

**Cancer Therapy: Preclinical****A High-Throughput Pharmaceutical Screen Identifies Compounds with Specific Toxicity against BRCA2-Deficient Tumors**

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**Abstract**

**Purpose:** Hereditary breast cancer is partly explained by germline mutations in *BRCA1* and *BRCA2*. Although patients carry heterozygous mutations, their tumors have typically lost the remaining wild-type allele. Selectively targeting BRCA deficiency may therefore constitute an important therapeutic approach. Clinical trials applying this principle are underway, but it is unknown whether the compounds tested are optimal. It is therefore important to identify alternative compounds that specifically target BRCA deficiency and to test new combination therapies to establish optimal treatment strategies.

**Experimental Design:** We did a high-throughput pharmaceutical screen on BRCA2-deficient mouse mammary tumor cells and isogenic controls with restored BRCA2 function. Subsequently, we validated positive hits *in vitro* and *in vivo* using mice carrying BRCA2-deficient mammary tumors.

**Results:** Three alkylators—chlorambucil, melphalan, and nimustine—displayed strong and specific toxicity against BRCA2-deficient cells. *In vivo*, these showed heterogeneous but generally strong BRCA2-deficient antitumor activity, with melphalan and nimustine doing better than cisplatin and the poly-(ADP-ribose)-polymerase inhibitor olaparib (AZD2281) in this small study. *In vitro* drug combination experiments showed synergistic interactions between the alkylators and olaparib. Tumor intervention studies combining nimustine and olaparib resulted in recurrence-free survival exceeding 330 days in 3 of 5 animals tested.

**Conclusions:** We generated and validated a platform for identification of compounds with specific activity against BRCA2-deficient cells that translates well to the preclinical setting. Our data call for the re-evaluation of alkylators, especially melphalan and nimustine, alone or in combination with the poly-(ADP-ribose)-polymerase inhibitors, for the treatment of breast cancers with a defective BRCA pathway.

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Breast cancer is the most common malignancy in women. Approximately 10% to 15% of patients are reported to have a family history of breast cancer (1). Mutations in the two hereditary breast cancer genes *BRCA1* and *BRCA2*, identified in the mid-1990s (2–5), are responsible for 15% to 20% of familial cases (1). Although more

than a decade of research has significantly increased our knowledge on *BRCA* gene function, this has not yet led to specific guidelines for the treatment of hereditary breast cancer patients (6).

*BRCA1* and *BRCA2* play major roles in the error-free repair of DNA double-strand breaks (DSB) through the process of homologous recombination (7–9). Breast and ovarian tumors in *BRCA* mutation carriers are typically deficient for *BRCA* function due to loss of heterozygosity (10, 11). This inspired researchers to exploit the DNA repair defects of *BRCA*-mutated tumors by treating them with DNA-damaging agents that either directly or indirectly induce DSBs.

Two classes of drugs that recently gained momentum in the treatment of *BRCA*-associated patients are platinum-based DNA cross-linking agents and inhibitors of poly-(ADP-ribose) polymerase (PARP) activity. Cisplatin and carboplatin mostly induce interstrand and intrastrand cross-links, but the processing of these lesions results in intermediates of which the resolution depends on intact

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

We present a cell line-based platform for high-throughput identification of chemical compounds with specific toxicity towards BRCA2-deficient tumor cells. This method can be used to assess the specific toxicity of known drugs and to identify new classes of compounds targeting as yet unknown aspects of BRCA-deficient cancer. Using this platform, we find strong BRCA2-deficient antitumor activity of three alkylating agents, which were previously abandoned in clinical trials on unselected patient populations. These alkylating compounds have synergistic interaction with poly-(ADP-ribose)-polymerase inhibitors, which are currently in clinical trials for the treatment of hereditary breast cancer patients. Our results show the importance of re-evaluating alkylating agents in breast cancer patients with BRCA2-like defects. In addition to hereditary predisposed individuals, these may include patients with homologous recombination-deficient sporadic breast cancer.

homologous recombination. This is thought to explain the strong sensitivity of BRCA1- and BRCA2-deficient cells towards these drugs (12, 13).

PARP activity is necessary for efficient single-strand break repair by the base excision repair pathway. In the absence of PARP activity, these lesions are thought to be converted into DSBs when encountered by replication forks that are unable to proceed. Whereas wild-type cells can subsequently repair these DSBs by homologous recombination, BRCA-deficient cells will accumulate unrepaired DSBs or misrepaired lesions due to promiscuous activity of error-prone repair pathways, ultimately leading to mitotic catastrophe. Indeed, small molecule inhibitors of PARP enzymatic activity have been shown to be highly toxic to BRCA-deficient cells (14, 15).

Both platinum-based drugs and olaparib (AZD2281), a PARP inhibitor recently shown to kill BRCA-deficient mouse mammary tumor cells (16, 17), with the promising results from phase 0 and phase I trials (18, 19), are currently in phase II clinical trials in breast cancer patients (20). Although clinical trials usually take several years to complete, experimentation using animal models has already yielded several important insights. BRCA1-deficient mammary tumors responded well when treated with cisplatin, carboplatin, or olaparib (17, 21), and BRCA2-deficient mammary tumors regress upon treatment with olaparib (22). It seems unlikely, however, that these drugs will prove to be the ultimate answer to treating BRCA patients. In the same studies, Rottenberg et al. found that tumors develop resistance against olaparib and that treating with platinum drugs does not fully eradicate the tumor. Even combination treatment of olaparib with platinum agents resulted in tumor relapses, albeit with a longer la-

