Therapeutic Potential of Poly(ADP-ribose) Polymerase Inhibitor AG014699 in Human Cancers With Mutated or Methylated BRCA1 or BRCA2

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Background Mutations in BRCA1 and BRCA2 (BRCA1/2), components of the homologous recombination DNA repair (HRR) pathway, are associated with hereditary breast and ovarian cancers. Poly(ADP-ribose) polymerase (PARP) inhibitors are selectively cytotoxic to animal cells with defective HRR, but results in human cancer cells have been contradictory. We undertook, to our knowledge, the first comprehensive in vitro and in vivo investigations of the antitumor activity of the PARP inhibitor AG014699 in human cancer cells carrying mutated or epigenetically silenced BRCA1/2.

Methods We used nine human cell lines, four with nonmutated BRCA1/2 (MCF7, MDA-MB-231, and HCC1937-BRCA1 [breast cancer] and OSEC-2 [ovarian surface epithelial]), two with mutated BRCA1 (MDA-MB-436 and HCC1937 [breast cancer]), one with mutated BRCA2 (CAPAN-1 [pancreatic cancer]), one that was heterozygous for BRCA2 (OSEC-1 [ovarian surface epithelial]), and one with epigenetically silenced BRCA1 (UACC3199 [breast cancer]), and two Chinese hamster ovary cell lines, parental AA8 and XRCC3 mutated IRS 1Sf. We assessed cytotoxicity, DNA damage, and HRR function. Antitumor activity of AG014699 was determined by growth of xenograft tumors (five mice per treatment group). Long-term safety of AG014699 was assessed.

Results AG014699 (≤10 μM) was cytotoxic to cells with mutated BRCA1/2 or XRCC3 and to UACC3199 cells with epigenetically silenced BRCA1 but not to cells without BRCA1/2 or XRCC3 mutations or that were heterozygous for BRCA2 mutation. AG014699 induced DNA double-strand breaks in all nine cell lines studied. HRR was observed only in cells with functional BRCA1/2 proteins. Growth of xenograft tumors with BRCA1/2 mutations or with epigenetically silenced BRCA1 was reduced by AG014699 treatment, and combination treatment with AG014699 plus carboplatin was more effective than either drug alone. AG014699 was not toxic in mice with nonmutated or heterozygous BRCA2.

Conclusion Human cancer cells or xenograft tumors with mutated or epigenetically silenced BRCA1/2 were sensitive to AG014699 monotherapy, indicating a potential role for PARP inhibitors in sporadic human cancers.

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Mutations in BRCA1 and BRCA2 genes are associated with a high lifetime risk of breast and ovarian cancers (1,2). BRCA1 and BRCA2 proteins play a major role in the response to and repair of DNA double-strand breaks through the homologous recombination DNA repair (HRR) pathway (3). HRR, a complex error-free process, repairs double-strand breaks occurring in late S and/or G2 phase of the cell cycle and stalled replication forks after unrepaired single-strand breaks. In HRR defective, that is, BRCA1 and/or BRCA2 (BRCA1/2)-mutated cells, failure to execute error-free repair can result in the chromosomal aberrations and genomic instability characteristic of tumors (3). Preclinical studies (4,5) have reported that cells with mutated BRCA1/2 genes are sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors. PARP-1 and -2 are abundant nuclear proteins that form poly(ADP-ribose) (PAR) chains in response to DNA damage. They play a crucial role in DNA single-strand break repair (6). PARP inhibition leads to accumulation of single-strand breaks that during replication are converted to double-strand breaks, which are normally repaired by the HRR pathway (7). In HRR-defective cells with BRCA1/2 mutations, PARP inhibitors are synthetically lethal by inactivating the single-strand break repair pathway and the subsequent accumulation of irreparable double-strand breaks (8). Synthetic lethality, defined as the lethal effect of inactivating two enzymes or pathways when inactivation of either alone is tolerated, has major potential for cancer therapy.
Cells that lack other components of the HRR pathway or in which such components have been experimentally depleted (eg, RAD51, RAD54, ATR, or XRCC3 protein) have also been shown to be sensitive to PARP inhibitors (4,9), indicating that sensitivity may require defective HRR in general, not just BRCA1/2 mutations. Moreover, epigenetic gene inactivation by promoter CpG island methylation is a well-recognized mechanism for silencing tumor suppressor genes (10), and BRCA1 methylation has been reported in approximately 15% of sporadic breast and ovarian cancers (11,12). Dysfunction of BRCA1 is also a feature of basal-like triple-negative breast cancers, which are phenotypically similar to BRCA1-associated breast cancers (ie, estrogen receptor negative, high nuclear grade and high Ki-67 staining, and express cytokeratin 5/6 and epidermal growth factor receptor) (13,14). In addition, overexpression of EMTY mRNA, which represses BRCA2 protein function, has been observed in 13% of sporadic breast cancers and 17% of high-grade sporadic ovarian cancers (15). Therefore, a substantial proportion of sporadic breast and ovarian cancers may have defective HRR and thus be sensitive to PARP inhibitors (16).

In contrast to results from studies with noncancerous BRCA2-2 negative embryonic stem cells, BRCA2-deficient Chinese hamster lung fibroblasts, or genetically modified human cell lines, results from studies examining the sensitivity of human cancer cell lines to PARP inhibitors have been negative. De Soto et al. (17) found that human BRCA1-positive, BRCA1 heterozygous, and BRCA1-negative breast cancer cell lines were not sensitive to three different PARP inhibitors (ie, 3-aminozobenzamide, NU1025, and AG14361). In addition, Gallmeier and Kern (18) found that BRCA2-defective human pancreatic cancer CAPAN-1 cells were not sensitive to the PARP inhibitor NU1025. In view of these conflicting preclinical data, we undertook, to our knowledge, the first comprehensive study of the therapeutic potential of AG014699 in a panel of nine human cell lines with proficient or deficient HRR.

