Novel treatment strategies for hereditary breast cancer

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Chapter 2

Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin

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Selective inhibition of BRCA2-deficient mammary tumor cell growth by the novel PARP inhibitor AZD2281 in synergy with cisplatin

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Purpose
To assess efficacy of the novel, selective poly-(ADP-ribose)-polymerase-1 (PARP) inhibitor AZD2281 against newly established BRCA2-deficient mouse mammary tumor cell lines and to determine potential synergy between AZD2281 and cisplatin.

Experimental Design
We established and thoroughly characterized a panel of clonal cell lines from independent BRCA2-deficient mouse mammary tumors and BRCA2-proficient control tumors. Subsequently, we assessed sensitivity of these lines to conventional cytotoxic drugs and the novel PARP-inhibitor AZD2281. Finally, in vitro combination studies were performed to investigate interaction between AZD2281 and cisplatin.

Results
Genetic, transcriptional and functional analyses confirmed the successful isolation of BRCA2-deficient and BRCA2-proficient mouse mammary tumor cell lines. Treatment of these cell lines with 11 different anti-cancer drugs or with γ-irradiation showed that AZD2281, a novel and specific PARP inhibitor, caused the strongest differential growth inhibition of BRCA2-deficient vs. BRCA2-proficient mammary tumor cells. Finally, drug combination studies showed synergistic cytotoxicity of AZD2281 and cisplatin against BRCA2-deficient cells but not against BRCA2-proficient control cells.

Conclusion
We have successfully established the first set of BRCA2-deficient mammary tumor cell lines, which form an important addition to the existing preclinical models for BRCA-mutated breast cancer. The exquisite sensitivity of these cells to the PARP inhibitor AZD2281, alone or in combination with cisplatin, provides strong support for AZD2281 as a novel targeted therapeutic against BRCA-deficient cancers.
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Introduction

Mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 are responsible for the majority of hereditary breast cancers. Tumors in patients with heterozygous BRCA1 or BRCA2 germ-line mutations typically show somatic loss of heterozygosity (LOH) at the BRCA1 or BRCA2 locus, respectively, resulting in loss of the wild-type allele. Functionally, involvement of BRCA2 in the repair of DNA damage, especially DSBs has been firmly established by several groups. The primary role of BRCA2 in this process appears to be the regulation of damage-induced RAD51 protein filaments that are required for DSB repair by homologous recombination (HR). In the absence of BRCA2, repair of DSBs by error-prone mechanisms and chromosomal instability due to improper centrosome maintenance result in genomic instability, which renders cells susceptible to acquiring additional cancer initiating genetic lesions. The absence of error-free DSB repair mechanisms may prove to be the Achilles heel of BRCA2-deficient tumors, as increased sensitivity to γ-irradiation or DNA damaging agents is observed in cells with dysfunctional BRCA2. Clinical trials exploiting the sensitivity of BRCA-mutated breast cancers to platinum-based DNA damaging drugs have been started recently.

In vitro analysis of PARP inhibitors in CAPAN-1 tumor cells, derived from a liver metastasis of a human BRCA2 defective pancreas carcinoma and thus far the only tumor cell line available with abrogated BRCA2 function, yielded conflicting results. These experiments in pancreatic tumor cells call for evaluation of PARP inhibitor efficacy in additional BRCA2-deficient tumor cells, specifically of mammary epithelial origin. The latter is important considering the cell type-intrinsic differences between mammary epithelial cells and other cells, which have been postulated to - at least partially - explain the tumor spectrum in BRCA-carriers. Unfortunately, no BRCA2-deficient mammary tumor cell lines have been published until now.

Here, we report the successful establishment of clonal cell lines from two independent BRCA2-deficient mouse mammary tumors. Thorough characterization of these cell lines confirmed complete loss of BRCA2 function and increased sensitivity towards DNA damaging agents was shown for BRCA2-deficient cells as compared to...
BRCA2-proficient control cells. Using a novel, specific and potent inhibitor of PARP enzymatic activity, AZD2281 (Menear KA et al., manuscript submitted), we show growth inhibition of BRCA2-deficient mammary tumor cells. Since both PARP inhibition and cisplatin confer selective toxicity to BRCA2-deficient mammary tumor cells, and since PARP inhibition was recently shown to potentiate cisplatin-mediated cytotoxicity30, we also performed drug combination studies. AZD2281 synergized with cisplatin in inhibiting the growth of BRCA2-deficient mammary tumor cells, while this combination was additive in the BRCA2-proficient tumor cells. These data warrant further preclinical evaluation of AZD2281 as monotherapy or in combination with cisplatin in animal models for BRCA-deficient breast cancer.

