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Chapter 4

Novel compounds with specific toxicity against BRCA2-deficient tumor cells

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Novel compounds with specific toxicity against BRCA2 deficient tumor cells

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Breast cancer is a devastating disease, affecting millions of women worldwide every year. Hereditary breast cancer is explained in large by germline mutations in the BRCA1 and BRCA2 genes, which encode proteins involved in the repair of double strand DNA breaks. Targeting the repair defect of BRCA-mutated and sporadic homologous recombination deficient (HRD) tumors by induction of DNA damage is the strategy of several therapeutics which are currently being tested in clinical trials. Since resistance mechanisms are likely to develop, however, there remains a need for alternative compounds specifically targeting HRD tumor cells. We describe a high-throughput pharmacological screen, using 22,720 structurally diverse compounds, to identify novel compounds with specific toxicity against BRCA2-deficient mammary tumor cells. Two novel compounds were identified and validated to exert strong growth inhibition on BRCA2-deficient cells. While this sensitivity is not unambiguously restored upon re-expression of BRCA2, comparison with ‘normal-like’ HC11 cells supports the existence of a strong clinical window for these compounds. Both compounds are shown to induce DNA damage, but not through inhibition of poly-(ADP-ribose) polymerase activity. The exact mechanisms of toxicity remain a topic of further investigation.

Introduction

Nearly 1% of all women die of breast cancer, the most common malignancy in females worldwide (WHO World Cancer Report 2003⁵). Apart from environmental factors, family history strongly influences susceptibility to mammary tumor development. Linkage analysis has now identified several genes to confer susceptibility to breast cancer. Mutations in CHK2, ATM, PALB2 and BRIP1 only marginally increase the chance to developing breast cancer, while mutations in BRCA1, BRCA2, TP53, PTEN, STK11 and CDH1 are associated with a much higher risk⁶. The most prevalent high-penetrance breast cancer genes, BRCA1 and BRCA2, are both implicated in the faithful repair of damaged DNA⁷,⁸. While in recent years BRCA1 has been assigned many different functions, the role of BRCA2 seems much more defined⁹. The best understood role of BRCA2 involves regulation of homology directed repair (HDR) through recruitment of the RAD51 recombinase to sites of double strand break (DSB) DNA damage⁶,⁷. Absence of BRCA2 impairs DSB repair through homologous recombination (HR), leading to genomic instability and increased sensitivity to DNA damage inducing agents⁵. This is the basis of current clinical trials with agents such as platinum containing drugs, which directly generate DNA lesions, and inhibitors of poly-(ADP-ribose)-polymerase (PARP) activity, which generate DSBs after conversion of single-
strand breaks normally repaired by PARP. In addition, we have recently shown strong and specific activity of three bifunctional alkylators against BRCA2 deficient mouse mammary tumors (Evers et al., submitted). However, intrinsic and acquired resistance remains problematic. Even combinations of alkylators and PARP inhibitors failed to eradicate tumors (Evers et al., submitted).

It is currently unknown whether BRCA2 has other functions in addition to its reported role in HDR, although the protein has been implicated in processes such as transcriptional regulation and cytokinesis. Uncovering additional functions may be of key importance to finding additional therapeutic targets in BRCA-mutated tumors.

To uncover possible additional functions of BRCA2 and to search for additional treatment strategies, we performed a high-throughput screen (HTS) on our previously isolated and characterized isogenic pair of BRCA2-deficient and –proficient cell lines (Evers et al., submitted) (Figure 1). To this aim, we used a diverse chemical library consisting of 22,720 compounds adhering to Lipinski’s “Rule of Five” for drug-like properties and constituting a large structural variation. After pre-selection of compounds inducing significant toxicity at 5µM, we determined IC50 values of the 640 most cytotoxic compounds in both BRCA2-deficient and isogenic BRCA2-proficient cells. Three compounds with strong activity against BRCA2-deficient mammary tumor cells were identified, two of which could be validated in follow-up experiments. Both validated compounds induced DSBs as evidenced by the formation of nuclear foci positive for γH2A.X. To test the ability of these compounds to inhibit PARP activity, we have performed a PAR-ylation assay, showing that these compounds form DSBs through a different mechanism than inhibition of PARP. Additional experiments will be required to elucidate the exact mechanism of action of these compounds and to determine whether they are suitable candidates for further drug development.