We investigated the cytotoxicity of the PARP inhibitor, AG014699, by colony formation and sulforhodamine B assays, the mechanism of AG014699 cytotoxicity by DNA damage and repair immunofluorescence assays, the safety of AG014699 in mice that were heterozygous for BRCA2 after six cycles of AG014699 treatment and 12 months of follow-up, and the antitumor activity of AG014699 by measuring growth of MDA-MB-436, CAPAN-1, and UACC3199 xenograft tumors in mice.

Materials and Methods

Reagents

AG014699 was provided by Pfizer GRD (La Jolla, CA) and is a potent inhibitor of PARP-1 and -2 proteins (with an inhibition constant of <5 nM). AG014699 was dissolved in dimethyl sulfoxide to give a stock solution of 10 mM AG014699, which was stored at –20°C for in vitro studies. For in vivo experiments, AG014699 was dissolved in water at 1 mg/mL. All other chemicals and tissue culture reagents were from Sigma-aldrich (Sigma-aldrich, Gillingham, Dorset, UK), unless otherwise stated.

Cell Lines

CAPAN-1 cells are human pancreatic cancer cells with mutated BRCA2 (ie, 6174delT) in one allele and loss (or deletion) of the other allele (ie, loss of heterozygosity) (19). We used the following five human breast cancer cell lines, which were obtained from the American Type Culture Collection (Manassas, VA): MCF7 cells carry nonmutated BRCA1/2; MDA-MB-231 cells are hemizygous in BRCA1, with loss of one allele and the remaining nonmutated allele containing two nonpathogenic single-nucleotide polymorphisms; MDA-MB-436 cells carry mutated BRCA1 (5396 +1G>A in the splice donor site of exon 20) (http://research.nhgri.nih.gov/bic/) (20); HCC1937 cells carry mutated BRCA1 (5382insC) (21); and HCC1937-BRCA1 cells were derived from HCC1937 by correcting the BRCA1 mutation (22). UACC3199, a human breast cancer cell line, has BRCA1 genes that have been silenced by methylation (23); the identity of these cells was confirmed by Valerie Deregowski (University of Leven) by methylation-specific polymerase chain reaction. UACC3199 cells were purchased from the University of Arizona Cancer Research Center, Tucson. We used the following two immortalized human ovarian surface epithelial cells: OSEC1 cells are heterozygous for BRCA2 (ie, with 4630insA in exon 11 as one allele and nonmutated BRCA2 as the other allele; R. J. Edmondson, unpublished data) and OSEC2 cells carry nonmutated BRCA1/2 (24). The following two Chinese hamster
ovary cell lines were used: XRCC3-deficient IRS 1SF cells and for comparison AA8 cells with wild-type XRCC3. XRCC3-deficient IRS 1SF cells are established models for homologous recombination dysfunction (25) and were provided by Professor Penny Jeggo (Sussex University, Brighton, UK). Early passages (<30) of all cell lines were maintained in exponential growth at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. All cells were handled separately with their own unique reagents and were confirmed to be mycoplasma free by regular testing (MycoAlert Mycoplasma detection kit; Lonza, Rockland, ME). MCF7, MDA-MB-231, OSEC1, OSEC2, UACC3199, HCC1937, HCC1937-BRCA1, AA8, and IRS-1SF cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (1.0 mg/mL). CAPAN-1 cells were cultured as above but supplemented with 15% fetal bovine serum. MDA-MB-436 cells were cultured in RPMI 1640–Leibovitz medium (50:50 vol/vol), 4 mM L-glutamine, 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (1.0 mg/mL). All cell lines, described above, were used in all in vitro assays, unless otherwise indicated.

**Clonogenic Assay for Cell Survival**

All human cell lines (except the HCC1937 and HCC1937-BRCA1) and both Chinese hamster ovary cell lines were used for this assay. Exponentially proliferating cells were plated into six-well plates and incubated for 48 hours to allow cells to reach their optimum proliferation rate. AG014699 at 0, 0.1, 1.0, 10.0, 30.0, 50.0, or 100 µM in 1% dimethyl sulfoxide was added to the wells and incubated for 24 hours. Control cells received no AG014699 but were treated with medium containing 1% dimethyl sulfoxide for 24 hours. Cells were harvested and cultured in drug-free medium in 90-mm Petri dishes for up to 21 days, depending on the proliferation rate of the individual cell line. Colonies were fixed in methanol and acetic acid (3:1 vol/vol), stained with methyl violet 10B, and counted with an automated colony counter (Oxford Optronix, Oxford, UK). Data are expressed as the percentage of colonies in AG014699-treated cultures compared with that in control cultures. The concentration that results in death of 50% of cell population (LC₅₀) was calculated for each cell line in each independent experiment. Each assay contained triplicate samples for each concentration. Results represent data from at least three independent experiments.

**Sulforhodamine B Assay for Cell Growth Inhibition**

We used the following breast cancer cell lines for this assay: HCC1937, HCC1937-BRCA1, and MDA-MB-231. The HCC1937 and HCC1937-BRCA1 cell lines have poor cloning efficiency and thus are not appropriate for the clonogenic cell survival assay. As an alternative, we measured cell growth with the sulforhodamine B protein dye assay (26). MDA-MB-231 cells were also evaluated with this assay so that a direct comparison could be made between results from the clonogenic survival assay and the sulforhodamine B assay. Briefly, cells were seeded into 96-well plates at 2000 cells per well and allowed to attach overnight. AG014699 at 0.0, 1.0, 10.0, 30.0, 50.0, or 100 µM in 1% dimethyl sulfoxide was added, and cells were incubated for 140 hours at 37°C, fixed in 50% (wt/vol) trichloroacetic acid, and stained with 0.4% sulforhodamine B solution (diluted in 1% acetic acid) for 30 minutes. Absorbance of sulforhodamine B was measured at 520 nm with a SpectroMax 250 microplate spectrophotometer system (MDS Analytical Technologies, Toronto, ON, Canada) to determine cell density. The concentration required to produce 50% inhibition of cell growth (GL₅₀) was calculated for each cell line in each independent experiment. Each assay contained triplicate samples for each concentration. Results represent data from at least three independent experiments.