Materials and methods

Establishment and maintenance of tumor cell lines

Tumor bearing female mice of the K14-Cre;Brca2F11/F11; p53F2-10/F2-10 (KB2P) or K14-Cre;Brca2w.t/w.t.; p53F2-10/F2-10 (KP) genotype31 were sacrificed and the tumors were isolated. Small (3x3 mm) pieces were subsequently minced and digested for 1 hour in Leibovitz L15 medium with 3g/l Collagenase A and 1.5g/l porcine pancreatic trypsin with rigorous shaking at 37°C. Aggregates were plated out and cultured under low oxygen conditions (3% O₂, 5% CO₂, 37°C) using DMEM-F12 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum, 50U/ml penicillin, 50µg/ml streptomycin (Gibco, Carlsbad, CA), 5µg/ml insulin (Sigma, St. Louis, MO), 5ng/ml EGF (Gibco, Carlsbad, CA) and 5ng/ml cholera toxin (Gentaur, Brussels, Belgium). To remove contaminating fibroblasts, cultures were differentially trypsinized until homogeneous cell morphology indicated pure epithelial cultures.

Detection of Brca2 expression by qPCR

Total RNA (1.25µg) isolated from cell cultures using a Qiagen RNeasy kit, was used as input for a first strand reaction (Invitrogen, Carlsbad, CA, according to manufacturer’s protocol). Subsequently, 12ng cDNA was used for a qPCR reaction using the SYBR-Green PCR Mastermix (Applied biosystems, Foster City, CA), performed on an ABI Prism 7000. HPRT Levels were used as internal control. Brca2 primer sequences were as follows: Exon2-3 FW: gcattcttgaagagtgcttg; Exon2-3 RV: ccatttgctttatcgg; Exon10-11 FW: gaagcagtttttaag; Exon10-11 RV: caggaagaatctggtatacctg; Exon18-19 FW: ctctgattcgtgctg; Exon18-19 RV: cacggagagcccagccct.

Detection of p53 protein

Protein extraction of cultured cells was performed using ELB buffer (150mM NaCl, 50mM Hepes pH 7.5, 5mM EDTA, 0.1% NP-40) complemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). Primary antibodies used in subsequent Western blot assays: polyclonal sheep anti p53 (1:5000, Calbiochem, San Diego, CA); polyclonal goat anti β-Actin (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody: Rabbit-anti-Goat HRP (1:2000, Dakocytomation, Glostrup, Denmark).

γH2A.X/RAD51 Colocalization

Cells grown on cover slips were exposed to 20Gy γ-irradiation and fixed eight hours later using 1% paraformaldehyde in PBS. Cells were permeabilized 5’ in 0.1% Triton and preincubated for 1 hour at RT in staining buffer (PBS/0.5% BSA/0.15% Glycine), which was used as solvent in all subsequent steps. Incubation with polyclonal rabbit-anti-RAD51 antibody (a generous gift by Roland Kanaar, Erasmus medical center, Rotterdam, the
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Netherlands) followed for 2 hours. Secondary goat-anti-rabbit Alexa 568 (Molecular Probes, Carlsbad, CA, 1:400 dilution) was then co-incubated with FITC-conjugated monoclonal mouse-anti-γH2A.X antibody (Upstate, Billerica, MA, clone JBW301, 1:50 dilution) for 1 hour at RT. Finally, DNA was stained using 1:5000 To-Pro-3 (Molecular Probes, Carlsbad, CA). All incubations were followed by at least three wash steps using staining buffer. Slides were mounted using Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired on a Leica TCS TNT system (Leica Microsystems, Wetzlar, Germany).

Array CGH
Genomic DNA isolated from primary tumors or cell lines was labeled and hybridized to 3K mouse BAC microarrays using spleen DNA originating from the same animal as a reference, as described32.