**Figure 1 - Overview of the screening strategy.** Using BRCA2-deficient KB2P3.4 cells, a pre-selection of toxic compounds was made by testing the growth inhibition of 22,720 compounds at 5µM concentration. Of these, 403 were shown to have significant toxicity. Next, the 640 most cytotoxic compounds were assayed for growth inhibition at several concentrations to determine IC50s on both the KB2P3.4 cells and their reconstituted counterparts, KB2P3.4R3. Examples of compounds with (wells encircled with blue) or without (wells encircled with red) differential toxicity are indicated, along with their IC50 curves. Yellow wells represent growing cells, pink wells represent cells that suffered growth inhibition.

**Materials and methods**

**Cell lines and growth inhibition assays**

KP3.33, KP6.3, KB2P1.21, KB2P1.21R2, KB2P3.4 and KB2P3.4R3 mouse mammary tumor cell lines (Evers et al., submitted) 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 strep-
tomycin (Life Technologies), 5 μg/ml insulin (Sigma), 5 ng/ml epidermal growth factor (Life Technologies) and 5 ng/ml cholera toxin (Gentaur).

High-throughput growth inhibition assay
Growth inhibition assays were performed in high-throughput format using a proprietary library containing a diverse set of 22,720 drug-like small molecules, obtained from SPECS (Delft, The Netherlands). This library was screened at 5μM final concentration against BRCA2 deficient cell line KB2P3.4 using a CellTiter-Blue® viability assay, as described (Evers et al., submitted). For a selection of 640 of the most cytotoxic compounds IC50 values were determined on cell lines KB2P3.4 and KB2P3.4R3 by generating dose-response curves of 5-fold serial dilutions covering a range of 5μM to 8nM using the CellTiter-Blue® viability assay. Data were analyzed by using Matlab software (The Mathworks, MA).

Compounds
Screening hits α, β and γ were re-ordered from SPECS (Delft, The Netherlands) and used for subsequent validation experiments. Compound identity was confirmed by LC/MS analysis (data not shown). Cisplatin was obtained from Mayne Pharma. Olaparib (AZD2281) was kindly provided by KuDOS Pharmaceuticals.

DNA Damage induction assays
Staining KP3.33 cells grown on cover slips during 24 hours in the presence of different concentrations α, γ or cisplatin in order to determine γH2A.X nuclear focus formation was performed as described. Inhibition of poly-(ADP-ribose)-ylation activity was performed using the HT universal chemiluminescent PARP assay kit, according to the guidelines of the manufacturer (Trevigen, MD).

Results
High-throughput pharmacological screen
To identify novel drugs with specific activity against BRCA2-deficient tumor cells, we chose to screen a chemical library of 22,720 compounds on isogenic pairs of BRCA2-deficient and –proficient mammary tumor cell lines. For this, we used clonal BRCA2-deficient mouse mammary tumor cell lines (KB2P1.21 and KB2P3.4) and BRCA2-proficient isogenic control cell lines (KB2P1.21R2 and KB2P3.4R3), produced by reconstitution of KB2P1.21 and KB2P3.4 cells with a BAC clone encompassing the entire mouse Brca2 locus (Evers et al., submitted). Since determining IC50 values for 22,720 compounds would be extremely laborious, we decided to preselect compounds with significant toxicity in the BRCA2 deficient cell line KB2P3.4 at 5μM concentration. This represents the highest testable concentration of library compounds, without suffering from DMSO toxicity. Growth inhibition compared to DMSO controls was measured for all compounds in the presence of 100nM of the PARP inhibitor Olaparib (AZD2281)13.
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To correct for systemic row and column effects and to be able to compare results from different runs, B-Scores were calculated. A total of 403 compounds reported significant toxicity with a multiple-testing corrected P-value threshold of 0.01, corresponding to B-Score values lower than -5.05 (Figure 2). For the 640 most cytotoxic drugs, IC50 values were next determined in the BRCA2-deficient KB2P3.4 cells and in BRCA2-proficient KB2P3.4R3 isogenic control cells, using a cell-titer blue assay on cells exposed to 5µM, 1µM, 200nM, 40nM and 8nM of compound (Figure 3). When growth inhibition in either of the cell lines was less than 25%, IC50 values for that cell line were fixed at 10µM. Thus, IC50 values could be determined for 215 compounds in both cell lines (Figure 3, blue dots), for 182 compounds in only one of the cell lines (Figure 3, green dots) and for 243 compounds in neither of the cell lines (Figure 3, red dot).