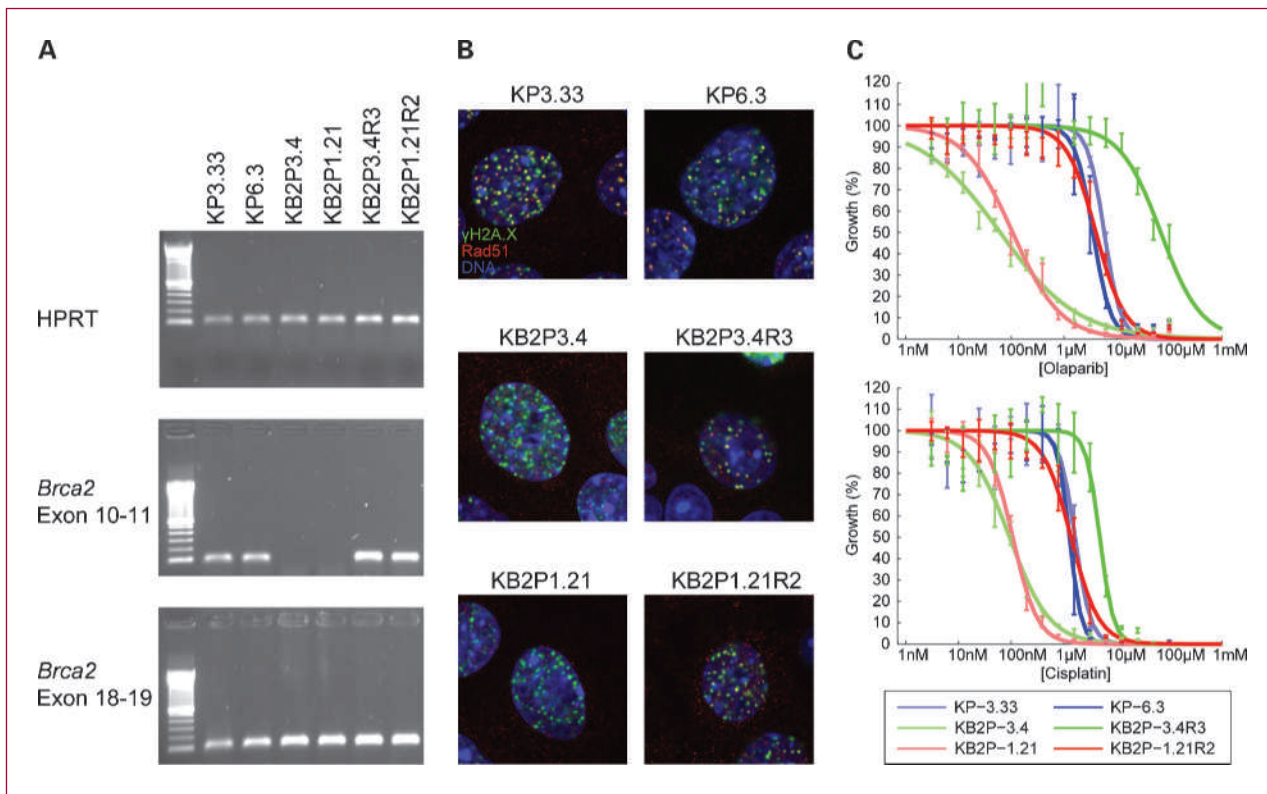
tenacy (17, 22). Hence, there is a continuing need for new drugs or drug combinations that selectively kill BRCA-deficient tumor cells.

In this article, we report the development of an *in vitro* high-throughput screening system to identify compounds with specific toxicity towards BRCA2-deficient cancer cells. We have used this system to probe a commercially available library of 1,258 drugs with known pharmacologic activity and found, besides carboplatin, three alkylating agents: chlorambucil, melphalan, and nimustine. *In vitro* validation experiments showed strong selective activity of these compounds against BRCA2-deficient cells and specific synergy with olaparib. *In vivo* studies using single-agent therapy showed favorable relapse-free survival times for two of the three compounds compared with cisplatin and olaparib in two different mouse models for BRCA2-deficient breast cancer. Combination therapy with nimustine and olaparib resulted in a relapse-free survival of >330 days in 3 of 5 animals tested. Besides offering a platform for the identification of additional compounds for the treatment of BRCA-related cancers, these results call for a re-evaluation of alkylating agents already known to the clinic but not generally used for the treatment of breast cancer and further assessment of combination therapies containing both alkylators and PARP inhibitors.

### Materials and Methods

**Cell line culturing and BRCA2 reconstitution.** The KP3.33, KP6.3, KB2P1.21, and KB2P3.4 mouse mammary tumor cell lines (16) were cultured at 37°C, 5% CO<sub>2</sub>, and 3% O<sub>2</sub> in complete medium [DMEM/F-12, (Life Technologies) supplemented with 10% FCS, 50 units/mL penicillin, 50 µg/mL streptomycin (Life Technologies), 5 µg/mL insulin (Sigma), 5 ng/mL epidermal growth factor (Life Technologies), and 5 ng/mL cholera toxin (Gentaur)]. To reconstitute the BRCA2-deficient cell lines, herpes simplex virus-1/EBV amplicons containing the complete mouse *Brc2* gene were produced as described previously (23). Concentrated (40×) *Brc2* infectious bacterial artificial chromosomes were aliquoted and stored at -80°C until use. After overnight infection and subsequent 48 h recovery, *Brc2* infectious bacterial artificial chromosomes containing KB2P cells were selected with 400 µg/mL hygromycin. Reverse transcriptase-PCR and RAD51/γH2A.X colocalization experiments to validate functional BRCA2 reconstitution were done as described (16).

**Growth inhibition assays.** All single-drug *in vitro* experiments were done in 96-well plates. Typically, 150 KP3.33, 100 KP6.3, 400 KB2P1.21, 400 KB2P1.21R2, 500 KB2P3.4, and 300 KB2P3.4R3 cells were plated in 160 µL complete medium on day 0. On day 1, 40 µL drug containing complete medium were added while maintaining DMSO concentrations similar in all wells and <0.4%. On day 5, the cells were incubated with 10 µL cell-titer blue (Promega) for 4 h at 37°C. Fluorescence was measured at 590 nm using an Envision plate reader (Perkin Elmer). Values from triplicate measurements were averaged, corrected



**Fig. 1.** Functional reconstitution of BRCA2-deficient mammary tumor cells. BRCA2-deficient mouse mammary tumor cells (KB2P1.21 and KB2P3.4) were reconstituted with transduction of an infectious bacterial artificial chromosome carrying the complete *Brca2* gene to result in KB2P1.21R2 and KB2P3.4R3 lines. **A**, reverse transcriptase-PCR experiments show that although the KB2P1.21 and KB2P3.4 cell lines lack expression of exon 11 due to cre-mediated recombination, full-length *Brca2* expression is restored upon stable introduction of a *Brca2*-containing infectious bacterial artificial chromosome. **B**, colocalization of  $\gamma$ H2A.X (green) with RAD51 (red) in the nucleus (blue) of cells exposed to 20 Gray  $\gamma$ -irradiation is restored in KB2P1.21R2 and KB2P3.4R3 versus KB2P1.21 and KB2P3.4 cell lines. BRCA2-proficient KP cells were shown as controls. **C**, cytotoxicity profiles of olaparib (top) and cisplatin (bottom) clearly show sensitivity associated with BRCA2 deficiency.