Karyotyping
Metaphases were prepared by culturing sub-confluent cells for 30 min. in the presence of 100ng/ml colcemid (Gibco, Carlsbad, CA). Cells were harvested and hypotonic swelling was induced for 10 min. in 0.075M KCl. Subsequently, cells were fixed using three short incubations in 3:1 dry methanol:glacial acetic acid solution and dropped on a microscope slide. Ploidy analysis was performed by mounting metaphase slides in DAPI containing Vectashield (Vector laboratories, Burlingame, CA) and images were acquired using a Zeiss Axiosvert 200M fluorescence microscope, mounted with a Zeiss Axiocam MRm Rev. 2 camera. Spectral karyotyping (SKY) analysis was performed as described33, using the spectracube 300. Between 6 and 9 metaphases were analyzed for each cell line using Skyview software version 2.1.1.

Drugs
AZD2281 was synthesized by KuDos Pharmaceuticals (Cambridge, UK) (Menear KA et al., manuscript submitted). Other compounds were obtained from Mayne Pharma, Brussels, Belgium (cisplatin); Sigma-Aldrich, St. Louis, MO (mitomycin-C [MMC], methylmethane sulphonate [MMS], 5-fluorouracil [5-FU], hydroxyurea [HU], nocodazole, valproic acid); Schering-Plough, Kenilworth, NJ (temozolomide); Pharmacia Netherlands, Woerden, The Netherlands (doxorubicin); and Aventis, Antony Cedex, France (docetaxel).

Temozolomide was dissolved in 10% ethanol (v/v) in saline to a concentration of 5 mg/ml.

Sulforhodamine B (SRB) growth inhibition assays
Cells were typically plated out in 96-well microplates on day 0 and either irradiated or supplied with twofold serial drug dilutions on day 1. All drugs were left on the cells for the duration of the experiment, except for MMS, which was removed from the cells after 1 hour incubation in serum-free medium. On day 5, the cells were fixed by adding trichloroacetic acid to a final concentration of 5% (v/v). After 1 hour at 4°C, plates were washed 5 times with demi water, dried and stained for 30 min. with 50µl sulforhodamine B, 0.4% (w/v). Following three wash steps with 1% acetic acid, 150µl 10mM Tris was added to dissolve the staining. Absorbance at 540nm was measured using a Tecan infinite m200 plate reader (Tecan, Salzburg, Austria). After correction for medium-only and no-drug controls, datapoints were fitted using the general formula for a sigmoid curve using the sig-

\[
\text{Survival} = \frac{1}{1 + \left(\frac{\text{Drug}}{\text{IC}_{50}}\right)^n}
\]
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A. Southern blot analysis of primary tumors and cell lines for Brca2. At the top is the genomic structure of the Brca2 locus, along with the probe location and Blnl(B)/NheI(N) restriction sites. LoxP sites are shown by triangles. Expected sizes of bands of all possible alleles detected by Southern blot analysis are indicated. All tumors analyzed showed strong Brca2Δ bands and no or weak Brca2F bands. In contrast, most of the resulting cell lines contained equal amounts of switched/unswitched DNA, as expected for heterozygous Brca2F11/Δ11 cells. Only KB2P-1 and KB2P-3 cell lines showed homozygous deletion of Brca2. B. Several clones of the KB2P-3 and KB2P-1 lines were re-analyzed using Southern blot analysis and still showed Brca2 loss. C. qPCR analysis of BRCA2-deficient clones KB2P-1.21 and KB2P-3.4 and BRCA2-proficient clonal line KP-6.3 confirm complete loss of full-length Brca2 expression. Three different regions of the Brca2 transcript were analyzed by real-time RT-PCR using different primer pairs spanning intron/exon boundaries. D. Western blot analysis showed that all clonal KP and KB2P cell lines have lost p53 expression. NIH-3T3 cells were used as a positive control for p53 detection. Immunoblotting for β-actin was used as loading control.

Results

Establishment of BRCA2-deficient mammary tumor cell lines

In vitro studies on BRCA2-associated breast cancer have been hampered by the lack of appropriate BRCA2-deficient mammary

mortality (m) and IC50 as fit parameters and Matlab software (The Mathworks, Inc., Natick, MA). At least three independent IC50 values were measured for each drug/cell line combination.
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We therefore set out to establish BRCA2-deficient mammary tumor cell lines by taking into culture 12 tumors from \(K14\text{Cre};\text{Brca2}^{F11/F11};\text{p53}^{F2-10/F2-10}\) (KB2P) mice that develop BRCA2-deficient mammary cancer\(^\text{31}\). As controls for functional assays, we also established cell lines from 3 BRCA2-proficient mammary tumors harvested from \(K14\text{Cre};\text{p53}^{F2-10/F2-10}\) (KP) mice\(^\text{34}\).