**Figure 3 - Differential screen.**
IC50 Values of the 640 most toxic drugs identified in the pre-screen were determined as described for KB2P3.4 and KB2P3.4R3 cells. Red lines delineate the maximum drug concentrations used in the screen. The blue diagonal indicates equal toxicity in both cell lines. Individual dots represent drugs with an IC50 determined in both cell lines (blue), either one of the cell lines (green), or in neither of the lines (red). In those cases where an IC50 could not be determined, IC50 values were arbitrarily set at 10µM, corresponding to a log10 value of -5. Encircled are those compounds with a greater than 10 differential IC50.

Strong and selective growth inhibition in BRCA2 deficient cells was observed for the poly-(ADP-ribose) polymerase (PARP) inhibitor Olaparib and cisplatin (Figure 4A-B), consistent with our previous work (12 and Evers et al., submitted). For compound α, the strong differential response between KB2P3.4 and KB2P3.4R3 was recapitulated and also KB2P1.21 cells were found to be very sensitive (Figure 4C). However, reconstitution of KB2P1.21 cells with BRCA2 had only a minor effect on their sensitivity to α. Similar effects were observed for compound γ, although differences in sensitivity are in general smaller (Figure 4E), consistent with the results from the primary screen. Solubility problems with compound β prevented testing at concentrations higher than 20µM and thus precluded precise determination of IC50 values. Nevertheless, at 20µM concentrations growth inhibition by β is around 50% in both BRCA2-deficient cell lines, whereas growth of BRCA2-proficient lines is hardly inhibited (Figure 4D).

**In vitro validation**
For further validation we selected three compounds with IC50 values differing more than a factor 10 between BRCA2-deficient and reconstituted cells (Figure 3, indicated with α, β and γ). Growth inhibition assays were performed on a more extensive panel of mouse mammary tumor cell lines, consisting of BRCA2-proficient lines KP3.33 and KP6.3, BRCA2-deficient lines KB2P1.21 and KB2P3.4, and the BRCA2-reconstituted isogenic control lines KB2P1.21R2 and KB2P3.4R3. In addition, HC11 cells were used which are derived from normal mammary epithelial tissue and therefore represent a close approximation of wild-type primary mammary epithelial cells.
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Figure 4 - Validation of α, β and γ.
Growth inhibition profiles are shown for the PARP inhibitor Olaparib (A), the DNA damage inducing drug cisplatin (B) and screen compounds α (C), β (D) and γ (E).

Figure 5 - α and γ induce DNA damage.
After 24 hour incubation in 10nM, 100nM or 1μM of cisplatin, α or γ, KP3.33 cells were fixed and stained for γH2AX (green) and DNA (blue). Induction of DNA double-strand breaks is visible at 100nM concentrations of all drugs.
Compounds α and γ induce DNA damage but do not inhibit PARP

The best known function of BRCA2 is its role in DSB repair. Indeed, in a pharmacological screen of 1258 pharmacologically active compounds in BRCA2-deficient and -proficient isogenic cells, we previously identified three DNA alkylation agents as compounds with the strongest differential cytotoxicity (Evers et al., submitted). Therefore, the capacity of compounds α and γ to induce DSBs was assessed at different concentrations of compound by measuring the amount of phospho-H2A.X positive nuclear foci (Figure 5). Indeed, 24-hour incubation with compounds α and γ induced nuclear phospho-H2A.X foci at 100nM concentrations, although for cisplatin and compound α, this was more pronounced at 1µM.

PARP enzymatic activity is necessary for properly resolving DNA single-strand breaks (SSBs), which is a frequently occurring endogenous type of DNA damage. When PARP enzymatic activity is inhibited, SSBs are thought to be converted to replication-associated DSBs, marked by the induction of phosphorylated H2A.X foci. Therefore, we sought to assess the PARP inhibitory capacity of compounds α, β and γ. Using an in vitro poly-(ADP-ribose)-ylation assay, we found that, although activity of recombinant PARP is almost fully inhibited by 1nM Olaparib, no PARP inhibitory effect could be measured for α, β or γ at 10µM (Figure 6). We thus conclude that the strong toxicity of α and γ against BRCA2-deficient cells is not due to PARP inhibition.

Discussion

In order to identify novel compounds with specific toxicity against BRCA2-deficient cells, we performed a high-throughput small molecule screen using BRCA2-deficient KB2P3.4 and isogenic reconstituted KB2P3.4R3 cells. To reduce the number of candidates, a pre-screen was performed to select compounds with significant toxicity at 5µM, the highest concentration to be used in the follow-up screen determining the differential IC50 values.