**Assay for PARP Activity**

We determined PARP activity after AG014699 treatment with the validated assay as used in the phase I clinical trial of AG014699 (27). Briefly, cells were incubated in medium containing 10 µM AG014699 and 1% dimethyl sulfoxide or 1% dimethyl sulfoxide (control) for 30 minutes at 37°C. Medium was removed and cells were washed in phosphate-buffered saline before use in this assay. The assay measured the amount of poly(ADP-ribose) synthesized by maximally stimulated PARP during a 6-minute reaction. Briefly, cells were permeabilized with digitonin (0.15 mg/mL) and subsequently exposed to blunt-ended oligonucleotide (5′-CGGATTCG-3′; Invitrogen, Paisley, UK) at 200 µg/mL in the presence of excess NAD⁺ (7 mM) in reaction buffer (100 mM Tris–HCl, 120 mM MgCl₂, pH 7.8) for 6 minutes at 27°C. PAR was detected with the mouse monoclonal anti-PAR 10H antibody (1.5 mg/mL, a gift from Professor Alex Bürkle, University Konstanz, Konstanz, Germany), and the anti-PAR antibody was detected with horseradish peroxidase–conjugated polyclonal goat anti-mouse IgG antibody (DAKO, Ely, Cambridgeshire, UK). Both antibodies were diluted 1:1000 in phosphate-buffered saline with 0.05% Tween 20 and 5% milk power). Secondary antibody was followed by Amersham ECL detection fluid (GE Healthcare Life Sciences, Buckinghamshire, UK) development and chemiluminescence detection by the Fujifilm LAS 3000 imager (Raytest, Sheffield, UK). PARP activity in drug-treated cells was expressed as the percentage of the activity in untreated control cells. Each cell line was assayed in triplicate, and results represent data from three independent experiments.

**Assay of DNA Double-Strand Breaks**

We used the phosphorylated histone H2AX (γH2AX) focus formation assay to measure DNA double-strand breaks. Within 1–3 minutes of the formation of a double-strand break, serine-139 in the unique carboxyl-terminal tail of histone H2AX is phosphorylated to generate γH2AX. Several γH2AX histone molecules then localize to the site of a double-strand break to form a focus; one focus corresponds to one double-strand break within the cell nucleus (28). Cells were cultured on glass coverslips and then exposed to medium containing no drug (vehicle control; ie, 1% dimethyl sulfoxide) or to 10 µM AG014699 in 1% dimethyl sulfoxide for 24 hours at 37°C before fixation in ice-cold 100% methanol for 5 minutes. As a positive control for DNA damage, cells were cultured on coverslips and then exposed to 2 Gy (2.5 Gy/min at 310 kV and 10 mA) of x-ray irradiation using the Gulmay Medical RS320 irradiation system (Gulmay Medical Limited, Surrey, UK); after this exposure, cells were incubated for 30 minutes in culture medium at 37°C to allow focus formation and then fixed as described above. Fixed control, AG014699-treated, or irradiated cells were blocked for 1 hour at room temperature in KCM buffer...
(120 mM KCl, 20 mM NaCl, 10 mM Tris–HCl at pH 8.0, 1 mM EDTA), 0.1% Triton X-100, 2% bovine serum albumin, and 10% milk powder and then incubated overnight at 4°C in primary anti-γH2AX histone IgG mouse monoclonal antibody (Millipore, Watford, UK). Cells were next incubated with secondary Alexa Fluor 546-conjugated goat anti-mouse IgG fluorescent antibody (Invitrogen, Paisley, UK) for 1 hour at room temperature but protected from light. Images of γH2AX foci were obtained with a Leica DMR microscope, RT SE6 Slider camera, and Advanced Spot software version 3.4.08 (Diagnostic Instruments, Inc, Sterling Heights, MI). γH2AX foci were counted in 30 nuclei from three different areas of each treatment slide. The number of γH2AX foci in treated cells was normalized to that in the corresponding untreated control cells, and results are expressed as the percentage of foci in untreated control cells. Three slides were prepared for each treatment, and three independent experiments were performed.

RAD51 Focus Formation Assay for Functional HRR

Nuclear RAD51 foci formation can be used as marker of functional HRR as localization of the RAD51 recombinase at DNA double-strand breaks is an essential component of HRR (29,30). After DNA double-strand break resection, RAD51 binds to the resulting over-hanging single-stranded DNA to create the nucleoprotein filament, which can be detected as RAD51 foci by immunofluorescence microscopy. To quantify RAD51 foci, cells were cultured on glass coverslips and then exposed to medium containing no drug (vehicle control; ie, 1% dimethyl sulfoxide) or to 10 µM AG014699 in 1% dimethyl sulfoxide for 24 hours at 37°C. Cells were fixed as described above and incubated overnight at 4°C with primary anti-RAD51 rabbit polyclonal antibody (Calbiochem now Merck Chemicals Ltd, Nottingham, UK), followed by incubation in the dark for 2 hours at room temperature with secondary Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Invitrogen). Cells were imaged and the number of RAD51 foci counted and normalized to untreated controls as described above. Three slides were prepared for each treatment, and three independent experiments were performed.

Characteristics of the Mice

CD-1 nude mice (female and aged 10–12 weeks) from Charles River laboratories (Wilmington, MA) were used in all xenograft experiments. These mice were maintained and handled in isolators under specific pathogen-free conditions, with five mice per cage and 20 cages per isolator. Wild-type 129/C57BL6/DBA mice and 129/C57BL6/DBA mice (female and aged > 10 weeks) that were genetically engineered to carry a heterozygous BRCA2 mutation (leading to a truncated nonfunctional protein) (31), a gift from Professor Alan Ashworth (Institute of Cancer Research, London), were used in the AG014699 toxicity experiments. These mice were maintained and handled in standard cages, with five mice per cage. All experiments involving mice were reviewed and approved by the relevant institutional animal welfare committee and then performed according to the UK Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia and UK law. For these experiments, mice were treated with drug or control vehicle between the hours of 09.00 and 11.00. Treatments were administered intraperitoneally in the animal’s home cage within the animal facility. Mice were killed by cervical dislocation for the following reasons: tumor size was greater than 10 x 10 mm, weight loss was more than 15% of baseline, or the study ended as defined a priori.