Histopathological examination of the parental tumors confirmed their resemblance to invasive ductal carcinomas (IDCs), the main tumor type in human BRCA2-mutation carriers (Supplementary Figure 1). Low oxygen (3%) culturing conditions were continuously applied to minimize oxidative DNA damage and thereby prevent specific depletion of BRCA2-deficient cells in KB2P cultures\(^\text{35}\). When cultures showed homogeneous epithelial characteristics upon visual inspection, the KB2P lines were probed for Cre-mediated recombination of the Brca2\(^{F11}\) alleles using Southern analysis (Figure 1A). Whereas all corresponding primary tumors showed homozygous switching of both Brca2\(^{F11}\) alleles (with a residual Brca2\(^{F11}\) band derived from non-switched stromal cells), one or two functional Brca2\(^{F11}\) alleles were retained in cell lines established from 10 out of 12 tumors, suggesting strong selection against BRCA2-deficient mammary epithelial cells during in vitro culture. Nevertheless, homozygous Brca2 loss was detected in 2 independent tumor cell lines (KB2P-1 and KB2P-3, Figure 1A). Limiting dilution culturing resulted in 5 clonal cell lines with homozygously switched Brca2\(^{F11}\) alleles (Figure 1B) and 3 clonal cell lines de-
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![Graphs and images depicting molecular analysis of tumor cell lines](image)
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Brca2-mutated cell lines display genomic instability. A. Array-CGH analysis was carried out on DNA of primary tumors and clonal cell lines derived from these. Top panels show Log2 ratios of DNA copy number changes as compared to spleen control DNA are indicated. Significant copy number gains are represented by red dots, significant copy number losses are indicated by green dots. Bottom panels show unsupervised hierarchical clustering of CGH profiles of all primary KB2P tumors and the clonal cell lines KB2P-1.21 (derived from tumor KB2P-1) and KB2P-3.4 (derived from tumor KB2P-3). Increasing vertical distance indicates larger differences in CGH profiles. B. Ploidy analysis of clonal KP and KB2P cell lines. A minimum of 18 DAPI-stained metaphases were photographed and chromosomes were counted. Error bars represent S.D. C. SKY analysis revealed large amounts of structural and non-clonal aberrations in KB2P cell lines compared to KP controls. Aberrations were considered clonal if more than 2 metaphases contained a specific aberration or gain, or more than 3 losses were detected. The graph depicts the average numbers of clonal and non-clonal aberrations per cell, both for numerical (red) and structural (blue) aberrations. D. A representative SKY example of BRCA2-deficient clone KB2P-3.4, showing both numerical and structural chromosomal aberrations.

BRCA2-deficient mammary tumor cell lines do not form IR-induced RAD51 foci

Cre-mediated recombination within the Brca2 allele results in loss of exon 11, which encodes the BRC repeats necessary for proper regulation of RAD51 protein filament formation during DSBS repair by HR. In BRCA2-proficient cells, induction of DSBS by ionizing radiation results in rapid re-localization of both RAD51 and γH2A.X to sites of DNA damage. In contrast, re-localization of RAD51 does not occur in irradiated CAPAN-1 cells with dysfunctional BRCA2. To ascertain that Brca2Δ11/Δ11;p53Δ1-10/Δ1-10 KB2P cells were incapable of carrying out HR-mediated DSBS repair, we assessed radiation-induced co-localization of RAD51 with γH2A.X in nuclear foci. BRCA2-proficient cells, subjected to 20Gy of γ-irradiation and harvested 8 hours later, showed profound co-localization of RAD51 and γH2A.X. In contrast, complete absence of IR-induced RAD51 foci was seen in the KB2P cells lacking wildtype BRCA2 (Figure 2).