Our pre-screen selection strategy was validated by comparing the results from the first screen with the IC50 values determined in the differential screen. From the 403 compounds determined to be cytotoxic in the pre-screen, 275 (68%) gave IC50 values <10µM suggesting that our pre-screen criteria were very sensitive. On the other hand, from the 237 compounds screened with low, but not significant, pre-screen B-values, only 23 (9.7%) resulted in an IC50 value of <10µM, indicating that our pre-screen criteria were quite selective. Together, we conclude that our pre-screen effectively identified the most interesting subset of compounds for a differential screen.

In the differential screen, the majority of compounds reported rather similar IC50 values for both cell lines, indicating that our BRCA2-deficient cell lines are not per se more sensitive to the effect of drugs.
results were obtained in a previous screen with a library of 1258 pharmacologically active compounds (Evers et al., submitted). Nevertheless, a significant amount of drugs reported measurable IC\textsubscript{50} values in the BRCA2-deficient KB2P3.4 cells, whilst not meeting IC\textsubscript{50} value determination criteria in the BRCA2-reconstituted KB2P3.4R3 cells. The exact significance of this observation is hard to determine, however, because limitations in compound solubility precluded cytotoxicity studies at higher drug concentrations.

Three compounds with strong differential cytotoxicity, designated α, β and γ, were selected for follow-up experiments. Differential sensitivity between KB2P3.4 and KB2P3.4R3 cells was clearly maintained when more precise IC\textsubscript{50} values were determined on a panel of 3 BRCA2 wild-type cell lines and 2 pairs of isogenic BRCA2-deficient and BRCA2-reconstituted mammary tumor cell lines. However, some IC\textsubscript{50} values were determined to be quite different from the screening results (e.g. for α in KB2P3.4 cells, and for β in both KB2P3.4 and KB2P3.4R3 cells). Follow-up experiments using different cytotoxicity assays should therefore be performed to more precisely determine IC\textsubscript{50} values.

In addition, while both pairs of BRCA2-deficient and -proficient cell lines reported quite similarly in response to Olaparib or cisplatin, more heterogeneity was observed for compounds α and γ. In both cases higher sensitivity was observed in KB2P1.21 cells compared to KB2P3.4 cells, and reconstitution of BRCA2 expression in KB2P1.21 cells only partially restored resistance to α and γ. This could indicate that KB2P1.21 cells contain additional mutations that further sensitize these cells for α and γ. This hypothesis is supported by the relative resistance of ‘normal-like’ HC11 cells to both compounds. Measuring differential toxicity of α and γ in HC11 cells with or without knock-down of BRCA2 should shed more light on the specific toxicity of these compounds against BRCA2-deficient cells.

Using immunohistochemical staining against phosphorylated H2A.X, we show that sub-lethal concentrations of α and γ induce DSBs in KP3.33 cells. This damage is unlikely the result of PARP inhibition as shown by an \textit{in vitro} poly-(ADP-ribose)-ylation assay. Although both α and γ induced nuclear phospho-H2A.X focus formation, γ appeared to induce fewer but larger foci than α. Whether this difference reflects different types of DNA damage remains the subject of further investigation.

Since the best known function of BRCA2 is to resolve DSB damage, and since this type of damage appears to be induced by α and γ, it seems likely that both compounds confer toxicity to BRCA2-deficient cells through induction of DSBs. DSB damage, however, can be induced in many different ways, \textit{e.g.} by inhibition of topo-isomerase activity, by alkylating activity or by inhibition of PARP activity. Because we have ruled out PARP-inhibitory activity, and because the chemical structures do not indicate alkylating activity, it will be important to perform topo-isomerase inhibition and DNA intercalation assays with compounds α and γ. Compared to cisplatin, alkylators and Olaparib, compounds α and γ were found to display a different cytotoxicity profile across our panel of cell lines. This might indicate that, compared to other chemotherapeutics, α and γ induce DSBs by distinct mechanisms. Since this may correspond with different toxicity and resistance mechanisms, further investigations on α and γ are certainly worthwhile.
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In conclusion, we report two novel compounds with strong cytotoxicity against BRCA2-deficient mammary tumor cells. Both compounds appear to induce DNA damage without inhibiting PARP activity. However, their mechanisms of action as well as their precise specificity towards BRCA2 deficiency remain to be determined.

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