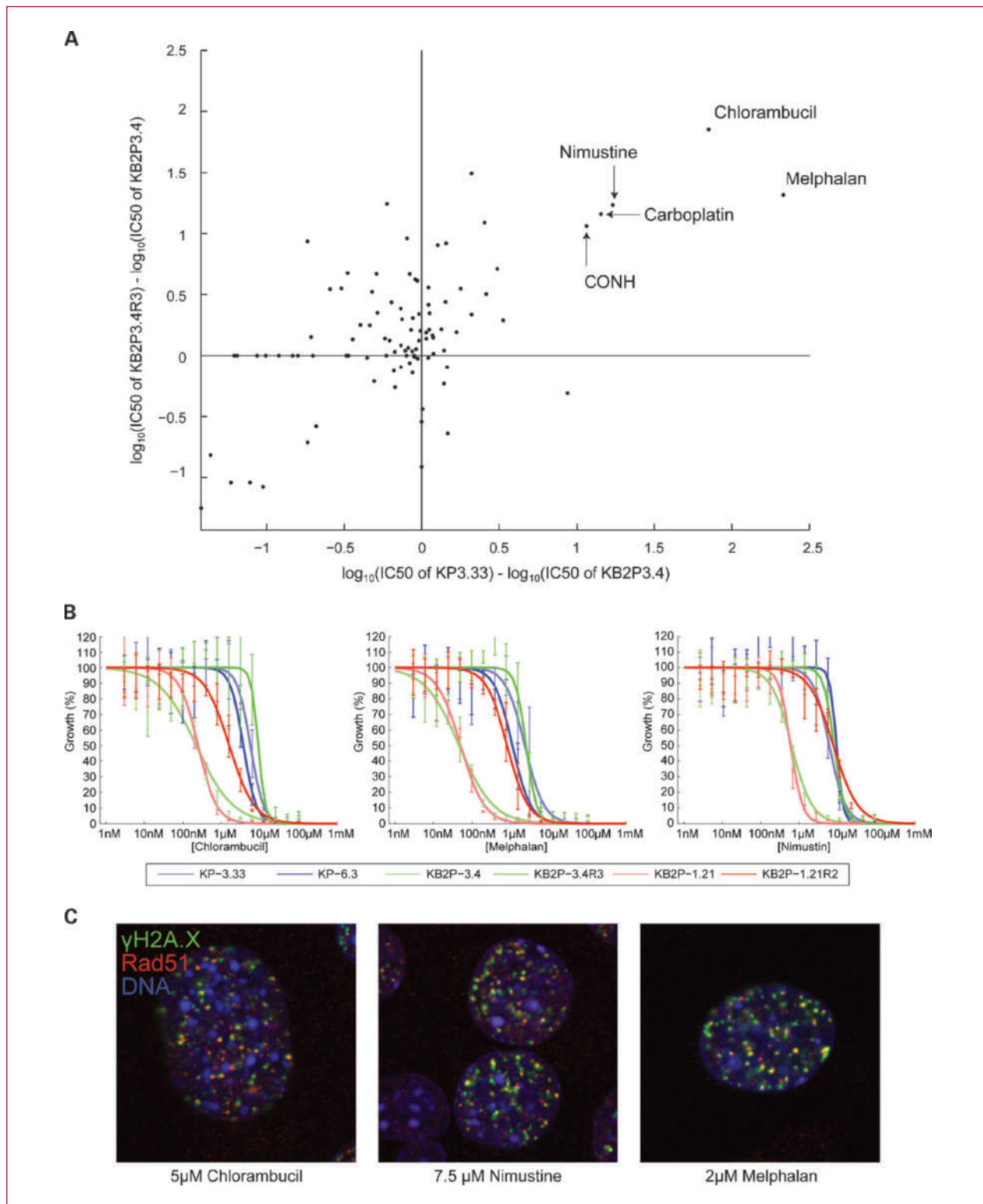
for background, and normalized against values from DMSO-only controls.  $IC_{50}$  values were calculated using Matlab software, as described (16). All drug interaction experiments were done with sulforhodamine B assays, because these were less prone to signal variation, which is crucial to accurate determination of combination indices. Combination index values were determined as described (16).

**Screen set-up.** DMSO stocks (10 mmol/L) of the LO-PAC1280 compound library (Sigma) were used to create 5 mmol/L and subsequent 10-fold dilutions in DMSO. Cells were seeded on day 0 in 384-well plates in 40  $\mu$ L complete medium. On day 1, 96-well plates were prepared with 100-fold diluted drugs in complete medium in columns 2 to 11. Columns 1 and 12 were used for DMSO-only negative controls and multiple olaparib concentrations as positive controls. Assay plates were supplied with 10  $\mu$ L diluted compound. On day 5, a 4-h incubation with 20  $\mu$ L 8 $\times$  diluted cell-titer blue was used to determine cell viability. Screening was done using five 10-fold concentration steps, ranging from 1 nmol/L to 10  $\mu$ mol/L. Mean values of technical duplicates were standardized against DMSO-only controls in the same plate and  $IC_{50}$  values were

calculated. When  $IC_{50}$  values were  $>50$   $\mu$ mol/L or when the maximum growth inhibition of any range did not exceed 25%,  $IC_{50}$  values were arbitrarily set at 50  $\mu$ mol/L.

**In vivo validation studies.** Chlorambucil (Sigma) was dissolved in 70% EtOH to 50 mg/mL. Immediately prior to use, this stock was diluted 40 $\times$  with 100 mmol/L phosphate buffer (pH6.8). Melphalan (Alkeran, Glaxo-SmithKline) was reconstituted immediately before administration, according to the manufacturer's protocol. Nimustine hydrochloride (1 g; Sigma) was dissolved in 6.5 mL DMSO and just before injection, diluted 25-fold with physiologic salt solution. Cisplatin was obtained from Mayne Pharma. Olaparib was kindly provided by KuDOS Pharmaceuticals (24). Olaparib, chlorambucil, melphalan, and nimustine were injected i.p. Cisplatin was injected i.v. in the tail vein.

Because all cells and tumors were derived from mice with a mixed FVB and 129/Ola background (FVB  $\times$  129/Ola), F1 hybrid females were used for all animal experiments reported in this article. Maximum tolerable doses were determined using dose escalation in at least three animals for all three alkylators and cisplatin using a



**Fig. 2.** A pharmacologic screen identifies three alkylators with specific toxicity to BRCA2-deficient cells. **A**, scatter-plot depicting differential toxicities of 97 compounds for which  $\text{IC}_{50}$ s could be determined. *X*-axis, differential toxicity between BRCA2-proficient (KP) and BRCA2-deficient cells; *y*-axis, differential toxicity between BRCA2-reconstituted (KB2P) and BRCA2-deficient cells. **B**, cytotoxicity profiles of chlorambucil, nimustine, and melphalan done on the complete cell line panel validate the alkylators picked up from the screen. **C**, immunofluorescence of  $\gamma\text{H2A.X}$  (green) and RAD51 (red) in the nucleus (blue) of cells exposed for 24 h to the alkylators at indicated concentrations. Colocalization of  $\gamma\text{H2A.X}$  and RAD51 indicates induction of recombinogenic lesions.