Assessment of AG014699 Toxicity in BRCA2 Wild-Type Mice and in BRCA2 Heterozygous Mutant Mice

Women who carry a mutation in BRCA2 have a high lifetime risk of developing breast and/or ovarian cancer. PARP inhibitors represent a novel treatment for these cancers; however, concerns about toxicity of these drugs in these patient populations remain. To investigate whether long-term administration of AG014699 had adverse side effects, we used mice that were heterozygous for BRCA2, a genotype that is similar to that of women who carry a mutated BRCA2 gene. A total of 34 mice were used in this experiment, including 20 BRCA2 heterozygous mice and 14 of the corresponding wild-type mice. Ten BRCA2 heterozygous mice and seven wild-type mice received AG014699 (25 mg/kg) intraperitoneally once daily on days 1–5 of a 21-day cycle for six cycles, and control groups (10 BRCA2 heterozygous mice and seven wild-type mice) received as control, saline (10 mL/kg) intraperitoneally once daily on days 1–5 of a 21-day cycle for six cycles. Mice were weighed, and their general health was observed daily during the six treatment cycles; then three times per week until mice were killed, for reasons described above, during the post treatment follow-up period of 1 year. A postmortem examination was carried out on each mouse at the time of its death, including a gross examination of the vital organs.

Efficacy of AG014699 in Xenograft Models of Tumors by BRCA1/2 Status

MDA-MB-436, CAPAN-1, and UACC3199 xenografts in CD-1 nude mice were used to test the efficacy of AG014699 because these models represent BRCA1-mutated, BRCA2-mutated, and BRCA1-silenced tumors, respectively. We subcutaneously injected 1 × 10⁶ exponentially growing MDA-MB-436 (BRCA1 mutated), CAPAN-1 (BRCA2 mutated), or UACC3199 (BRCA1 silenced) cells in 50 µL of phosphate-buffered saline into one site on the right flank of each mouse. When sufficient mice (to allow five mice per treatment group) had palpable tumors (ie, ≥5 x 5 mm), the mice were randomly assigned, to avoid treatment bias, to treatment groups (five mice per group). All treatments were administered intraperitoneally. Investigators were not blinded to the treatment groups.

UACC3199 Xenograft Tumors. Treatments for mice bearing UACC3199 xenograft tumors in experiment 1 were as follows: AG014699 (25 mg/kg) administered once daily for 10 days or a single dose of carboplatin (75 mg/kg) administered on day 1, and control mice received saline (10 mL/kg) administered once daily for 10 days. Treatment groups in experiment 2 were as follows: AG014699 (10 mg/kg) administered once daily for 5 days of a 7-day cycle for six cycles or a single dose of carboplatin (75 mg/kg), and control mice received saline (10 mL/kg) administered once daily for 5 days of a 7-day cycle for six cycles.

MDA-MB-436 Xenograft Tumors. Treatment groups were as follows: control saline (10 mL/kg) once daily for 5 days of a 7-day
cycle for six cycles or AG014699 (10 mg/kg) once daily for 5 days of a 7-day cycle for six cycles or a single dose of carboplatin (75 mg/kg) on day 1.

**CAPAN-1 Xenograft Tumors.** Treatment groups were as follows: saline control (10 mL/kg) daily for 10 days, AG014699 (10 mg/kg) once a day for 10 days, AG014699 (10 mg/kg) once daily for 5 days of a 7-day cycle for six cycles, a single dose of carboplatin (75 mg/kg) on day 1, a combination of carboplatin (75 mg/kg) on day 1 in combination with AG014699 (10 mg/kg) once a day for 10 days, or a combination of carboplatin (75 mg/kg) on day 1 with AG014699 (10 mg/kg) once a day for 5 days of a 7-day cycle for six cycles.

Mice were weighed and tumor volumes were determined daily from two-dimensional caliper measurements and the equation $a^2 \times b/2$ (where $a$ = width and $b$ = length of the tumor). Tumor data are presented as the dimension-less parameter, relative tumor volume (RTV). For example, RTV1 is the tumor volume on the first day of treatment (day 0), RTV3 is 3 times larger than RTV1, RTV4 is 4 times larger than RTV1, and RTV5 is 5 times that of RTV1.

**Statistical Analysis**

In vitro cell line data were analyzed by two-tailed Student $t$ tests. In vivo tumors data were analyzed by comparing the time taken to reach a specified RTV among groups randomly assigned to different treatments with a Mann–Whitney test and Minitab software, version 15 (Coventry, UK). All statistical tests were considered statistically significant if the $P$ value was less than .05. Statistical tests were two-sided.

![Figure 1. Cytotoxicity and growth inhibition by AG014699. A) Clonogenic survival. Cells were treated with AG014699 at various concentrations, as indicated, for 24 hours, and survival was determined with the clonogenic assay. Open symbols and dashed lines = cells with epigenetically silenced (UACC3199) or mutated BRCA1 (MDA-MB-436) or with BRCA2 (CAPAN-1) or XRCC3 (IRS-1SF) mutations; solid symbols and lines = cells with nonmutated BRCA1/2 (MCF7, OSEC2, AA8) or cells that are BRCA1 and/or BRCA2 heterozygous (OSEC1, MDA-MB-231). B) Cell proliferation. Cells were treated with AG014699 at various concentrations, as indicated, for 6 days. Cell proliferation was assessed by a sulforhodamine B assay. Open symbols and dashed lines = BRCA1-mutated HCC1937 cells; solid symbols and lines = BRCA1-reconstituted HCC1937-BRCA1 or MDA-MB-231 cells. Data in both panels are the mean values from three independent experiments, with triplicate values for each concentration. Error bars = 95% confidence intervals.](https://academic.oup.com/jnci/article-abstract/103/4/334/895898)

**Results**

**PARP Activity in Cells After Exposure to AG014699**

We investigated whether AG014699 could inhibit PARP activity in all nine human cell lines and both Chinese hamster ovary cell lines by use of a validated PARP activity assay (25). In all cell lines studied, a 30-minute exposure to 10 µM AG014699 caused greater than 94% inhibition of PARP activity, compared with untreated controls (Supplementary Figure 1, available online), demonstrating that AG014699 freely permeates the cells, binds to and inactivates PARP, and that the inhibition persisted during cell permeabilization and subsequent PARP enzyme stimulation. There was no statistically significant difference in PARP inhibition across the cell lines.

