Published in final edited form as:

Clin Cancer Res. 2010 January 1; 16(1): 99–108. doi:10.1158/1078-0432.CCR-09-2434.

A high throughput pharmaceutical screen identifies compounds with specific toxicity against BRCA2-deficient tumors

Bastiaan Evers¹, Eva Schut¹, Eline van der Burg¹, Tanya M. Braumuller¹, David A. Egan², Henne Holstege¹, Pauline Edser³, David J. Adams⁴, Richard Wade-Martins³, Peter Bouwman¹, and Jos Jonkers^{1,5}

¹Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands ²Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands ³Department of Physiology, Anatomy and Genetics, Le Gros Clark Building, University of Oxford, South Parks Road, Oxford, OX1 3QX, United Kingdom ⁴Department of Experimental Cancer Genetics, The Wellcome Trust Sanger Institute, Hinxton, Cambs, CB10 1SA, United Kingdom

Abstract

Purpose: Hereditary breast cancer is partly explained by germline mutations in BRCA1 and BRCA2. While 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 performed 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 outperforming cisplatin and the poly-(ADP-ribose)-polymerase (PARP) 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 out 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 PARP inhibitors for the treatment of breast cancers with a defective BRCA pathway.

⁵Corresponding author: Jos Jonkers, Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands, Phone: +31 20 5122000, Fax: +31 20 6691383, j.jonkers@nki.nl.

Introduction

Breast cancer is the most common malignancy in women. Approximately 10-15% 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 90's [2-5], are responsible for 15-20% 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 (DSBs) through the process of homologous recombination (HR) [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 inter- and intrastrand cross-links, but processing of these lesions results in intermediates of which the resolution depends on intact HR. 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 (SSB) repair by the base excision repair (BER) 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 HR, *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] and with promising results from phase 0 and phase 1 trials [18, 19], are currently in phase II clinical trials in breast cancer patients [20]. While 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 also *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 latency [17, 22]. Hence, there is a continuing need for new drugs or drug combinations that selectively kill *BRCA*-deficient tumor cells.

In this paper, 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 1258 drugs with known pharmacological 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 to 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 more than 330 days in 3 out of 5 animals tested. Besides offering a platform for the identification of additional compounds for 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

KP3.33, KP6.3, KB2P1.21 and KB2P3.4 mouse mammary tumor cell lines [16] were cultured at 37°C, 5% CO₂, 3% O₂ in complete medium (CM, 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, HSV-1/EBV amplicons containing the complete mouse *Brca2* gene were produced as described previously [23]. 40x Concentrated *Brca2* iBACs were aliquoted and stored at -80°C until use. After overnight infection and subsequent 48hr recovery, *Brca2* iBAC containing KB2P cells were selected with 400µg/ml hygromycin. RT-PCR and RAD51/γH2A.X co-localization experiments to validate functional BRCA2 reconstitution were performed as described [16].

Growth inhibition assays

All single-drug *in vitro* experiments were performed 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 160µl CM on day 0. On day 1, 40µl drug containing CM was added while maintaining DMSO concentrations similar in all wells and <0.4%. On day 5, cells were incubated with 10µl cell-titer blue (Promega) for 4 hours at 37°C. Fluorescence was measured at 590 nm using an Envision plate reader (Perkin Elmer). Values from triplicate measurements were averaged, corrected for background and normalized against values from DMSO only controls. IC50 Values were calculated using Matlab software, as described [16]. All drug interaction experiments were done with SRB assays, since 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

10mM DMSO Stocks of the LOPAC1280 compound library (Sigma) were used to create 5mM and subsequent ten-fold dilutions in DMSO. Cells were seeded on day 0 in 384-well plates in 40µl CM. On day 1, 96-well plates were prepared with 100-fold diluted drugs in CM in columns 2-11. Columns 1 and 12 were used for DMSO-only negative controls and multiple olaparib concentrations as positive controls. Assay plates were supplied with 10µl diluted compound. On day 5, a 4-hour incubation with 20µl 8x diluted cell-titer blue was used to determine cell viability. Screening was done using five 10-fold concentration steps, ranging from 1nM to 10µM. Mean values of technical duplicates were standardized against DMSO-only controls in the same plate and IC50 values were calculated. When IC50 values were >50µM or when the maximum growth inhibition of any range did not exceed 25%, IC50s were arbitrarily set at 50µM.