20% weight loss as a toxicity threshold. The following maximum tolerable doses were established: chlorambucil, 15 mg/kg i.p.; melphalan, 7.5 mg/kg i.p.; nimustine, 30 mg/kg i.p.; cisplatin, 6 mg/kg i.v. Olaparib was administered daily at 100 mg/kg i.p., at which no toxicity was observed, but higher amounts would exceed the maximum of 1 mL daily injection volume. For the tumor intervention studies, either 500,000 KB2P3.4 cells, or 1 mm<sup>3</sup> pieces of five independent mammary tumors from K14cre;*Brca2*<sup>F11/F11</sup>; *p53*<sup>F2-10;F2-10</sup> animals were engrafted orthotopically in the 4th mammary fat pad of adult F1 hybrid mice as described (21). Treatment at maximum tolerable dose levels was started once tumor volume, calculated as 0.5 × length × width<sup>2</sup>, exceeded 200 mm<sup>3</sup>. Because olaparib did not show any toxicity at 100 mg/kg and because after 24 h only about one third of the maximum PARP inhibition remained due to rapid elimination (17), we decided to dose olaparib daily for a period of 28 consecutive days. When the tumor volume was >1,500 mm<sup>3</sup>, the animal was sacrificed. All animal experiments were done in accordance with institutional guidelines and local regulations.

## Results

**Reconstitution of BRCA2-deficient mouse mammary tumor cells.** We previously isolated a set of BRCA2-proficient (KP) and BRCA2-deficient (KB2P) mouse mammary tumor cell lines with differential responses to several agents such as cisplatin and the PARP inhibitor olaparib (16). These cell lines are not suitable to carry out pharmaceutical screens, because many other mutations may account for any specific toxicity found. Reconstituting the BRCA2-deficient KB2P-1.21 and KB2P-3.4 cell lines, resulting in isogenic cell line pairs with differential BRCA2 functionality, should overcome this problem. However, artificial *BRCA2* expression from cDNA constructs was shown to severely impair cell growth (25). We therefore chose to reconstitute the complete *Brca2* gene including its endogenous promoter by stable introduction of a bacterial artificial chromosome clone encompassing the mouse *Brca2* locus. Both KB2P lines were reconstituted with a herpes simplex virus-1-based *Brca2* infectious bacterial artificial chromosome (23), which led to reconstitution of full-length *Brca2* expression as shown by reverse transcriptase-PCR analysis (KB2P3.4R3 and KB2P1.21R2; Fig. 1A). Array comparative genomic hybridization analysis confirmed the isogenicity of the BRCA2-deficient cell lines and their reconstituted counterparts (Supplementary Fig. S1). Rescue of RAD51 colocalization with  $\gamma$ H2A.X in  $\gamma$ -irradiated KB2P3.4R3 and KB2P1.21R2 cells showed that the reconstitution was functional (Fig. 1B). To further test the utility of these cell lines for pharmaceutical screens, we did growth inhibition assays for olaparib and cisplatin on the KB2P, KB2PR, and KP cell lines (Fig. 1C). BRCA2 reconstitution completely reversed sensitivity to these compounds to similar levels as in the BRCA2-proficient KP lines (KB2P1.21R2) or to even

higher levels (KB2P3.4R3). This hyperresistance correlated with higher *Brca2* expression levels in KB2P3.4R3 cells compared with KP control cells as measured by quantitative reverse transcriptase-PCR (data not shown). Because the window of differential sensitivity seemed highest in the KB2P3.4/KB2P3.4R3 combination, we used this isogenic pair of cell lines to carry out a high-throughput pharmacologic screen. As an independent control, we used the BRCA2-proficient KP3.33 cell line (16).

**A high-throughput screen for compounds with specific cytotoxicity against BRCA2-deficient cells.** Using a high-throughput robotic screening system, we exposed KP3.33, KB2P3.4, and KB2P3.4R3 cells to a library of clinically active compounds (LOPAC, Sigma) containing 1,258 compounds. Ninety-seven drugs had an IC<sub>50</sub> < 50  $\mu$ mol/L in any of the cell lines used (Fig. 2A). To determine the influence of *Brca2* expression on the IC<sub>50</sub> values, a combined specificity index was calculated by addition of the log<sub>10</sub> differential IC<sub>50</sub> values for KB2P3.4 versus KB2P3.4R3 and KB2P3.4 versus KP3.33, respectively (see Supplementary Table S1). Carboplatin, which is currently used in clinical trials in BRCA-associated breast cancer patients, was the fifth best hit with an IC<sub>50</sub> of 4.4  $\mu$ mol/L in the KB2P3.4 cells, but with no significant growth inhibition in the BRCA2-proficient cells. Also the topoisomerase inhibitors camptothecin and ellipticine showed a considerable differential cytotoxicity, which is in accordance with previously published work (26). Inhibition of topoisomerase activity is known to result in DNA strand breaks, which could explain the activity of these drugs.

**In vitro validation of compounds.** Four compounds (chlorambucil, melphalan, nimustine, 2-cyclooctyl-2-hydroxyethylamine) reported an even higher combined specificity index than carboplatin. Except for 2-cyclooctyl-2-hydroxyethylamine, which did not validate in subsequent experiments (data not shown), these compounds were alkylating agents not currently part of the standard of care for treating breast cancer patients (27). To further validate the efficacy of these drugs, we determined growth inhibition curves of freshly dissolved compounds on our complete panel of cell lines. Indeed, for all three alkylators, both BRCA2-deficient cell lines showed much greater sensitivity than the BRCA2-reconstituted and -proficient controls (Fig. 2B). Immunofluorescent staining revealed clear colocalization of  $\gamma$ H2A.X and RAD51 foci after treating KP3.33 cells for 24 hours with the alkylators at IC<sub>50</sub> concentrations (Fig. 2C). This confirms that these drugs are capable of inducing recombinogenic DNA breaks, which are known substrates for BRCA2 function.

