Cancer Therapy: Preclinical

Development of a Functional Assay for Homologous Recombination Status in Primary Cultures of Epithelial Ovarian Tumor and Correlation with Sensitivity to Poly(ADP-Ribose) Polymerase Inhibitors

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Abstract

Purpose: Poly(ADP-ribose) polymerase (PARP) inhibitors selectively target homologous recombination (HR)–defective cells and show good clinical activity in hereditary breast and ovarian cancer associated with BRCA1 or BRCA2 mutations. A high proportion (up to 50%) of sporadic epithelial ovarian cancers (EOC) could be deficient in HR due to genetic or epigenetic inactivation of BRCA1/BRCA2 or other HR genes. Therefore, there is a potential for extending the use of PARP inhibitors to these patients if HR status can be identified. We developed a functional assay of HR status in primary cultures of EOCs based on Rad51 focus formation that correlates well with sensitivity to the potent PARP inhibitor AG014699.

Experimental Design: Primary cultures were derived from ascitic fluid from patients with EOCs. HR status was investigated by γH2AX and Rad51 focus formation by immunofluorescence. Cytotoxicity to PARP inhibitors was tested by sulforhodamine B and survival assay.

Results: Twenty-five cultures were evaluated for HR status and cytotoxicity to PARP inhibitor. Following exposure to AG014699, there was an increase in Rad51 foci (HR competent) in 9 of 24 (36%) but no increase (HR deficient) in 16 of 24 (64%) cultures. Cytotoxicity was observed in 15 of 16 (93%) HR-deficient samples but not in 9 of 9 HR-competent samples following 24-hour exposure to 10 μmol/L AG014699.

Conclusion: HR status can be determined in primary cancer samples by Rad51 focus formation, and this correlates with in vitro response to PARP inhibition. Use of this assay as a biomarker now needs testing in the setting of a clinical trial. Clin Cancer Res; 16(8); 2344-51. ©2010 AACR.
Poly(ADP-ribose) polymerase inhibitors are emerging as an exciting class of novel chemotherapeutic agents. Their use is currently restricted to tumors known to be deficient in homologous recombination as a result of mutations of the BRCA genes. In this study, we propose that an assay of homologous recombination status in tumors may be of use in predicting response to poly(ADP-ribose) polymerase inhibition, thus potentially widening the use of these drugs.

**Translational Relevance**

Poly(ADP-ribose) polymerase inhibitors are emerging as an exciting class of novel chemotherapeutic agents. Their use is currently restricted to tumors known to be deficient in homologous recombination as a result of mutations of the BRCA genes. In this study, we propose that an assay of homologous recombination status in tumors may be of use in predicting response to poly(ADP-ribose) polymerase inhibition, thus potentially widening the use of these drugs.

**Materials and Methods**

AG014699 (Pfizer Oncology), a potent inhibitor of PARP-1 and PARP-2 (K<sub>i</sub> of <5 nmol/L), was dissolved in DMSO and stored at −20°C for in vitro studies.

Cell lines. The following cell lines were used: OSEC1-immortalized (BRCA2 heterozygote) and OSEC2-immortalized (BRCA wild-type) human ovarian surface epithelial cells (15).

AA8 and IRS-1SF (XRCC3-deficient Chinese hamster ovarian cells) were kindly provided by Prof. Penny Leggo (Sussex University, Brighton, United Kingdom). VC8 (BRCA2-mutant Chinese hamster lung fibroblast) and VC8-B2 (BRCA2 reconstitut) were kindly provided by Prof. Malgorzata Z. Zdzienicka (Leiden University, Leiden, the Netherlands).

OSEC1, OSEC2, AA8, IRS-1SF, VC8, and VC8-B2 cell lines were grown in RPMI 1640 supplemented with 20% fetal bovine serum. All cells were confirmed as Mycoplasma-free by regular testing (Mycoplasma Detection kit, Lonza).

**Development of primary cultures.** Ethical approval and specific consent were obtained for collection of clinical material. Primary cultures were obtained from ascitic/plural fluid from patients undergoing primary surgery for ovarian cancer at the Northern Gynaecological Oncology Centre, Queen Elizabeth Hospital. None of the patients had been screened for BRCA mutations or selected on the basis of a family history of breast or ovarian cancer. From the operating theatre, clinical material was transported to the laboratory immediately. Tissue handling was carried out in accordance with human tissue act (UK) regulations and local guidelines.

