MDR transporters had occurred (Fig. 3*A*). Furthermore, Western blotting of proteins from these tumors with an antibody against Bcrp-1 showed up-regulation of the protein in three of the tumors suggested by real-time multiplex ligation-dependent probe amplification, most notably in tumor R14, which also showed the highest increase in expression at the RNA level (Fig. 3*B*). Although these results suggest that we are seeing up-regulation of MDR in a subset of AZD2281-resistant tumors, our model system does not allow us to directly compare levels between these tumors and the untreated tumors from which they originated. However, our data do suggest that altered MDR levels may be a significant mechanism of resistance in these tumors and that additional treatment with MDR inhibitors may further improve the effectiveness of PARP inhibitor therapy in such resistant tumors in the clinic.

If elevated MDR activity does mediate resistance within this model, one might hypothesize cross-resistance to other therapeutics. To address this, we assessed platinum sensitivity in 9 tumors, which developed resistance during continuous AZD2281 treatment. Eight of 9 responded to subsequent carboplatin treatment (Fig. 3*C*), suggesting that resistance to AZD2281 does not usually confer resistance to carboplatin. The MDR proteins that we have shown to be up-regulated are rarely implicated in platinum resistance (21); therefore, the fact that the majority of tumors retained carboplatin sensitivity can probably be explained by drug-specific MDR activity.

Our data therefore implicate MDR up-regulation in resistance to AZD2281. However, this does not rule out other potential mechanisms, such as altered DNA repair activity, which has been shown previously to mediate resistance to platinum (22).

Analysis of AZD2281/carboplatin combinatorial therapy. We next wanted to analyze the effect of a combination treatment of PARP-1 inhibition with a DNA-damaging chemotherapeutic agent. Recently, AZD2281 has been shown to synergize with platinum compounds against cells derived from tumors deficient in both *Brca2* and *p53* (17). To test this in a true *in vivo* setting, we treated *Brca2/p53*-deficient tumor-bearing mice with daily injections of AZD2281 or vehicle combined with weekly injections of carboplatin on days 1, 8, 15, and 22 (1 h following AZD2281/vehicle treatment) and measured tumor size over a 28-day period. Tumors in both cohorts (CARB and COMBO) responded to treatment in a similar way (Fig. 4*A* and *B*) and we found that there was very little BrdUrd staining in any of these tumors at day 28, although occasional foci of BrdUrd positivity were observed in tumors from both treatment cohorts (data not shown). In addition, we stained day 28 tumor sections for the proliferation marker Ki-67 and scored for positivity and also scored H&E-stained sections of the same tumors for mitosis. Although both therapy regimens showed a reduction in both Ki-67 staining and mitosis compared with AZD2281 alone (Supplementary Fig. S2), there was no significant difference between the two therapeutic regimens at this time point. In terms