BRCA2-deficient mammary tumor cell lines display genomic instability

Absence of proper DSBS repair, centrosome regulation and cytokinesis upon loss of BRCA2 is thought to result in a mutator phenotype driving additional oncogenic events. Indeed, BRCA2-deficient tumors have been shown to contain high amounts of genetic aberrations. To investigate the extent of genomic gains and losses, we performed genome-wide array CGH analysis of both the parental BRCA2 tumors and the clonal cell lines derived thereof (Figure 3A). Many genomic regions were found to be significantly amplified (red) or deleted (green) as compared to control DNA harvested from the spleen of the tumor-bearing animal. In general, the BRCA2-deficient tumors showed much more aberrations...
than the BRCA2-proficient control tumors, similar to the situation in BRCA1-deficient tumors. Unsupervised hierarchical clustering of all parental tumors and KB2P cell lines showed co-clustering of the KB2P-1 clones together with their ancestral tumor 1, but not of KB2P-3 clones with tumor 3 (Figure 3A). For subsequent ploidy analysis, metaphase spreads were prepared and stained with DAPI to count at least 18 spreads. All but one cell line (KP-6.3) were shown to be aneuploid, a general hallmark of solid tumors. For subsequent analysis, metaphase spreads were prepared and stained with DAPI to count at least 18 spreads. All but one cell line (KP-6.3) were shown to be aneuploid, a general hallmark of solid tumors (Figure 3B). To determine the contribution of individual chromosomes to the increased ploidy as well as the amount of chromosome rearrangements, we performed spectral karyotyping (SKY) analysis. BRCA2-deficient KB2P cells showed large amounts of complex translocations and in addition displayed many other structural aberrations such as deletions and the presence of satellite and marker chromosomes (Figure 3C, D). Besides the expected increase in structural chromosomal aberrations in the KB2P cells vs. the KP cells, we observed more clonal variation within the BRCA2 negative cells than in the controls (Figure 3C). The complete composite karyotype of several cells analyzed is shown in Supplementary Table 1.

Selective sensitivity of BRCA2-deficient mammary tumor cell lines to DNA damaging drugs

Absence of proper repair of damaged DNA leads to hypersensitivity to agents affecting DNA structure. Indeed, the specific hypersensitivity of BRCA2-deficient tumor cells to DNA damaging drugs is the basis for several clinical trials in BRCA2 mutation carriers with breast or ovarian cancer. To evaluate the utility of our mammary tumor cell lines to model BRCA2-deficient breast cancer, sensitivity to both γ-irradiation and cisplatin was determined in growth inhibition assays. Both treatments clearly re-
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Recent experiments support the idea that inhibiting PARP-1 activity, and thus SSB repair, may induce DSBs that are specifically toxic to cells with defective HR repair, such as BRCA2-deficient cells. To test the utility of PARP inhibitors for treating BRCA2-associated breast cancer, we investigated selective growth inhibition of a novel PARP inhibitor, AZD2281, in our panel of BRCA2-proficient and BRCA2-deficient cell lines (Figure 4, Table 1). To compare the activity of AZD2281 with various classes of cytotoxic agents, we tested a set of 10 drugs, including compounds directly inducing DNA-strand lesions that result in DSBs (mitomycin C [MMC], methylmethane sulfonate [MMS], temozolomide), antime-

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<tr>
<th>Table 1 - Differential IC50 Values of 12 agents on Brca2 proficient vs Brca2 deficient cells.</th>
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<tr>
<td><strong>Agent</strong></td>
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<tr>
<td><strong>KP3.33</strong></td>
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<tr>
<td>AZD2281 (nM)</td>
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<td>Temozolomide (µM)</td>
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<td>Cisplatin (nM)</td>
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NOTE: Mean IC50 values of at least 3 independent experiments were determined for 11 drugs and γ-irradiation. Values between brackets represent s.e.m. of the independent IC50 determinations. Average IC50 values determined for the KP lines were compared to average IC50 values of the KB2P lines and displayed as their ratio. Statistical significance was determined using a t-test. *Significant with p<0.01. #For temozolomide, no accurate IC50 values could be obtained for KP-7.7 due to deviation of actual datapoints from a sigmoid curve.

resulted in strong specific growth inhibition in the BRCA2 mutant KB2P lines as compared to the KP controls (Figure 4).