**Clonogenic Cell Survival and Growth Inhibition Assays to Determine Sensitivity of Cell Lines to AG014699**

The impact of AG014699 on survival and/or cell growth was determined in all cell lines. Using clonogenic cell survival assays, we found that AG014699 was more cytotoxic to cell lines examined with mutations in BRCA1/2 (CAPAN-1 and MDA-MB-436 cells) or XRCC3 (IRS-1SF cells) than to those with functional HRR (AA8, MCF7, MDA-MB-231, and OSEC2 cells) (Figure 1, A). Table 1 presents the mean LC$_{50}$ data for all cell lines demonstrating a marked difference in the range for the HRR functional cell lines (range of LC$_{50}$ values = 20.2–50.7 µM) and the HRR mutated cells (range of LC$_{50}$ values = 1.3–5.5 µM). The sensitivity of the epigenetically silenced BRCA1 UACC3199 cells (mean LC$_{50}$ = 7.6, 95%
Table 1. Cell survival after a 24-hour exposure to AG014699*

| Cell line                  | BRCA1/2 or HRR status | Mean LC₅₀ for AG014699, µM (95% CI) |
|---------------------------|-----------------------|-------------------------------------|
| Functional HRR            |                       |                                     |
| MCF7                      | Wild type             | 20.2 (14.6 to 25.8)                 |
| MDA-MB-231                | BRCA1 hemizygous      | 21.9 (17.8 to 26.1)                 |
| OSEC1                     | BRCA2 heterozygote    | 44.8 (9.3 to 80.3)                  |
|                           | (4630insA in exon 11) |                                     |
| OSEC2                     | Wild type             | 31.6 (4.1 to 59.1)                  |
| AA8                       | Wild type             | 50.7 (33.7 to 67.8)                 |
| Defective HRR             |                       |                                     |
| MDA-MB-436                | BRCA1 mutation        | 1.3 (−0.12 to 2.8)                  |
|                           | 5396 + 1G>A           |                                     |
| Capan-1                   | BRCA2 mutation        | 5.5 (2.4 to 8.7)                    |
|                           | 6174delT              |                                     |
| UACC3199                  | BRCA1 silenced by methylation | 7.6 (6.1 to 9.1)                   |
| IRS-1SF                   | XRCC3 deficient       | 1.4 (0.4 to 2.4)                    |

* CI = confidence interval; HRR = homologous recombination DNA repair; LC₅₀ = dose that results in death of 50% of cell population; wild type = nonmutated BRCA1 and/or BRCA2.

The sensitivity of cells to 10 µM AG014699 appeared to discriminate between cells with a functional HRR system and cells with a dysfunctional HRR system. Thus, we used 10 µM AG014699 in subsequent in vitro experiments.

**Induction of DNA Double-Strand Breaks After Exposure to AG014699**

We investigated whether AG014699 induced DNA double-strand breaks by use of a γH2AX focus formation assay. After a 24-hour treatment, we found an increased level of γH2AX foci in all 11 cell lines treated with 10 µM AG014699 compared with that in untreated control cells (Figure 2, A). Variation in the levels of γH2AX foci between cell lines was unlikely to be the result of their HRR status because similar levels were found in parental BRCA1-mutated HCC1937 and in BRCA1-corrected HCC1937–BRCA1 cell lines. Differences in focus number could be secondary to differences in DNA content, S-phase cell fraction, and cell number doubling times between individual cell lines. In individual cell lines, levels of γH2AX foci after AG014699 treatment were similar to those resulting from exposure to 2 Gy of ionizing radiation.
RAD51 Focus Formation and Exposure to AG014699

A 24-hour exposure to 10 µM AG014699 caused a statistically significant increase in the number of RAD51 foci for all cell lines examined with functional HRR (MCF7, MDA-MB-231, OSEC-2, AA8, and HCC1937-BRCA1 cells) and in a cell line that is heterozygous for BRCA2 (OSEC1 cells) compared with the corresponding untreated control cells (P < .02). In contrast, AG014699 exposure did not result in a statistically significant increase in the number of RAD51 foci in BRCA1/2-mutated MBA-MB-436, CAPAN-1 and HCC1937 cells, epigenetically silenced UACC3199 cells, or XRCC3-mutated IRS-1SF cells compared with corresponding untreated controls (Figure 2, B).

Toxicity of AG014699 in BRCA2 Heterozygous and Wild-type Mice

We used in vivo experiments to investigate whether AG014699 is nontoxic to BRCA2 heterozygous mice. With a follow-up of 365 days, no difference was observed in mean body weight or survival between BRCA2 heterozygous and control treated groups (Figure 3, A and B). Ten heterozygous and 10 wild-type mice were each treated with a total AG014699 dose of 750 mg/kg. Postmortem examination revealed no abnormalities in the major organs of any mouse, except that a BRCA2 heterozygous mouse treated with AG014699 at 25 mg/kg (aged 426 days) had multiple tumors in its liver. One BRCA2 heterozygous mouse treated with AG014699 at 25 mg/kg (aged 473 days) died of an ear infection. In addition, two wild-type mice treated with AG014699 at 25 mg/kg (aged 407 and 445 days), a control wild-type mouse (aged 341 days), and a control heterozygous mouse (aged 272 days) died of unknown causes.