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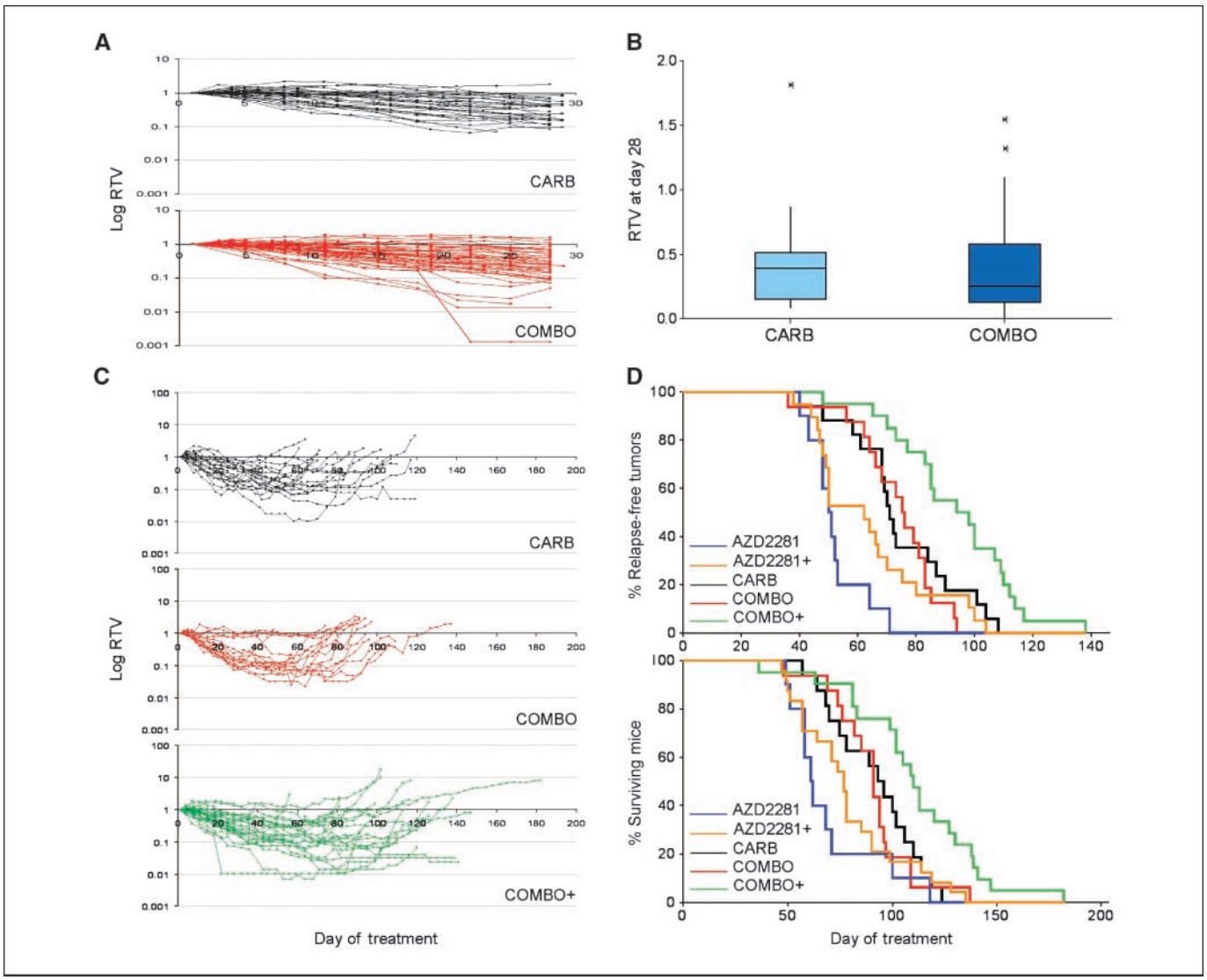

Figure 4. Responses of *Brca2/p53*-deficient tumors in mice treated with a combination of AZD2281 and carboplatin. *A,* treatment of tumors for 28 d with weekly 50 mg/kg carboplatin (*CARB*) or a combination of daily 50 mg/kg AZD2281 and weekly 50 mg/kg carboplatin (*COMBO*). *X axis,* day of treatment; *Y axis,* log RTV; each line represents the response of an individual tumor. *B,* box plot comparing RTV (*Y axis*) at day 28 of the treatments shown in *A*. *C,* long-term responses of tumors treated with weekly 50 mg/kg carboplatin for 28 d or a combination of daily 50 mg/kg AZD2281 and weekly 50 mg/kg carboplatin for 28 d or a combination of weekly 50 mg/kg carboplatin for 28 d and continuous daily 50 mg/kg AZD2281 (*COMBO+*). *X axis,* day of treatment; *Y axis,* log RTV; each line represents the response of an individual tumor. *D,* Kaplan-Meier statistical analysis of tumor relapse (*top*) and animal survival (*bottom*) in response to treatments represented graphically in *C* and in Fig. 2*B* and *C*. In both graphs, *X axis,* day of treatment. *Top, Y axis,* percentage of tumors that have not relapsed, where relapse is defined as the point at which the tumor has doubled in size from its lowest volume. *Bottom, Y axis,* percent surviving mice.

of mechanism of interaction between the two therapeutics, it is possible that PARP inhibitors and platinum are both dependent on functional HR, although this is far less clear for platinum than it is for PARP inhibitors (23).