The PARP inhibitor AZD2281 strongly inhibits BRCA2-deficient cell growth

Recent experiments support the idea that inhibiting PARP-1 activity, and thus SSB repair, may induce DSBs that are specifically toxic to cells with defective HR repair, such as BRCA2-deficient cells. To test the utility of PARP inhibitors for treating BRCA2-associated breast cancer, we investigated selective growth inhibition of a novel PARP inhibitor, AZD2281, in our panel of BRCA2-proficient and BRCA2-deficient cell lines (Figure 4, Table 1). To compare the activity of AZD2281 with various classes of cytotoxic agents, we tested a set of 10 drugs, including compounds directly inducing DNA-strand lesions that result in DSBs (mitomycin C [MMC], methylmethane sulfonate [MMS], temozolomide), antime-
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PARP inhibition synergizes with cisplatin in growth inhibition of BRCA2-deficient mammary tumor cells

Since both platinum drugs and the PARP inhibitor AZD2281 give rise to DSBs, combination therapy with platinum and AZD2281 may lead to a reduced effective dose of both single agents. This can be favorable with regard to toxic side-effects of platinum drugs. To study possible drug synergy or additivity, growth inhibition induced by combinations of 16 different concentrations of AZD2281 and 9 different concentrations of cisplatin was determined for both KB2P cell lines and a KP control cell line (Figure 5A). Subsequently, using the growth inhibition curves of both single agents, combination index values as explained in^42 were calculated using the formula

\[
Cl = \frac{m_{AZD2281}}{IC_{50(AZD2281)} \times IC_{50(Cisplatin)}} - \frac{1}{IC_{50(AZD2281)}} + \frac{m_{Cisplatin}}{IC_{50(Cisplatin)} \times IC_{50(AZD2281)}} - \frac{1}{IC_{50(Cisplatin)}}
\]

Thus, log10 CI values < -0.15 indicate synergy at any given combination of drug concentration, values >0.15 indicates antagonism, and values between -0.15 and

Figure 5 - Cisplatin and AZD2281 show additivity in KP lines and synergism in KB2P lines.

A. Two-dimensional plots, showing growth inhibitory effects exerted by combinations of 15 different AZD2281 concentrations with 9 different cisplatin concentrations. The color scale indicates growth ratios of treated cells compared to mock-treated control cells. B. For all drug combinations tested, log10 values of the combination indices are shown. All data points resulting in <20% or >80% growth are arbitrarily set to 0, since predictions of single-agent concentrations for such effects are intrinsically inaccurate.
0.15 represent additive drug interaction\(^4\). Whereas most cisplatin and AZD2281 combinations only exerted additive growth inhibitory effects on the KP-6.3 cell line, synergistic growth inhibition was observed for both BRCA2-deficient cell lines (Figure 5B).

**Discussion**

Tumor derived cell lines are likely to recapitulate several cell-intrinsic properties of tumor cells *in vivo*\(^4\) and have been used extensively as models for human cancer. For studying human hereditary breast cancer associated with BRCA2 loss-of-function, researchers have thus far relied upon the CAPAN-1 line, which carries a 6174delT mutation in one BRCA2 allele accompanied by loss of the wild-type allele\(^4\). This cell line originates from a pancreatic tumor and may therefore be less qualified as a model system for human BRCA2-mutated breast cancer. Growth arrest, probably caused by rapid accumulation of unrepaired DNA damage, makes it very difficult to culture cells with dysfunctional BRCA2\(^4\). Consequently, no established BRCA2-deficient mammary tumor cell lines have been reported until now. Since supraphysiological oxygen levels can increase DNA damage\(^3\), we reasoned that cells with dysfunctional BRCA2 should be cultured at low oxygen conditions, comparable to the levels present \(in\ situ\). Although this strategy resulted in the successful establishment of BRCA2-deficient cell lines from two independent primary mammary tumors, cultures derived from 10 additional tumors displayed depletion of BRCA2-deficient cells, suggesting strong selection against BRCA2 dysfunction during \(in\ vitro\) culture. The fact that all mammary tumors and tumor cell lines derived from \(K14\text{Cre;Brca2}^{F11/F11};p53^{F2-10/F2-10}\) female mice are p53 deficient shows that inactivation of p53-mediated cell-cycle checkpoints is not sufficient to permit \(in\ vitro\) growth of BRCA2-deficient mammary tumor cells. Apparently, other factors present in the tumor stroma are required for unhindered proliferation in the absence of functional BRCA2. Endogenous or culture-induced mutations in the successfully established BRCA2-deficient lines may underlie the evasion of stromal dependency. Identification of such mutations might be of therapeutic relevance.