For primary cultures, 20 mL of ascites were added to 20 mL of warmed medium (RPMI 1640 supplemented with 20% fetal bovine serum, 20 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μL/mL streptomycin) in a 75-cm<sup>2</sup> flask and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air (16). All experiments were carried out on early-passage (up to two passages) cells and medium was replaced every 48 to 72 h.

**Cytokeratin staining.** Direct immunofluorescence using a FITC-conjugated anti-cytokeratin antibody (MNF116; Dako) and 4′,6-diamidino-2-phenylindole to visualize nuclei was used to characterize EOC cells. Cultures containing >95% cytokeratin-positive cells were considered to be epithelial and used in further assays.

**γH2AX/Rad51 immunofluorescence.** A key component in DNA repair is the histone protein H2AX, which becomes rapidly phosphorylated to form large numbers of γH2AX at nascent DSB, creating a focus where proteins involved in DNA repair and chromatin remodeling accumulate. This amplification makes it possible to detect DSBs with an antibody to γH2AX, with the number of DSBs estimated from the number of foci (17, 18). Rad51 is a crucial downstream protein involved in HR repair, which is relocalized within the nucleus in response to DNA damage to form distinct foci that can be visualized by immunofluorescent microscopy and are thought to represent assemblies of proteins at these sites of HR repair. Therefore, quantification of Rad51 could serve as a marker of HR function to distinguish between HR-proficient and HR-deficient cell lines (19).

Cells were seeded on coverslips in six-well plates and incubated in 2 mL medium containing 10 μmol/L AG014699 for 24 h. Coverslips were washed twice with ice-cold PBS, fixed with methanol at −20°C for 10 min, rehydrated with two changes of PBS for 20 min, and transferred to covered 90-mm Petri dishes. Cells were permeabilized with 150 to 200 μL of blocking buffer (KGM buffer: 120 mmol/L KCl, 20 mmol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA plus 0.1% Triton X-100, 2% bovine serum albumin, 10% milk powder, and 10% normal goat serum) and incubated with primary anti–phospho-histone H2AX (Ser<sup>139</sup>) antibody (clone JBW301, mouse monoclonal antibody; Upstate, Millipore Corp.) diluted in blocking buffer (1:200) at room temperature for 1 h.
After washing the coverslips (four times; 0.1% Triton X-100 in KCM buffer), primary antibody for Rad51 (anti-Rad51 rabbit polyclonal antibody; Calbiochem, EMD Biosciences, Inc.) at 1:200 dilution in blocking buffer + 0.1% Triton X-100, four times, 5 min per wash, was used after each change of antibodies. Coverslips were mounted on slides using 4′,6-diamidino-2-phenylindole (Vectorshiel Hard Set, Vector Laboratories, Inc.) Slides were air dried at room temperature before storing at 4°C. Images were obtained using a Leica DMR microscope and RT SE6 Slider Camera Advanced Spot software version 3.408 (Diagnostic Instruments, Inc.).

Counting foci. Both manual counting and automated analysis (Image), version 1.42, available from Research Services Branch of NIH) of γH2AX/Rad51 foci were carried out. The average number of foci per cell was expressed as percentage of untreated controls. When assessed manually, γH2AX and Rad51 foci were quantified in 30 nuclei from three different fields of each coverslip. Reliability, reproducibility, and validity of our data were confirmed by repeated tests across different fields, comparison of two counting methods (manual and software), and counting by two independent reviewers.

Clonogenic cell survival assays to determine sensitivity to AG014699. Exponentially growing cells were seeded into six-well plates and incubated for 24 to 48 h to allow optimum growth before exposure to medium containing 0, 1, 10, or 50 μmol/L AG014699 for 24 h. Following harvesting, cells were seeded in drug-free medium into 90-mm Petri dishes (2,500/5,000/10,000 per dish). Cells were fixed (methanol/acetic acid, 3:1) and stained by methyl violet 10B after 14 to 21 d. In cases where distinct colonies were not formed, cells in monolayer were counted across three fields by three independent reviewers and percentage cell survival was calculated in relation to untreated controls.