**In vitro synergism between alkylators and PARP inhibitor olaparib.** Combinations of the DNA cross-linker cisplatin with the PARP inhibitor olaparib were previously shown to synergistically inhibit the growth of BRCA2-deficient cancer cells (16). We assessed drug interactions between olaparib and chlorambucil, melphalan, or nimustine in our panel of cell lines by determining combination index values for a range of combined drug concentrations. The means of all combination indices of two independent

**Table 1. Drug interactions between olaparib and alkylators**

	KP-3.33	KP-6.3	KB2P-3.4	KB2P-3.4R3	KB2P1.21	KB2P-1.21R2
Olaparib+						
Chlorambucil	-0.06	-0.05	-0.21	-0.04	-0.17	0.02
Melphalan	-0.05	-0.07	-0.14	-0.08	-0.16	-0.06
Nimustine	-0.05	-0.02	-0.22	-0.07	-0.17	-0.06

NOTE: Averaged combination index values of olaparib and alkylators, tested on the complete cell-line panel. Synergistic interactions, with an average combination index of  $<-0.15$ , are highlighted.

experiments were calculated and averaged (Table 1). Synergistic drug interactions, corresponding to combination indices of  $<-0.15$  (28), were found for chlorambucil, melphalan, and nimustine in the BRCA2-deficient KB2P1.21 cell line and are comparable with an average cisplatin/olaparib combination index of  $-0.16$  (16). For chlorambucil and nimustine, synergy was also found in the KB2P3.4 line at an even higher level than for cisplatin, which was previously shown to have an average combination index of  $-0.17$  (16). Interestingly, combinations of alkylating agents with olaparib in the BRCA2-proficient cell lines resulted in additive effects rather than synergistic interactions. Because drug synergism was thus found to be specific for BRCA2-deficient cells, combining these drugs could further increase an existing clinical window in patients with BRCA-deficient tumors.

**In vivo responses of orthotopically transplanted BRCA2-deficient mammary tumor cells.** To further substantiate our findings, we carried out *in vivo* validation studies with chlorambucil, melphalan, and nimustine in mice carrying BRCA2-deficient tumors. Upon orthotopic transplantation of 500,000 KB2P3.4 cells in the 4th mammary fat-pad of young adult female mice, palpable tumors developed in approximately 1.5 months. Drug treatment was started when tumors exceeded 200 mm<sup>3</sup>. Alkylators were administered only once, whereas olaparib was administered daily for 28 consecutive days.

Tumor-bearing animals that were left untreated had to be sacrificed after 1 to 2 weeks, when tumors reached a size of approximately 1,500 mm<sup>3</sup> (Fig. 3). The efficacy of PARP inhibition as an approach to target BRCA2-deficient tumor cell growth is clearly shown, although responses were heterogeneous and tumors progressed quite rapidly after initial response to olaparib (Fig. 3). On the contrary, treatment with a single dose of cisplatin or any of the alkylators invariably resulted in partial or even complete tumor remission (Fig. 3). Relapses nevertheless occurred in all cisplatin- and chlorambucil-treated animals within a 45-day period. Melphalan-treated animals, however, survived longer (61 days on average versus 38 for cisplatin and 45 for chlorambucil). Strikingly, all animals treated with nimustine remained relapse free for at least 100 days.

**Treatment responses of allografted BRCA2-deficient mammary tumors.** Although orthotopic allografting of BRCA2-deficient mammary tumor cell lines into the mouse

mammary fat pad constitutes an efficient way of determining targeted drug efficacy *in vivo*, differences between spontaneous tumors and tumor cell line outgrowths may exist due to prolonged *in vitro* culturing and the potential absence of an intact stromal compartment. These issues may be overcome by direct engraftment of tumor pieces into multiple syngeneic animals, resulting in secondary tumors that closely mimic the parental tumors (21). Similar to these studies, we engrafted tissue pieces originating from a panel of five independent mammary tumors from K14cre;Brca2<sup>F11/F11</sup>;p53<sup>F2-10;F2-10</sup> mice (29) into the mammary gland fat pads of multiple recipients (Fig. 4A). When left untreated, the mice needed to be sacrificed 1 to 2 weeks after diagnosis of a palpable tumor, due to rapid, expansive growth of the local tumor. The engrafted tumors showed varying responses to the PARP inhibitor olaparib. Although some tumors (Fig. 4A, tumors 1 and 4) hardly responded and even needed to be sacrificed before the end of the 28-day treatment schedule, other tumors showed a partial response (Fig. 4A, tumors 3 and 5) or even complete remission before eventual relapse (Fig. 4A, tumor 2). In line with the results of the cell line allografts, tumor grafts generally showed stronger responses to cisplatin, chlorambucil, melphalan, and nimustine than to olaparib. Again, melphalan and nimustine resulted in better relapse-free survival after treatment compared with olaparib and cisplatin (Fig. 4B). Of note, two animals of our nimustine-treated tumor graft cohort needed to be sacrificed before having relapses, approximately 6 months after treatment. Histologic analyses showed renal failure to be the most likely cause of death. Despite the relatively small size of the tumor cohort, borderline significant log-rank *P* values were determined for olaparib versus melphalan/nimustine and for cisplatin versus melphalan.

Besides these favorable results, complete cure seems still infrequent. Only one animal, treated with melphalan, was still recurrence free 290 days after the start of treatment. To see whether the *in vitro* synergy between the alkylators and olaparib translates *in vivo*, we treated mice harboring allografts of the same tumors as used in the single-agent experiments with combination therapy. Unfortunately, combining single-dose melphalan at maximum tolerable doses with a 28-day olaparib course was associated with increased toxicity, precluding analysis of this combination at dosages comparable with their single-agent treatment