AG014699 Treatment of Mice Bearing Xenograft Tumors With Epigenetically Silenced or Mutated BRCA1 or BRCA2 Epigenetically Silenced BRCA1 UACC3199 Xenograft Tumors. Of the 30 mice implanted with epigenetically silenced BRCA1 UACC3199 cells, only 15 developed tumors by 40 days after implantation. These 15 mice were then randomly assigned to the following treatment groups (five mice per group): AG014699 (25 mg/kg daily for 10 days), carboplatin (single dose of 75 mg/kg), or saline control. UACC3199 tumors grew relatively slowly, with the size of control tumors being an RTV of 2.6 at day 10 (Figure 4, A, and Table 2). Because of the variable lag time for tumor appearance (range = 21–32 days), there was an up to six-fold variation in tumor size (range = 36–214 mm³) when treatment was initiated. In addition, at day 12, two mice or more in each group had to be killed because their tumor was larger than 10 × 10 mm. For this reason, data up to and including day 12 are presented (with individual tumor measurements being presented in Supplementary Figure 2, available online). During the 10-day dosing period, AG014699 at 25 mg/kg appeared to arrest tumor growth, and the RTV in treated groups was less than that in the corresponding untreated control mice (RTV = 1.1 on day 10 in AG014699-treated mice vs RTV = 2.6 in control mice; difference in RTV = 1.1, P = .04). However, when AG014699 treatment stopped, tumors began to grow again. In contrast, the response to carboplatin (single dose on day 1) appeared to be delayed, with a slowing of tumor growth that was detectable only after day 9 (RTV on day 10 = 1.7).

Because the growth of UACC3199 xenograft tumors appeared only to be retarded during AG014699 therapy, we next evaluated a prolonged AG014699 schedule. In this study, 40 mice were implanted with UACC3199 cells and randomly assigned to receive: saline (10 mL/kg), AG014699 (10 mg/kg) daily for 5 days of a 7-day cycle for six cycles, or carboplatin (75 mg/kg) single dose on day 1. Because of the variable lag time for the appearance of the tumors observed in the previous experiment, mice were randomly assigned to the different treatment groups and treatment was initiated on day 42 after implantation when nine mice had confirmed tumors (ie, when there were sufficient mice for at least two mice per treatment group). Additional mice were randomly assigned to the treatment groups as their tumors appeared. In this study, tumor growth was more consistent (Supplementary Figure 2, available online). The mean time to RTV5 in saline-treated control mice was 25.2 days (95% CI = 14.0 to 36.0). In AG014699-treated mice, the time to RTV5 was extended to 35.8 days (95%
Figure 4. Efficacy of AG014699 treatment in mice bearing BRCA1/2-defective xenografts. The efficacy of AG014699 in two different dosing schedules was evaluated in comparison to carboplatin therapy in mice bearing UACC3199 (BRCA1 methylated), MDA-MB-436 (BRCA1 mutated), and CAPAN-1 (BRCA2 mutated) xenografts by determining the mean relative tumor volume. There were five mice per group in all experiments. Mean relative tumor volume (RTV) data are given (results for individual mice are given in Supplementary Figures 2-4, available online).

A) UACC3199. i) Experiment 1. Solid circles and line = saline vehicle alone; open circles and solid line = AG014699 (25 mg/kg per day for 10 days); or solid triangles and dashed line = carboplatin (single dose of 75 mg/kg on day 1). ii) Experiment 2. Solid circles and line = saline vehicle alone; open circles and solid line = AG014699 (10 mg/kg, day 1–5 on a 7-day cycle for six cycles); solid triangles and dashed line = carboplatin (75 mg/kg, single dose on day 1).

B) MDA-MB-436. Solid circles and line = saline vehicle alone; open circles and solid line = AG014699 (10 mg/kg days 1–5 on a 7-day cycle for six cycles); or solid triangles and dashed line = carboplatin (75 mg/kg, single dose on day 1).

C) CAPAN-1. i) Experiment 1. Solid circles and line = saline vehicle alone; open circles and solid line = AG014699 (10 mg/kg daily for 10 days); or solid triangles and dashed line = carboplatin (75 mg/kg intraperitoneally); open triangles and dashed line = combination of carboplatin and AG014699 (10 mg/kg (continued))
Tumor growth delay (TGD) was calculated as the mean time to relative tumor volume (RTV) 4 or 5 after the specified treatment minus the mean time to RTV 4 or 3 (22.8 to 55.4). Data include two transient tumor regressions on days 17 (60, 63) and 24 (7 to 44.8). P = .01.

BRCA1-Mutated MDA-MB-436 Xenograft Tumors. MDA-MB-436 cells formed xenograft tumors poorly; only 15 of the 50 mice that were implanted with these cells developed tumors. Treatment of these 15 MDA-MB-436 tumor-bearing mice began 44 days after implantation. Because the AG014699 schedule of a once daily dose of 10 mg/kg for 5 days of a 7-day cycle for six cycles resulted in greater tumor growth delay in the UACC3199 xenografts, we compared this schedule with a single dose of carboplatin and a matched saline control in mice bearing MDA-MB-436 xenograft tumors. Tumors in untreated control mice reached RTV 4 after 19 days (95% CI = 9.4 to 25.0). Tumor growth was delayed in mice treated with AG014699 (P = .03), with a mean time to RTV 4 of 29 days (95% CI = 13.0 to 45.0), which was equivalent to a tumor growth delay of 10 days (Figure 4, B, and Table 2). Among this group, one mouse had a transient complete tumor regression (ie, no tumor detectable) between days 15 and 17. MDA-MB-436 tumors were very sensitive to carboplatin treatment, with transient complete tumor regressions observed in two mice (one on days 17–50 and the other on days 20–34) and with a third mouse experiencing a sustained durable complete tumor regression from day 15 until it was killed at the end of the study. Times to RTV 4 in the remaining two mice with tumors were 57 and 63 days. AG014699 was also non-toxic in this study, with the maximum weight loss among treated mice being 4% and that among control mice being 6.4%; however, carboplatin caused measurable toxicity, with the maximum weight loss being 13%.