Detailed functional characterization of our newly established BRCA2-deficient mammary tumor cell lines showed that both KB2P cell lines are defective in IR-induced RAD51 foci formation. Together with the observed early embryonic lethality of homozygous \(\text{Brca2}^{F11/F11}\) mouse mutants\(^3\), these data indicate that Cre-mediated switching of \(\text{Brca2}^{F11}\) results in a non-functional allele. In line with this, both BRCA2-deficient KB2P cell lines showed increased genomic instability, characterized by large numbers of clonal structural aberrations, compared to BRCA2-proficient KP cell lines. The high degree of genomic instability of the BRCA2-deficient KB2P cell lines is also reflected by the fact that many aberrations were not clonal, giving rise to \(de\ novo\) genetic heterogeneity within the clonal KB2P cell lines. This continuing genetic heterogeneity may, in combination with strong selective pressure for adaptation to \(in\ vitro\) growth conditions, also be the cause of the discordance between the CGH profiles of KB2P-3 clonal cell lines and the corresponding primary tumor.

Interestingly, whereas tri- and quadriradial chromosome structures have been described to be a direct effect of BRCA2 dysfunction\(^4\), these structures were not observed in any of the metaphase spreads prepared from our KB2P cell lines (data not shown).
Although it is known that DNA damaging agents show selective cytotoxicity against non-tumor cells with engineered Brca2 mutations, relatively few data are available about the effect of such agents on BRCA2-deficient mammary tumor cells. These tumor cells are either intrinsically less sensitive to the proliferative impediment that is induced by BRCA2 loss-of-function in non-tumor cells, or have somehow overcome this inhibition, perhaps by acquiring additional mutations. For this reason it is important to assess the efficacy of known cytotoxic agents in the context of steadily proliferating BRCA2-deficient cells. We have assessed, for the first time, the selective toxicity of γ-irradiation and 11 anticancer drugs on proliferating BRCA2-deficient breast cancer cells. Our in vitro cytotoxicity studies with BRCA2-proficient KP cell lines vs. BRCA2-deficient KB2P lines clearly showed selective sensitivity of KB2P cells to cytotoxic agents directly inducing DNA strand lesions (IR, Cisplatin, MMC, MMS, Temozolomide) but not to agents that do not, or only indirectly, induce DNA damage (doxorubicin, 5FU, HU, docetaxel, nocodazol, valproic acid). Temozolomide displayed an exquisite 46-fold higher sensitivity towards BRCA2-deficient cells. This strong selectivity may be explained by the fact that temozolomide indirectly induces DSBs as a result of futile mismatch repair of O6-methylguanine lesions. Our results highlight, for the first time, the potential efficacy of temozolomide against BRCA2-mutated breast cancer, and warrant further investigation. Still, the strongest selective sensitivity of KB2P cells was observed with the clinical PARP inhibitor AZD2281. One explanation for the unrivaled sensitivity of BRCA2-defective cells towards AZD2281 is that suppression of BER by PARP inhibition may result in the conversion of SSBs to DSBs during DNA replication, thus activating BRCA2 dependent recombination pathways. The two independently isolated BRCA2-deficient cell lines show very similar responses to all agents tested, as do the three independent BRCA2-proficient lines. Nevertheless, definitive proof of BRCA2 directly being responsible for the differential effects between these groups of cell lines should come from reconstitution experiments that should rule out that other mutations are causal to the observed sensitization spectrum.

The initial reports on the selective sensitivity of BRCA2-deficient cells to PARP inhibition were challenged by other studies, which claimed that known PARP inhibitors were not or only minimally selective to BRCA2 mutant CAPAN-1 tumor cells and BRCA1 mutant tumor cells. Although CAPAN-1 cells were subsequently shown to be highly sensitive to the PARP inhibitor used in one of the original studies, these experiments did not rule out the possibility that cell type related differences might compromise the utility of PARP inhibitors for treating BRCA-associated breast cancer. This concern is alleviated by the potent growth inhibition induced by the clinical PARP inhibitor AZD2281 in our BRCA2-deficient mammary cancer cell lines. Whether the strong growth inhibition induced by AZD2281 also applies to BRCA1-deficient tumor cells remains yet to be determined.

Most known PARP inhibitors inhibit PARP-1 as well as PARP-2 activity and, in contrast to PARP-1 single knockouts, PARP-1/ PARP-2 double knockout mice are embryonic lethal. Indeed, PARP-1 and PARP-2 have both overlapping and non-redundant functions in the maintenance of genomic stability. Nevertheless, both the large differential effect between BRCA2-deficient and -proficient cells and the absence of apparent toxic effects in mice treated with AZD2281 (data not shown) indicate that
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partial inhibition of PARP-1 and PARP-2 by AZD2281 does not cause notable toxicity in wild type cells.