These experiments show that, for the regression of tumors, additional AZD2281 treatment did not enhance carboplatin monotherapy during the initial treatment period of 28 days. This is most likely due to the high dose of carboplatin that we used, such that we were unable to see any additional effect of AZD2281. We therefore extended our observations by analyzing the length of time taken for tumors to relapse following five different treatment regimens: 28 days of AZD2281 treatment (AZD2281; Fig. 2*B*), continuous AZD2281 treatment (AZD2281+; Fig. 2*C*), 28 days of carboplatin treatment (CARB; Fig. 4*C*), 28 days of combination AZD2281/carboplatin (COMBO; Fig. 4*C*), or 28 days of carboplatin with continuous daily treatment with AZD2281 (COMBO+; Fig. 4*C*). Time to relapse was defined as the day at which the tumor had doubled in size from its lowest volume. Extended treatment with AZD2281 after the initial 28-day combination treatment (COMBO+) resulted in a significant delay to tumor relapse compared with all other treatments (Fig. 4*D*; Supplementary Table S1). At day 100 after treatment started, only 6 of 20 (30%) of CARB-treated tumors and 3 of 16 (19%) COMBO-treated tumors were still below their initial volume compared with 14 of 25 (56%) tumors treated with COMBO+ therapy. By day 120, only 1 of 20 (5%) CARB-treated and 1 of 16 (6%) COMBO-treated tumors were still below their initial volume compared with 8 of 25 (32%) tumors treated with COMBO+ therapy. Additionally, tumor-bearing mice that received the COMBO+ treatment survived significantly longer than those that received any of the other treatments (Fig. 4*D*; Supplementary Table S2). Taken

together, these results clearly show that continued PARP inhibition enhanced carboplatin monotherapy or PARP inhibitor/carboplatin combinatorial therapy in terms of tumor relapse and mouse survival.

Critically, this enhancement occurs without any increase in toxicity. Attempts to treat beyond the initial 28-day period with carboplatin resulted in significant mortality (data not shown). By contrast, continuous treatment with AZD2281 did not lead to any observed toxicity. Furthermore, although toxicity was observed in the combined therapy regimen, this was no higher than following carboplatin monotherapy (data not shown).

There is currently considerable excitement about the potential of novel anticancer strategies based on the notion of synthetic lethality. Principal among these is the use of PARP inhibition to sensitize HR-deficient tumor cells. To date, efficacy of these agents has only been established within defined experimental settings such as clinically irrelevant xenografts derived from ES cells or Chinese hamster cells (5–8) but not within spontaneously developing tumors arising in directly relevant preclinical models. Here, we show for the first time that mammary tumors arising in a genetically appropriate *Brca2*$^{-/-}$ setting show a dramatic response to treatment with a potent PARP-1 inhibitor.

Critically, this model has allowed us to go beyond simple assessment of monotherapy, as we have shown combined exposure to AZD2281 and carboplatin to significantly increase the length of time taken for tumors to relapse. This ability to rapidly assess combinatorial drug interactions in a true tumor setting should accelerate both the development of the optimal clinical regimens and our understanding of the basis of resistance. As such, by altering the doses and scheduling of the two compounds, we are now attempting to develop a combinatorial treatment regimen, which will maintain the efficacy that we have thus far shown but which may also reduce the toxicity of the platinum compound and further delay tumor relapse.

In conclusion, our data establish a paradigm for the successful tailoring of treatment to genetic status and provide further evidence that treatment of HR-defective tumors with a combination of PARP inhibition and standard chemotherapy will be clinically effective.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 6/23/08; revised 1/26/09; accepted 3/9/09; published OnlineFirst 4/21/09.

Grant support: Association for International Cancer Research grant 03-336 (T. Hay) and AstraZeneca (L. Pietzka).

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We thank Tak Mak and Anton Berns for generously donating mouse strains, Richard Clarkson for the donation of reagents, Robert Boulter, Mark Bishop, and Derek Scarborough for excellent technical assistance, and Elizabeth Mills and Lynsey Womersley at AstraZeneca for help with statistical analyses.

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Poly(ADP-Ribose) Polymerase-1 Inhibitor Treatment Regresses Autochthonous *Brca2/p53*-Mutant Mammary Tumors *In vivo* and Delays Tumor Relapse in Combination with Carboplatin

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Cancer Res 2009;69:3850-3855. Published OnlineFirst April 21, 2009.