In vivo validation studies

Chlorambucil (Sigma) was dissolved in 70% EtOH to 50mg/ml. Immediately prior to use, this stock was diluted 40x with 100mM Phosphate buffer pH6.8. Melphalan (Alkeran®, GlaxoSmithKline) was reconstituted immediately before administration, according to manufacturer's protocol. 1 Gram nimustine hydrochloride (Sigma) was dissolved in 6.5ml DMSO and just before injection, diluted 25-fold with physiological salt solution. Cisplatin was obtained from (Mayne Pharma). olaparib was kindly provided by KuDOS Pharmaceuticals [24]. Olaparib, Chlorambucil, Melphalan and nimustine were injected intraperitoneally. Cisplatin was injected intravenously in the tail vein.

Since all cells and tumors were derived from mice with a mixed FVB and 129/Ola background, (FVB × 129/Ola) F1 hybrid females were used for all animal experiments reported in this paper. Maximum tolerable doses (MTDs) were determined using dose escalation in at least three animals for all three alkylators and cisplatin using a 20% weight loss as a toxicity threshold. The following MTDs were established: chlorambucil: 15mg/kg *i.p.*; melphalan: 7.5mg/kg *i.p.*; nimustine: 30mg/kg *i.p.*; cisplatin: 6mg/kg *i.v.*. olaparib was administered daily at 100mg/kg *i.p.*, at which no toxicity was observed, but higher amounts would exceed the maximum of 1ml daily injection volume. For the tumor intervention studies, either 500.000 KB2P3.4 cells, or 1mm³ pieces of 5 independent mammary tumors from K14cre;*Brca2*^{F11/F11}; *p53*^{F2-10;F2-10} animals were engrafted orthotopically in the 4th mammary fat pad of adult F1 hybrid mice as described [21]. Treatment at MTD levels was started once tumor volume, calculated as $0.5 \times \text{length} \times \text{width}^2$, exceeded 200mm³. Since olaparib does not show any toxicity at 100mg/kg and since after 24 hours only about 1/3 of the maximum PARP inhibition remains due to rapid elimination [17], we decided to dose olaparib daily for a period of 28 consecutive days. When the tumor volume was greater than 1500mm³, 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 perform 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 BAC clone encompassing the mouse *Brca2* locus. Both KB2P lines were reconstituted with an HSV-1 based *Brca2* infectious BAC (iBAC) [23], which led to reconstitution of full-length *Brca2* expression as shown by RT-PCR analysis (KB2P3.4R3 and KB2P1.21R2, Figure 1A). Array CGH analysis confirmed the isogenicity of the BRCA2-deficient cell lines and their reconstituted counterparts (Supplementary Figure 1). Rescue of RAD51 colocalization with γ H2A.X in γ -irradiated KB2P3.4R3 and KB2P1.21R2 cells showed that the reconstitution was functional (Figure 1B). To further test the utility of these cell lines for pharmaceutical screens, we performed growth inhibition assays for olaparib and cisplatin on the KB2P, KB2PR and KP cell lines (Figure 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 to KP control cells as

measured by qRT-PCR (data not shown). Since the window of differential sensitivity appeared highest in the KB2P3.4/KB2P3.4R3 combination, we used this isogenic pair of cell lines to perform a high-throughput pharmacological 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 1258 compounds. Ninety-seven drugs had an IC₅₀ <50 μM in any of the cell lines used (Figure 2A). To determine the influence of BRCA2 expression on the IC₅₀ values, a combined specificity index (CSI) was calculated by addition of the log₁₀ differential IC₅₀ values for KB2P3.4 vs. KB2P3.4R3 and KB2P3.4 vs. KP3.33, respectively (see Supplementary Table 1). Carboplatin, which is currently used in clinical trials in BRCA-associated breast cancer patients, was the fifth best hit with an IC₅₀ of 4.4 μM 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 (CONH)) reported an even higher CSI than carboplatin. Except for CONH, 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 (Figure 2B). Immunofluorescent staining revealed clear co-localization of γH2A.X and RAD51 foci after treating KP3.33 cells for 24hrs with the alkylators at IC₅₀ concentrations. 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 (CIs) of two independent experiments were calculated and averaged (Table 1). Synergistic drug interactions, corresponding to CIs of less than -0.15 [28], were found for chlorambucil, melphalan and nimustine in the BRCA2-deficient KB2P1.21 cell line and are comparative to an average cisplatin/olaparib CI 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 CI 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. Since 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 performed *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 200mm³. Alkylators were administered only once, while olaparib was administered daily during 28 consecutive days.