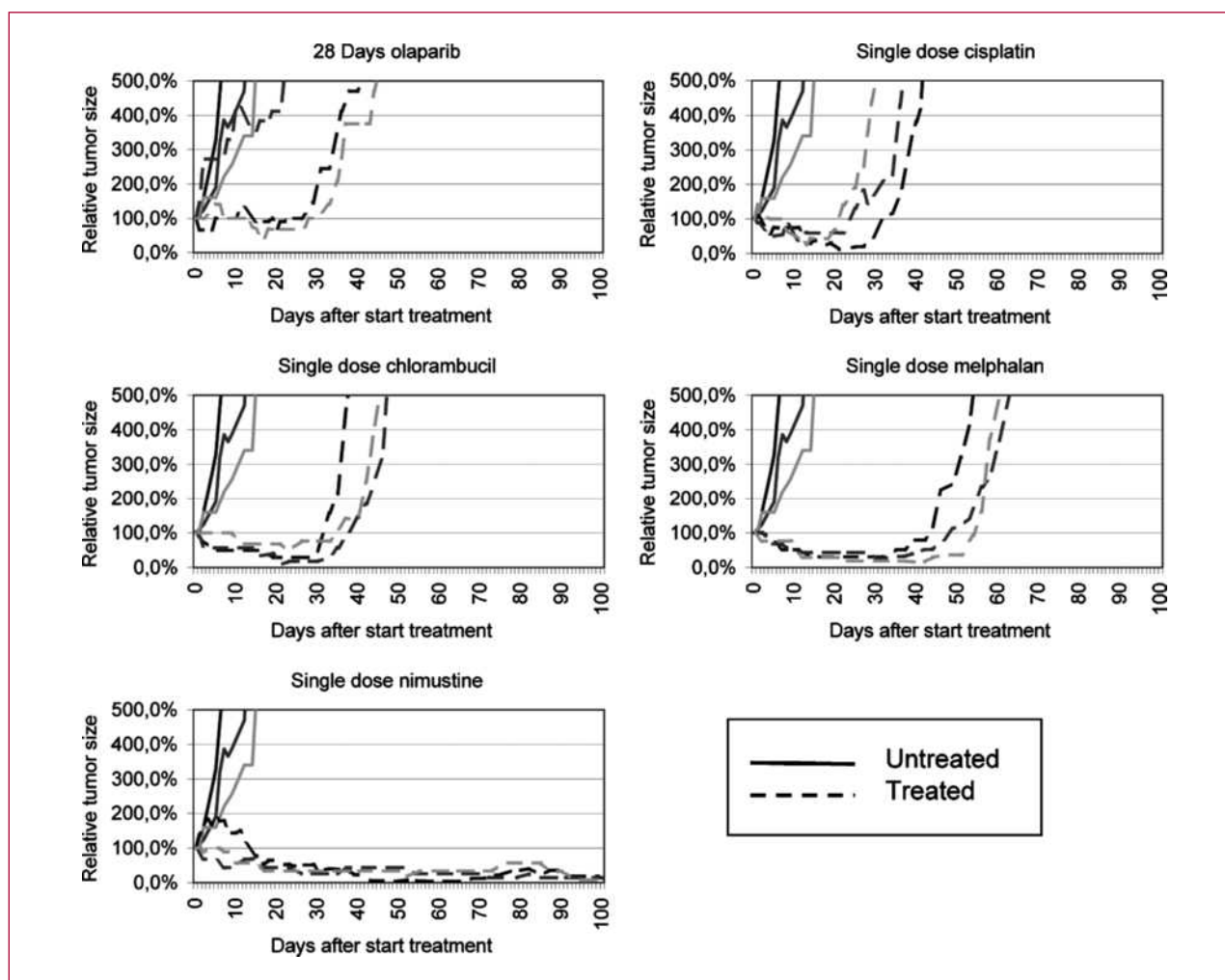
regimes. However, when single-dose nimustine treatments were combined with 28 days of olaparib treatment, only two of five animals suffered from recurrences in a period currently exceeding 330 days.

## Discussion

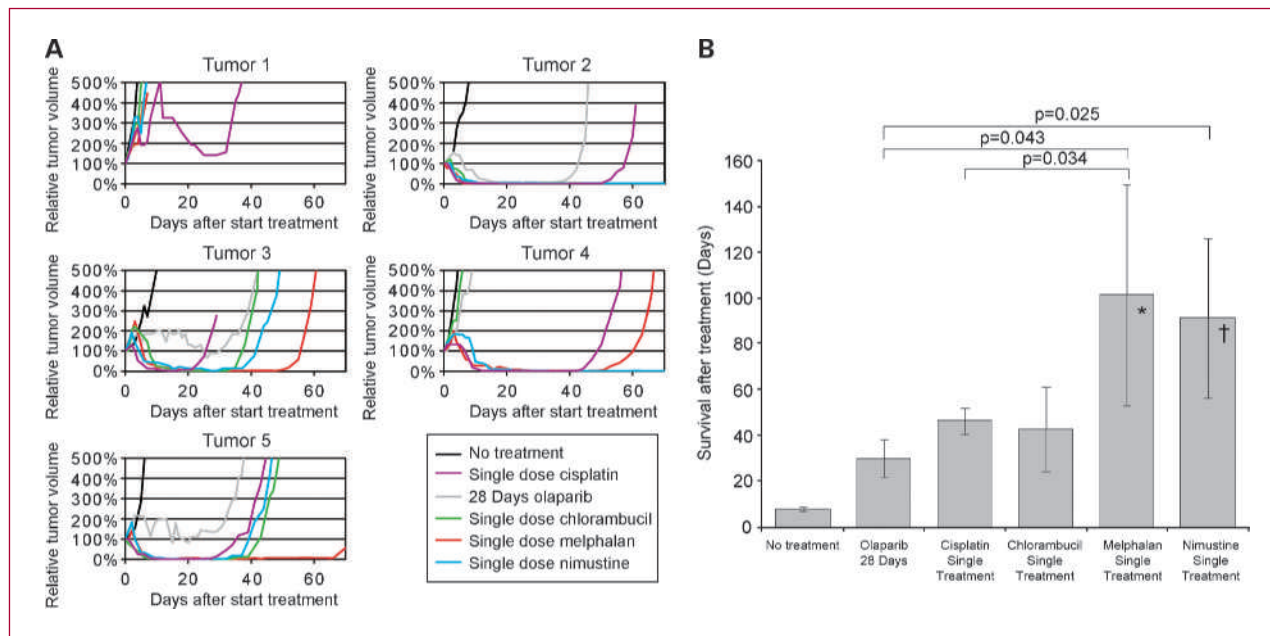
Knowledge of the role of BRCA1 and BRCA2 in DNA damage repair has greatly accumulated in recent years. It can be expected that this knowledge will ultimately translate into treatment protocols that discriminate between sporadic cancers and BRCA-associated or BRCA-like tumors. Indeed, clinical trials with conventional and novel anticancer drugs targeting homologous recombination deficiency (HRD) are currently ongoing (20). Both approaches taken in these clinical studies—inhibition of base excision repair by PARP inhibitors and DNA damage

induction by platinum-based cross-linkers—are thought to induce DSBs in replicating cells (14, 15, 30). It is unknown, however, whether these agents are the most suitable compounds to reach this goal. Ideally, HRD-targeting compounds should generate lesions that are only toxic to HRD cells without inducing DNA damage toxic to normal cells.

To identify novel compounds with specific toxicity towards BRCA2-deficient cells, we have developed a discovery pipeline consisting of isogenic cell lines suitable for high-throughput *in vitro* screening, as well as *in vivo* models for validation studies. We found that three alkylators, which are currently not used in the treatment of breast cancer patients, exert strong differential *in vitro* cytotoxicity against BRCA2-deficient mammary tumor cells versus isogenic BRCA2-proficient control cells. *In vivo* validation studies in mice engrafted with BRCA2-deficient tumor cell lines or primary tumors showed very strong antitumoral activity of all three alkylators especially given the fact that



**Fig. 3.** *In vivo* validation of drug activity on orthotopically transplanted cell lines. FVB × 129/Ola F1 hybrid animals were injected with 500,000 KB2P3.4 cells in the 4th fat pad. When tumor volume exceeded 200 mm<sup>3</sup>, daily treatment for 28 d with olaparib was started, or single-dose injections with cisplatin, chlorambucil, melphalan, or nimustine were given. For each treatment and for untreated controls, transplantations in three mice were done.