Sulforhodamine B assay. Cytotoxicity was also determined using sulforhodamine B (SRB) assays to provide a direct comparison with the clonogenic survival. Cells were seeded into 96-well plates (1,000-2,000 per well), allowed to attach overnight, and then incubated in medium containing 0.0, 0.3, 1.0, 10, 30, or 100 μmol/L AG014699. Cells were fixed with 50% (w/v) trichloroacetic acid after the controls reached 80% to 90% confluence and stained with SRB as previously described (20). Absorbance was measured at 570 nm using SpectraMax 250 Microplate Spectrophotometer System.

Statistical analyses. Correlation and k statistics were calculated to measure interobserver variability. Two-tailed P values were generated using SPSS (version 15) statistical software.

Results

Establishing primary culture of EOC. A 90% success rate was obtained in developing primary cultures from ascitic/plerual fluid in patients with EOC. Primary cultures were named as primary culture ovary (PCO) followed by the number of collection. Serous and papillary serous adenocarcinomas were the predominant histologic subtypes (44%).

The most commonly observed cellular pattern was a monolayer of cells, which were cobblestone in appearance (Supplementary Fig. A), as described by Dunfield et al. (21). Late-passage cells developed a much more mesenchymal phenotype, becoming more elongated. Moreover, as the cells were passaged further, the cultures became senescent, exhibiting a markedly reduced growth rate. Cultures varied in their growth potential: the first passage was carried out between 5 and 30 days of collection. Senescence occurred between the second and eighth passages, most commonly between fourth and fifth. Eight of 45 (17%) cultures were rejected for lack of cytokeratin expression (Supplementary Fig. B). By directly plating ascitic fluid onto coverslips in equal proportion to full medium, it was possible to test for HR status in cultures that had a particularly slow growth rate or became senescent after being passaged only once (Supplementary Fig. C).

γH2AX/Rad51 assay to assess HR status in cell lines and primary cultures. The basis of this assay is to induce
DNA damage to produce DSB and stalled/collapsed replication forks that specifically require repair by HR. Therefore, quantification of this damage and its repair by HR-specific proteins could provide a basis for a test for HR function.

Validation of γH2AX/Rad51 foci formation in cell lines with known HR status. Treatment with 10 μmol/L AG014699 for 24 hours resulted in an increase in γH2AX foci in all cell lines showing DNA damage accumulation (Fig. 1). The HR-competent cell lines (OSEC1, OSEC2, AA8, and VC8-B2) all showed at least 2-fold increase in Rad51 foci in response to AG014699 over the untreated controls. In comparison, HR-deficient cell lines (IRS-1SF and VC8) did not show an increase in Rad51 foci formation (Fig. 1).

HR status in primary cultures. Twenty-five cultures were assessed for both HR status and cytotoxicity to PARP inhibitor. There was an increase in γH2AX foci in all cultures treated with AG014699, confirming DNA damage (data not shown). Rad51 foci increased following treatment by ~2-fold compared with controls in 9 of 25 primary cultures (PCO 44/51/61/63/64/66/69/72/76), which were therefore deemed to be HR competent (Figs. 2A and 3A). In the remaining 16 cultures, there was no increase or even a decrease in Rad51 foci in response to AG014699, and therefore, they were deemed to be HR deficient (Fig. 2A). Baseline Rad51 did not seem to affect the outcome of induction with AG014699.

There was good correlation in Rad51 foci counting between two reviewers (Pearson’s correlation coefficient = 0.941; two-tailed P < 0.01) and good agreement in prediction of HR status (κ = 1.00; P = 0.002).

Cytotoxicity assay in primary cultures. Results from our previous work showed that <50% survival in the presence of 10 μmol/L AG014699 seems to distinguish HR-deficient from HR-competent cell lines (14). The cultures predicted to be HR competent on the basis of Rad51 focus formation remained viable in the presence of 10 μmol/L AG014699 by both clonogenic and SRB assays (positive predictive value = 100%; Fig. 2B; Table 1).
In contrast, there was an \( \sim 2 \)-fold reduction in cell survival (median survival of 47\%) by at least one cytotoxicity assay in 15 of 16 (positive predictive value = 93\%) primary cultures predicted to be HR deficient (Fig. 2B; Table 1). Although it was difficult to visualize discrete colonies in all the cultures by clonogenic assay, the cell density and morphology after treatment with 10 \( \mu \)mol/L AG014699 were distinctly different between the HR-competent and the HR-deficient cultures (Fig. 3B). There was a good correlation between three reviewers in calculating percentage survival of cells in monolayers (Pearson’s correlation, two-tailed significance \( P < 0.01 \)).