Tumor bearing animals that were left untreated had to be sacrificed after 1-2 weeks, when tumors reached a size of approx. 1500 mm³ (Figure 3). 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 (Figure 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 (Figure 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 vs. 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 5 independent mammary tumors from K14cre;*Brca2*^{F11/F11}; *p53*^{F2-10;F2-10} mice [29] into the mammary gland fat pads of multiple recipients (Figure 4A). When left untreated, mice needed to be sacrificed 1-2 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. While some tumors (Figure 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 (Figure 4A, tumors 3 and 5) or even complete remission before eventual relapse (Figure 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 to olaparib and cisplatin (Figure 4B). Of note, two animals of our nimustine-treated tumor graft cohort needed to be sacrificed before having relapses, approximately 6 months after treatment. Histological 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 vs. melphalan/nimustine and for cisplatin vs. melphalan.

Besides these favorable results, complete cure seems still infrequent. Only one animal, treated with Melphalan, is 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 have treated mice harboring allografts of the same tumors as used in the single agent experiments with combination therapy. Unfortunately, combining single dose Melphalan at MTD with a 28-day olaparib course was associated with increased toxicity, precluding analysis of this combination at dosages comparable to their single agent treatment regimes. However, when

single dose nimustine treatments were combined with 28-days of olaparib treatment, only two out 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 BER 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 these compounds were administered only once. In these mouse models, melphalan and nimustine outperformed olaparib and cisplatin, although tumor cohorts were relatively small, precluding definitive statistical conclusions. However, these findings may have clinical importance since 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 inter- 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) [32]. Thus, the differential sensitivity of HR-deficient vs. HR-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 HR, 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, also BRCA2 tumors exist that have wild-type *TP53* [35]. Treating these HR-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 showed favorable response to single-agent melphalan therapy[36]. However, regimens using melphalan or chlorambucil did not perform any better than the standard of care combination treatment at the time,

cyclophosphamide, methotrexate and 5-fluorouracil (CMF)[37, 38], and these alkylators were therefore abandoned as treatment options. While 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 μ M 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 absence of response in the majority of (HR-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 (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 (aCGH) classifier able to identify BRCA1 tumors ([42, 43]). Recently, this BRCA1-like aCGH 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 (Vollebergh et al, submitted and [44]).

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 3 out of 5 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.

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 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)-

polymeration (PARP) 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 (HRD) sporadic breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank KuDOS Pharmaceuticals, a wholly owned subsidiary of AstraZeneca, for providing olaparib. Dr Y. Saeki and Dr R. M. Sandri-Goldin are kindly acknowledged for providing reagents for iBAC production. This work was supported by grants from the Dutch Cancer Society (2002-2635 to J.J. and A. Berns; 2007-3772 to J.J., S. Rottenberg and J.H.M. Schellens), the Netherlands Organization for Scientific Research (NWO Vidi 917.036.347 to J.J.) and by the Biotechnology and Biological Science Research Council UK (BB/D012910/1 to RWM). D.J.A. is supported by Cancer Research-UK and the Wellcome Trust. Hanneke van der Gulden is acknowledged for her technical assistance. We thank Piet Borst, Michael Hauptmann, Huib Ovaa, Sjoerd Rodenhuis, Boris Rodenko and Marieke Vollebergh for providing useful comments on the manuscript.

Reference List

1. Easton DF. Familial risks of breast cancer. *Breast Cancer Res.* 2002; 4:179–81. [PubMed: 12223120]
2. Futreal PA, Liu Q, Shattuck-Eidens D, et al. BRCA1 mutations in primary breast and ovarian carcinomas. *Science.* 1994; 266:120–2. [PubMed: 7939630]
3. 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. [PubMed: 7545954]
4. Wooster R, Bignell G, Lancaster J, et al. Identification of the breast cancer susceptibility gene BRCA2. *Nature.* 1995; 378:789–92. [PubMed: 8524414]
5. 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. [PubMed: 8589730]
6. Lux MP, Fasching PA, Beckmann MW. Hereditary breast and ovarian cancer: review and future perspectives. *J Mol Med.* 2006; 84:16–28. [PubMed: 16283147]
7. Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. *Mol Cell.* 1999; 4:511–8. [PubMed: 10549283]
8. Moynahan ME, Pierce AJ, Jasin M. BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol Cell.* 2001; 7:263–72. [PubMed: 11239455]
9. 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. [PubMed: 11447276]
10. 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. [PubMed: 1303261]
11. 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. [PubMed: 7731724]
12. 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. [PubMed: 10843985]
13. Overkamp WJ, Rooimans 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. [PubMed: 8291021]