In summary, we have generated the first set of BRCA2-deficient mammary tumor cell lines, which may prove useful for mechanistic studies and for preclinical evaluation of novel anti-cancer drugs. In vitro cytotoxicity studies with these cell lines showed clear selective sensitivity of BRCA2-deficient cells to drugs that induce DNA strand lesions or compounds that target the BER pathway. Moreover, potent synergy between the clinical PARP inhibitor AZD2281 and cisplatin was observed. Taken together, our results provide strong evidence for the use of selective PARP inhibitors alone, or in combination with platinum drugs for the treatment of BRCA-associated cancers and BRCA-like tumors with defective HR repair.

Acknowledgments
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References


Chapter 2: Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin

Supplementary information

Figure S1 - H&E slides of parental tumors.
Hematoxylin and eosin stained sections of the tumors from which cell lines are derived. All tumors are classified as solid adenocarcinomas resembling human infiltrating ductal carcinoma not otherwise specified (IDC-nos) grade III, although the expansive growth and partial spindle cell morphology may suggest a more metaplastic phenotype. Scale bars are 200μm.

Figure S2 - Clonal cell lines are epithelial.
Cells seeded on coverslips and harvested 24 hours later were stained for cytokeratin 8 (green, A) or E-cadherin (green, B), and DNA (blue) as described¹. Subsequently, pictures were taken using a Leica TCS TNT microscope (Leica Microsystems, Wetzlar, Germany).

### Supplementary table 1 - Cell-line karyotypes as determined by SKY-analysis

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ploidy</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP-3.33</td>
<td>4n</td>
<td>-X(8), -14, -9(3), -10(8), -12(7), -13(3), -14(4), -15(3), -18(8), -19(3), der(4)t(4;19)[7], der(9)t(9;17)[2], del(X)[6], der(11)dup(11)[5], +sat[4][cp9]</td>
</tr>
<tr>
<td>KP-6.3</td>
<td>2n</td>
<td>+19(8), der(16)t(16;3)[8], der(11)dup(11)[3][cp9]</td>
</tr>
<tr>
<td>KP-7.7</td>
<td>4n</td>
<td>-X*<a href="7">2</a>, -1(8), -2(7), -4(7), -5(3), -7(8), +8(5), +9(8), +11(6), -12(7), -13(6), -14(8), +15(2), -16(8), -17(3), +19*[2][6][cp8]</td>
</tr>
<tr>
<td>KB2P-1.21</td>
<td>3n</td>
<td>-X(4), -2(3), -10(5), -11(4), -13(5), -15(3), -16(3), -18(4), +del(19)[2], der(X)t(10;18)[2], der(X)t(X;15)[2], der(1)t(1;11)[4], der(6)t(6;X)[2], der(11)t(11;6)[5], der(15)t(15;X)[3], der(18)t(18;11)[2], der(18)t(18;3)[2], del(5)[2], del(12)[5], del(12)*2[2], del(19)[2], der(19)dup(19)[2], +sat[4][2][cp6]</td>
</tr>
<tr>
<td>KB2P-3.4</td>
<td>3n</td>
<td>-1(4), -8<em>2[3], -9(4), -10(5), -10</em>2[3], -11(4), -13(3), -14(3), -17(4), +del(18)[4], der(X)t(X;11)[3], der(4)t(4;14)[2], der(4)t(4;14)[2], der(5)t(5;1)[2], der(5)t(5;17)[3], der(7)t(7;4;16;4;19)[2], der(12)t(12;19;6;18)[2], der(13)t(13;12)[4], der(14)t(14;12)[2], der(14)t(14;18)[2], der(16)t(16;5)[2], del(X)[4], del(2)[2], del(3)[2], del(4)[2], del(6)[2], del(7)[5], del(8)[2], del(12)[3], del(13)[3], del(16)[4], del(18)*2[2], del(10)dup(10)[2], +sat[37][cp6]</td>
</tr>
</tbody>
</table>

**NOTE:** A minimum of 6 cells per cell-line are analyzed by spectral karyotyping analysis. The composite karyotype of all clonal variations is described while using the assigned ploidies as a starting point.