**Fig. 4.** *In vivo* validation of drug activity on allografted BRCA2-deficient tumors. **A**, small pieces of five independent mammary tumors from K14cre;Brca2<sup>F11/F11</sup>;p53<sup>F2-10;F2-10</sup> mice were transplanted orthotopically into multiple wild-type recipient mice. When tumor volumes exceeded 200 mm<sup>3</sup>, control animals were left untreated and either 28 consecutive daily olaparib treatments were given, or single-dose injections of cisplatin, chlorambucil, melphalan, or nimustine. **B**, average survival time after treatment is indicated for the complete cohort of five tumors. Error bars, SE. Log-rank *P* values between olaparib and cisplatin versus chlorambucil, melphalan, and nimustine are indicated when <0.05. \*, one animal in the melphalan treatment group was still alive 290 d after the start of treatment. †, two animals in this group were sacrificed at day 143 and 204 due to other health problems (see Discussion).

these compounds were administered only once. In these mouse models, melphalan and nimustine did better than olaparib and cisplatin, although tumor cohorts were relatively small, precluding definitive statistical conclusions. However, these findings may have clinical importance because olaparib and the platinum compound carboplatin are currently in phase II clinical trials.

All three compounds identified are bifunctional alkylating agents, with melphalan and chlorambucil belonging to the class of nitrogen mustards and nimustine being a nitrosourea compound. Bifunctional alkylators create both interstrand and intrastrand cross-links in double-stranded DNA. Platinum compounds such as cisplatin and carboplatin have similar activity but are thought to be different from most bifunctional alkylators because they mainly produce intrastrand cross-links instead of interstrand cross-links (30, 31). Intrastrand lesions are repaired by nucleotide excision repair (NER; ref. 32). Thus, the differential sensitivity of homologous recombination-deficient versus homologous recombination-proficient cells towards platinum compounds may actually be explained by a minority of the lesions produced, with many intrastrand cross-links as "collateral damage." These intrastrand cross-links may be selectively toxic in some HRD tumors, but not in others. Besides its role in homologous recombination, BRCA1 is also involved in NER (33). BRCA1-associated tumors may thus be sensitive to intrastrand cross-linking agents. BRCA2, on the other hand, is not implicated in NER. Although p53 is involved in NER (34) and

many BRCA2 tumors have *TP53* mutations, BRCA2 tumors also exist that have wild-type *TP53* (35). Treating these homologous recombination-deficient but NER-proficient BRCA2-associated tumors with platinum compounds may thus prove to be suboptimal due to the specific spectrum of DNA adducts formed. In addition, resistance mechanisms may differ between platinum compounds and bifunctional alkylators.

The use of melphalan and chlorambucil in the treatment of breast cancer is not entirely new. Especially poorly differentiated tumors have shown a favorable response to single-agent melphalan therapy (36). However, regimens using melphalan or chlorambucil did not do any better than the standard of care combination treatment at the time of cyclophosphamide, methotrexate, and 5-fluorouracil (37, 38), and these alkylators were therefore abandoned as treatment options. Although nimustine is mainly used in Japan for the treatment of malignant glioma, we are not aware of any clinical trials with this compound in breast cancer patients. Related compounds such as BCNU (carmustine) and CCNU (lomustine) have been part of drug combinations used in several clinical trials. Interestingly, carmustine was part of the LOPAC library but did not result in significant toxicity in BRCA2-deficient KB2P3.4 cells, even at 10  $\mu\text{mol/L}$  concentration.

Importantly, early clinical trials with melphalan, chlorambucil, and nitrosoureas have only been carried out in cohorts of breast cancer patients not selected for certain molecular properties. Strong specific response in patients



with HRD tumors may thus have been obscured by the absence of response in the majority of (homologous recombination-proficient) patients. In light of the strong preclinical responses reported in this study, re-evaluation of “classical” alkylators in HRD tumors should be considered. Apart from BRCA-associated hereditary breast cancers, hormone receptor- and HER2-negative sporadic breast tumors (the so-called “triple negative” tumors) are also believed to harbor a considerable fraction of HRD tumors with BRCA-like characteristics (39–41). Although identification of sporadic HRD tumors remains problematic, promising results have been obtained with an array-based comparative genomic hybridization classifier able to identify BRCA1 tumors (42, 43). Recently, this BRCA1-like array-based comparative genomic hybridization pattern was shown to be significantly associated with a prolonged progression-free survival of metastatic breast cancer patients treated with high-dose alkylating chemotherapy with cisplatin, thiotepa, and cyclophosphamide (44).<sup>5</sup>

Our results show that mammary tumor cells are sensitive to cisplatin or the PARP inhibitor olaparib in a BRCA2-dependent fashion, further supporting the rationale for ongoing clinical trials with these compounds. In addition to favorable single-agent activity of olaparib against BRCA2-deficient mammary tumors, we also observe *in vitro* synergy between olaparib and the alkylators, specifically in BRCA2-deficient cells. *In vivo*, we observed very long recurrence-free survival—suggestive of complete tumor eradication—in three of five animals treated with a combination of nimustine and olaparib. Combining olaparib with bifunctional alkylators may thus further increase the clinical window for the treatment of BRCA-deficient cancers.

We introduce here a cell-based screening and validation system for identification and preclinical *in vitro* and *in vivo* validation of compounds with specific toxicity against mammary tumor cells with a defined genetic lesion, i.e., BRCA2 deficiency. Our strategy, based on isogenic

pairs of tumor cell lines, is in principle applicable to other tumor suppressor genes, provided that the reconstitution does not affect proliferation and/or survival of tumor cells. Our results obtained with isogenic BRCA2-deficient and -proficient mammary tumor cell lines call for a re-evaluation of melphalan and nimustine as single agents or in combination with PARP inhibitors, in the clinical management of BRCA2-deficient breast cancer.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### References

- Easton DF. Familial risks of breast cancer. *Breast Cancer Res* 2002; 4:179–81.
- Futreal PA, Liu Q, Shattuck-Eidens D, et al. BRCA1 mutations in primary breast and ovarian carcinomas. *Science* 1994;266:120–2.
- Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66–71.
- Wooster R, Bignell G, Lancaster J, et al. Identification of the breast cancer susceptibility gene BRCA2. *Nature* 1995;378:789–92.
- Tavtigian SV, Simard J, Rommens J, et al. The complete BRCA2 gene and mutations in chromosome 13q-linked kindreds. *Nat Genet* 1996;12:333–7.
- Lux MP, Fasching PA, Beckmann MW. Hereditary breast and ovarian cancer: review and future perspectives. *J Mol Med* 2006;84: 16–28.
- Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. *Mol Cell* 1999;4:511–8.
- Moynahan ME, Pierce AJ, Jasin M. BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol Cell* 2001;7:263–72.
- Xia F, Taghian DG, DeFrank JS, et al. Deficiency of human BRCA2 leads to impaired homologous recombination but maintains normal nonhomologous end joining. *Proc Natl Acad Sci U S A* 2001;98:8644–9.
- Smith SA, Easton DF, Evans DG, Ponder BA. Allele losses in the region 17q12–21 in familial breast and ovarian cancer involve the wild-type chromosome. *Nat Genet* 1992;2:128–31.
- Collins N, McManus R, Wooster R, et al. Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12–13. *Oncogene* 1995;10:1673–5.
- Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR, Bishop DK. The breast cancer susceptibility gene BRCA1 is required for

- subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J Biol Chem* 2000; 275:23899–903.
13. Overkamp WJ, Rooyans MA, Neuteboom I, Telleman P, Arwert F, Zdzienicka MZ. Genetic diversity of mitomycin C-hypersensitive Chinese hamster cell mutants: a new complementation group with chromosomal instability. *Somat Cell Mol Genet* 1993;19:431–7.
  14. Bryant HE, Schultz N, Thomas HD, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 2005;434:913–7.
  15. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 2005;434:917–21.
  16. Evers B, Drost R, Schut E, et al. Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin. *Clin Cancer Res* 2008;14:3916–25.
  17. Rottenberg S, Jaspers JE, Kersbergen A, et al. High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc Natl Acad Sci U S A* 2008;105:17079–84.
  18. Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* 2009;361:123–34.
  19. Kummar S, Kinders R, Gutierrez ME, et al. Phase 0 clinical trial of the poly (ADP-ribose) polymerase inhibitor ABT-888 in patients with advanced malignancies. *J Clin Oncol* 2009;27:2705–11.
  20. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* 2008;8:193–204.
  21. Rottenberg S, Nygren AO, Pajic M, et al. Selective induction of chemotherapy resistance of mammary tumors in a conditional mouse model for hereditary breast cancer. *Proc Natl Acad Sci U S A* 2007;104:12117–22.
  22. Hay T, Matthews JR, Pietzka L, et al. Poly(ADP-ribose) polymerase-1 inhibitor treatment regresses autochthonous Brca2/p53-mutant mammary tumors *in vivo* and delays tumor relapse in combination with carboplatin. *Cancer Res* 2009;69:3850–5.
  23. Wade-Martins R, Smith ER, Tyminski E, Chiocci EA, Saeki Y. An infectious transfer and expression system for genomic DNA loci in human and mouse cells. *Nat Biotechnol* 2001;19:1067–70.
  24. Menear KA, Adcock C, Boulter R, et al. 4-[3-(4-cyclopropanecarbonylpiperazine-1-carbonyl)-4-fluorobenzyl]-2H-phthalazin-1-one: a novel bioavailable inhibitor of poly(ADP-ribose) polymerase-1. *J Med Chem* 2008;51:6581–91.
  25. Wang SC, Shao R, Pao AY, Zhang S, Hung MC, Su LK. Inhibition of cancer cell growth by BRCA2. *Cancer Res* 2002;62:1311–4.
  26. Rahden-Staron I, Szumilo M, Grosicka E, Kraakman van der Zwet M, Zdzienicka MZ. Defective Brca2 influences topoisomerase I activity in mammalian cells. *Acta Biochim Pol* 2003;50:139–44.
  27. Eifel P, Axelson JA, Costa J, et al. National Institutes of Health Consensus Development Conference Statement: adjuvant therapy for breast cancer, November 1–3, 2000. *J Natl Cancer Inst* 2001;93:979–89.
  28. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006;58:621–81.
  29. Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nat Genet* 2001;29:418–25.
  30. McHugh PJ, Spanswick VJ, Hartley JA. Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet Oncol* 2001;2:483–90.
  31. Rice JA, Crothers DM, Pinto AL, Lippard SJ. The major adduct of the antitumor drug cis-diamminedichloroplatinum(II) with DNA bends the duplex by approximately equal to 40 degrees toward the major groove. *Proc Natl Acad Sci U S A* 1988;85:4158–61.
  32. Huang JC, Zamble DB, Reardon JT, Lippard SJ, Sancar A. HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proc Natl Acad Sci U S A* 1994;91:10394–8.
  33. Hartman AR, Ford JM. BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair. *Nat Genet* 2002; 32:180–4.
  34. Ford JM, Hanawalt PC. Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription-coupled repair and enhanced UV resistance. *Proc Natl Acad Sci U S A* 1995;92:8876–80.
  35. Holstege H, Joosse SA, van Oostrom CT, Nederlof PM, de Vries A, Jonkers J. High incidence of protein-truncating TP53 mutations in BRCA1-related breast cancer. *Cancer Res* 2009;69:3625–33.
  36. Fisher B, Fisher ER, Redmond C. Ten-year results from the National Surgical Adjuvant Breast and Bowel Project (NSABP) clinical trial evaluating the use of L-phenylalanine mustard (L-PAM) in the management of primary breast cancer. *J Clin Oncol* 1986;4:929–41.
  37. Muss HB, Cooper MR, Brockschmidt JK, et al. A randomized trial of chemotherapy (L-PAM vs CMF) and irradiation for node positive breast cancer. Eleven year follow-up of a Piedmont Oncology Association trial. *Breast Cancer Res Treat* 1991;19:77–84.
  38. Senn HJ, Maibach R, Castiglione M, et al. Adjuvant chemotherapy in operable breast cancer: cyclophosphamide, methotrexate, and fluorouracil versus chlorambucil, methotrexate, and fluorouracil-11-year results of Swiss Group for Clinical Cancer Research trial SAKK 27/82. *J Clin Oncol* 1997;15:2502–9.
  39. Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. *Nat Rev Cancer* 2004;4:814–9.
  40. Turner NC, Reis-Filho JS, Russell AM, et al. BRCA1 dysfunction in sporadic basal-like breast cancer. *Oncogene* 2007;26:2126–32.
  41. Cleator S, Heller W, Coombes RC. Triple-negative breast cancer: therapeutic options. *Lancet Oncol* 2007;8:235–44.
  42. Wessels LF, van Welsem T, Hart AA, van't Veer LJ, Reinders MJ, Nederlof PM. Molecular classification of breast carcinomas by comparative genomic hybridization: a specific somatic genetic profile for BRCA1 tumors. *Cancer Res* 2002;62:7110–7.
  43. van Beers EH, van Welsem T, Wessels LF, et al. Comparative genomic hybridization profiles in human BRCA1 and BRCA2 breast tumors highlight differential sets of genomic aberrations. *Cancer Res* 2005;65:822–7.
  44. Linn SC, Vollebergh M, Hauptmann M, et al. Selective benefit of high-dose, platinum-containing, alkylating chemotherapy with stem cell rescue for patients with breast cancers with an expansive growth pattern on histology. *J Clin Oncol* 2009;15s:suppl:abstr 587.

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