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. [PubMed: 15829966]
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. [PubMed: 15829967]
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. [PubMed: 18559613]
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
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. [PubMed: 19553641]
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. [PubMed: 19364967]
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. [PubMed: 18256616]
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. [PubMed: 17626183]
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. [PubMed: 19383921]
23. Wade-Martins R, Smith ER, Tyminski E, Chiocca EA, Saeki Y. An infectious transfer and expression system for genomic DNA loci in human and mouse cells. *Nat Biotechnol*. 2001; 19:1067–70. [PubMed: 11689853]
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. [PubMed: 18800822]
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. [PubMed: 11888897]
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. [PubMed: 12673354]
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. [PubMed: 11438563]
28. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev*. 2006; 58:621–81. [PubMed: 16968952]
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. [PubMed: 11694875]
30. McHugh PJ, Spanswick VJ, Hartley JA. Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. *Lancet Oncol*. 2001; 2:483–90. [PubMed: 11905724]
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. [PubMed: 3380785]
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. [PubMed: 7937961]
33. Hartman AR, Ford JM. BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair. *Nat Genet*. 2002; 32:180–4. [PubMed: 12195423]
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. [PubMed: 7568035]

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. [PubMed: 19336573]
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. [PubMed: 3519883]
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. [PubMed: 1756271]
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. [PubMed: 9215818]
39. Turner N, Tutt A, Ashworth A. Hallmarks of ‘BRCAness’ in sporadic cancers. *Nat Rev Cancer.* 2004; 4:814–9. [PubMed: 15510162]
40. Turner NC, Reis-Filho JS, Russell AM, et al. BRCA1 dysfunction in sporadic basal-like breast cancer. *Oncogene.* 2007; 26:2126–32. [PubMed: 17016441]
41. Cleator S, Heller W, Coombes RC. Triple-negative breast cancer: therapeutic options. *Lancet Oncol.* 2007; 8:235–44. [PubMed: 17329194]
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. [PubMed: 12460933]
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. [PubMed: 15705879]
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.

Figure 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 iBAC carrying the complete *Brca2* gene to result in KB2P1.21R2 and KB2P3.4R3 lines. **A.** RT-PCR Experiments show that while 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 iBAC. **B.** Co-localization of γ H2A.X (green) with RAD51 (red) in the nucleus (blue) of cells exposed to 20 Gray γ -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 (upper graphs) and cisplatin (lower graphs) clearly show sensitivity associated with BRCA2 deficiency.

Figure 2. A pharmacological screen identifies three alkylators with specific toxicity to BRCA2-deficient cells

A. Scatter-plot depicting differential toxicities of 97 compounds for which IC₅₀s could be determined. On the x-axis, differential toxicity between BRCA2-proficient (KP) and BRCA2-deficient cells is depicted. The y-axis indicates differential toxicity between BRCA2-reconstituted (KB2P) and BRCA2-deficient cells. **B.** Cytotoxicity profiles of chlorambucil, nimustine and melphalan performed on the complete cell-line panel validate the alkylators picked up from the screen. **C.** Immunofluorescence of γH2A.X (green) and RAD51 (red) in the nucleus (blue) of cells exposed for 24 hours to the alkylators at indicated concentrations. Co-localization of γH2A.X and RAD51 indicates induction of recombinogenic lesions.

Figure 3. *In vivo* validation of drug activity on orthotopically transplanted cell-lines

FVB $\times$ 129/Ola F1 Hybrid animals were injected with 500,000 KB2P3.4 cells in the 4th fat pad. When tumor volume exceeded 200mm³, daily treatment for 28-days 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 performed.

Figure 4. In vivo validation of drug activity on allografted BRCA2-deficient tumors

A. Small pieces of 5 independent mammary tumors from K14cre;*Brca2*^{F11/F11}; *p53*^{F2-10;F2-10} mice were transplanted orthotopically into multiple wild-type recipient mice. When tumor volumes exceeded 200mm³, 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 5 tumors with error bars indicating s.e.m. Log-rank p-values between olaparib and cisplatin vs. chlorambucil, melphalan and nimustine are indicated when <0.05. *One animal in the melphalan treatment group was still alive 290 days after the start of treatment. †Two animals in this group were sacrificed at day 143 and 204 due to other health problems (see Discussion).

Table 1

Drug interactions between olaparib and alkylators

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.

	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	0.02
	Melphalan	-0.05	-0.07	-0.14	-0.08	-0.06
	Ninustine	-0.05	-0.02	-0.22	-0.07	-0.06