### Table 1. Combined HR status and cytotoxicity to AG014699 (n = 25)

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<th>HR+ (n = 9)</th>
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<td>Cytotoxicity−</td>
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Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

**Discussion**

We have shown that irrespective of BRCA mutation status, EOCs can be classified into two groups based on their ability to form Rad51 foci in response to DNA damage,
suggesting that these cells are either HR competent or HR deficient (Fig. 4A). This test is relatively simple and can be carried out in a time frame compatible with its use as a biomarker to direct subsequent therapy. Although it could be considered premature to extrapolate these data obtained from 25 cultures that had parallel cytotoxicity assessed, it seems that ~60% of EOCs might have a defect in HR function, but this proportion is consistent with previous estimates of HR dysfunction in ovarian cancer (11). The high proportion of HR-deficient cultures found in our series could also be due to selection bias, as the majority of our samples from ascitic fluid come from patients with advanced-stage and high-grade epithelial cancers and will tend to bias toward serous tumors, the subtype known to be most responsive to platinum chemotherapy. It has been shown that BRCA1/BRCA2-deficient cancer cells are hypersensitive to DNA cross-linking agents, including platinum (22). It is therefore likely that a high proportion of HR deficiency in these subsets of cancers attributing to a BRCA-ness phenotype could explain the platinum sensitivity as well.

The current study has been restricted to primary cultures derived from ascitic fluid from patients with ovarian cancer. This provides predominantly homogeneous cultures with minimal stromal contamination. Future work will need to consider the possibility of carrying out this assay in primary cultures derived from solid tissue.

About the determination of HR status based on increase in Rad51 foci after DNA damage, the findings are consistent both in cell lines and in primary cultures. In concordance with the data obtained in cell line models (14), 10 μmol/L AG014699 seemed to discriminate between HR-competent and HR-deficient cultures by both cytotoxicity assays (Fig. 4A and B). EOC cells that showed a 2-fold induction of Rad51 in response to AG014699 remained viable in the presence of 10 μmol/L AG014699 (Fig. 2A and B). These data suggest that the Rad51 assay is a good indicator of HR function and sensitivity to PARP inhibition, and further support the proposed mechanism underlying this sensitivity (Fig. 5; refs. 23, 24).

The only inconsistent result obtained in our experiment was the case of PCO 65, which seemed to be HR deficient by the immunofluorescence assay yet showed no cytotoxicity (90% cell survival). Probable explanations could be either a false-negative HR assay result or a resistance to PARP inhibition. Resistance to PARP inhibitors has been described in BRCA-deficient tumors due to secondary intragenic mutations (25–27). A false-negative assay result could be attributed to slow-growing cells not in S phase. In addition, the problem encountered while repeating experiments in primary cultures should be taken into account, as often cultures start senescing after early passages, making repeat experiments unreliable or difficult to complete.

To summarize, results from our pilot data indicate that there might be a role for PARP inhibitors in a significantly greater proportion of EOCs than those associated with BRCA mutations alone, and it may be
possible to identify this proportion with the use of a cell-based assay of HR function. As current phase I/II clinical trials continue to investigate the beneficial role of PARP inhibitors in patients with advanced ovarian cancers with known BRCA mutations (10), our findings provide a platform for extending the potential use of PARP inhibitors to a wider group of ovarian cancer patients based on their HR status, whatever the underlying mechanism and irrespective of their BRCA status. Our study also provides an example on how the principle of targeted chemotherapy may be exploited in clinical practice. It seems that it is possible to correctly identify patients who will not benefit from PARP inhibitors (∼40% of EOCs in our series). On the other hand, a significant proportion (>90% from our results obtained thus far) of patients predicted to be HR deficient by this assay are likely to benefit from PARP inhibitors. We propose to continue our work to further validate these findings as well as investigate the mechanisms responsible for the acquired deficiencies in HR.

**Disclosure of Potential Conflicts of Interest**

G. Los and Z. Hostomsky: development of the PARP inhibitor AG014699, Pfizer, Inc.

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**References**


**Fig. 5.** Inhibition of PARP (PARPi) leads to failure of SSB repair through the BER pathway, which when encountered by a DNA replication fork results in a DSB or fork stalling and γH2AX formation. In HR-competent cells, these DSBs will be repaired by error-free HR accompanied by increase in Rad51 foci formation, whereas in HR-deficient cells repair does not occur, resulting in DSB accumulation and cell death.
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