**BRCA2-mutated CAPAN-1 xenograft tumors.** All 40 CD-1 nude mice implanted with CAPAN-1 cells developed tumors. At 15 days after implantation, mice were randomly assigned to the following treatment groups: saline control (10 mL/kg) daily for 10 days, AG014699 (10 mg/kg) once a day for 10 days, AG014699 (10 mg/kg) once daily for 5 days of a 7-day cycle for six cycles, a single dose of AG014699 (10 mg/kg, d1–5) on a 7-day cycle for six cycles; AG014699 (10 mg/kg, d1–10), plus carboplatin at 75 mg/kg, d1; AG014699 (10 mg/kg, d1–5 every 7d) for 6 cycles; carboplatin at 75 mg/kg, d1; control saline at 10 mL/kg, d1–5 every 7d for 6 cycles.

**Table 2. Summary of in vivo efficacy data, after treatment with AG014699, carboplatin, or vehicle control of mice bearing UACC3199, MDA-MB-436, and CAPAN-1 xenograft tumors.**

| Xenograft and treatment group | Mean time to RTV5, d (95% CI) | Mean time to RTV4, d (95% CI) | Mean TGD, d† | % E | No. CRs/No. of mice in group |
|------------------------------|-------------------------------|-------------------------------|---------------|-----|-----------------------------|
| **UACC3199**                |                               |                               |               |     |                             |
| Control saline at 10 mL/kg, d1–5 every 7 d for 6 cycles | 25.2 (14 to 36) | N/A |                     0/5 |
| AG014699 at 10 mg/kg, d1–5 every 7 d for 6 cycles | 35.8 (25 to 46) | .02 | 11 | 0/5 |
| Carboplatin at 75 mg/kg, d1 | 35.7 (26 to 44) | .01 | 11 | 0/5 |
| **MDA-MB-436**              |                               |                               |               |     |                             |
| Control saline at 10 mL/kg, d1–5 every 7 d for 6 cycles | 19 (9.4 to 25) | N/A |                     0/5 |
| AG014699 at 10 mg/kg, d1–5 every 7 d for 6 cycles | 29 (13 to 45) | .03 | 10 | 1/5 |
| Carboplatin at 75 mg/kg, d1 | 60 (57, 63) | .001 | 41 | 3/5 |
| **CAPAN-1**                 |                               |                               |               |     |                             |
| Control saline at 10 mL/kg, d1–5 every 7 d for 6 cycles | 11.5 (4 to 20) | N/A |                     0/5 |
| AG014699 at 25 mg/kg, d1–10 | 24 (7 to 44.8) | .016 | 12 | 0/5 |
| AG014699 at 10 mg/kg, d1–5 every 7 d for 6 cycles | 39 (27 to 50) | .016 | 27 | 0/5 |
| Carboplatin at 75 mg/kg, d1 | 18 (1 to 35) | .026 | 6 | 1/5 |
| AG014699 at 10 mg/kg, d1–10, plus carboplatin at 75 mg/kg, d1 | 39 (22.8 to 55.4) | .035 | 27 | 323 | 2/5 |
| AG014699 at 10 mg/kg, d1–5 every 7 d for 6 cycles plus carboplatin at 75 mg/kg, d1 | 48 (10.9 to 86.3) | .01 | 36 | 1/5 |

* CI = confidence interval; CR = complete regressions.
† P values compared treatment groups with untreated control groups (unpaired t test). All statistical tests were two-sided.
‡ Tumor growth delay (TGD) was calculated as the mean time to relative tumor volume (RTV) 4 or 5 after the specified treatment minus the mean time to RTV 4 or 5 in the control group. Enhancement (E) was calculated as [TGD of the combination treatment/TGD of cytotoxic alone] × 100 = 100.
§ A transient tumor regression on days 15–17.
¶ 95% CIs are shown unless there were fewer than three tumors for evaluation because of tumor regression. For these experiments, individual values are shown.
†† Data include two transient tumor regressions on days 17–50 and days 20–34.
‡‡ These P values compared the combination treatment group with the carboplatin alone group.

**Figure 4 (continued).**

- Daily for 10 days.
- Experiment 2. Solid circles and line = saline vehicle alone; open circles and solid line = AG014699 (10 mg/kg, days 1–5 on a 7-day cycle for six cycles); solid triangles and dashed line = carboplatin (75 mg/kg, single dose on day 1); open triangles and dashed line = combination of carboplatin and AG014699 (10 mg/kg, days 1–5 on a 7-day cycle for six cycles).
of carboplatin (75 mg/kg) on day 1, a combination of carboplatin (75 mg/kg) on day 1 in combination with AG014699 (10 mg/kg) once a day for 10 days, or a combination of carboplatin (75 mg/kg) on day 1 with AG014699 (10 mg/kg) once a day for 5 days of a 7-day cycle for six cycles

Tumors in untreated mice reached mean RTV4 at 11.5 days (95% CI = 4.0 to 20.0) (Figure 4, C, Table 2, and Supplementary Figure 4, available online) consistent with previous reports (32). A single dose of carboplatin (on day 1) resulted in one complete tumor regression and an increase in mean time to RTV4 of 18 days (95% CI = 1.0 to 35.0) (P = .026) compared with that of untreated control mice. Treatment with AG014699 for 10 days resulted in an increase in time to RTV4 of 24 days (95% CI = 7.0 to 44.8) (P = .016), which was equivalent to a 12.5-day growth delay (Figure 4, C, and Table 2). The regimen of AG014699 (10 mg/kg) once daily for 5 days of a 7-day cycle for six cycles resulted in a 27.5-day growth delay (ie, time to RTV4 of 39 days, 95% CI = 27.0 to 50.0, P = .016) compared with control mice (Figure 4, C, and Table 2). The combination of carboplatin and the 10-day AG014699 schedule resulted in two complete tumor regressions (one at day 28 and the other at day 35), with a tumor growth delay of 27.5 days (time to RTV4 = 39 days; 95% CI = 22.8 to 55.4) that was greater than either treatment alone (Figure 4, B, and Table 2). The combination of carboplatin with AG014699 given once daily at a dose of 10 mg/kg for 5 days of a 7-day cycle for six cycles was the most effective treatment, with two complete regressions (one at day 7 and the other at day 12) and a tumor growth delay of 36.5 days (mean time to RTV4 = 48 days; 95% CI = 10.9 to 86.3) in the remaining tumors (Figure 4, C, and Table 2), which was greater than that achieved with carboplatin alone (P = .014). None of the treatments caused statistically significant toxicity (P = 0.08), with maximum body weight loss being less than 5% in all groups, except for mice receiving the carboplatin and AG014699 combination schedule described above, in which the maximum weight loss was 6.3%.

Discussion

The most important data to emerge from this study were from the epigenetically silenced BRCA1 UACC3199 model. Cytotoxicity of AG014699 was similar in the epigenetically silenced BRCA1 UACC3199 cells and in BRCA1/2 or XRCC3 mutated cells (CAPAN-1, IRS-1SF, and MDA-MB-436). AG014699 inhibited the growth of UACC3199 xenograft tumors as well as or better than carboplatin.

One limitation of this study to consider is that we did not authenticate, by fingerprinting, the cell lines used. However, cells were obtained from their originators or from reputable suppliers, they were isolated from each other by use of medium and reagents exclusive to each cell line, and only low-passage numbers of cells were used for experiments. Moreover, their behavior (eg, ability or not to form RAD51 foci) was consistent with their reported genotypes. The UACC3199 cells were confirmed to have methylated BRCA1, and their defect in HRR was confirmed by the observation that prolonged exposure to AG014699 failed to induce an increase in RAD51 foci.

We have also shown, to our knowledge, for the first time that PARP inhibition is selectively toxic to human cancer cell lines with mutated BRCA1 and BRCA2 and that xenograft tumors derived from these cell lines also responded to PARP inhibitors therapy. These results support those previously published showing selective sensitivity to PARP inhibitors in noncancer, nonhuman BRCA1/2-deficient models (4,5) and contrast with previous studies that failed to show such sensitivity in human cancer models (17,18). We also show that other defects in HRR (eg, through XRCC3 mutation) confer sensitivity to AG014699, indicating that, in addition to BRCA1/2-associated cancers, PARP inhibitors may have therapeutic potential in tumors with other defects in HRR. The antitumor activity clearly indicates that drug levels sufficient to suppress PARP activity and tumor cell viability can be achieved with non toxic doses of AG014699; doses that result in peak plasma concentrations of 3.3–5.6 μM and up to 95% PARP inhibition in other tumor models (H. D. Thomas, unpublished data). These data compare favorably with the peak plasma concentrations of 2.6 μM (837 ng/mL) and tumor PARP inhibition of 95% that have been reported after administration of AG014699 at 18 mg/m² to patients (27), the dose being used in the current phase II AG014699 BRCA study (http://www.clinicaltrials.gov/ct2/results?term=PARP), as well as the level of PARP inhibition observed in the cell lines described in this study.

The growth delay caused by the 10-day AG014699 schedule was at least equivalent to that after carboplatin treatment of CAPAN-1 xenograft tumors. A greater response was observed with more prolonged (once daily for 5 days of a 7-day cycle for six cycles) AG014699 therapy. Similarly, we observed a greater response in the UACC3199 xenograft model when treated on the prolonged schedule. These results indicate that continuous PARP inhibitor therapy might result in a superior clinical response than intermittent schedules such as that used for chemosensitization in the previous phase I AG014699 trial (days 1–5, every 28 days) (27). Cultured MDA-MB-436 cells were the most sensitive human cancer cell line to AG014699-induced cytotoxicity and also had fewer RAD51 foci in response to AG014699, suggesting that these cells had the most profound HRR defect. Xenograft tumors derived from these cells responded well to AG014699, with one transient complete tumor regression, and were also highly sensitive to carboplatin, with two transient and one durable complete responses, consistent with a severely HRR-defective phenotype. Similar studies, in mice bearing BRCA1- and p53-deleted transplanted mouse mammary tumors (33) and in mice bearing autochthonous BRCA2- and p53-deleted mouse mammary tumors (34), showed that the PARP inhibitor, AZD2281, on a 28- or 100-day cycle, increased survival but was less effective than cisplatin at 6 mg/kg or carboplatin at 100 mg/kg, a dose similar to that used in this study. In the transplantable, but not the autochthonous, mouse tumor studies, the combination of cisplatin and a PARP inhibitor resulted in prolonged recurrence-free and overall survival compared with mice treated with either drug alone. We also found that the combination of carboplatin and AG014699 was more effective that either drug alone in human pancreatic cancer cells carrying a BRCA2 mutation, resulting in complete tumor regressions in two of the five tumors treated.

These data have demonstrated that AG014699 inhibited growth in BRCA2 mutated (CAPAN-1), BRCA1 mutated (MDA-MB-436), and BRCA1 epigenetically silenced (UACC3199) tumors, with the
prolonged AG014699 treatment schedule and carboplatin combination being the most effective. However, in interpreting data from these xenograft studies, it should be noted that a statistically significant difference between treatment groups (determined by $P < .05$) could have been observed by chance, given the multiple statistical comparisons of the different treatment groups. The lack of adjustment for multiple statistical comparisons could be considered a limitation of the study.

Encouragingly, we did not observe any toxicity with AG014699 monotherapy in these xenograft studies and observed only marginally increased weight loss with the combination treatment of AG014699 and carboplatin. Preliminary phase II clinical data with the PARP inhibitor, BSI-201, support the increased response and lack of toxicity of the combination of PARP inhibitors plus carboplatin among patients with triple-negative breast cancer (35), and a phase III study of this combination is ongoing.

Recent reports (36–38) have demonstrated that sensitivity to PARP inhibitors can be lost by a secondary intragenic deletion of the original truncating mutation in BRCA1 or BRCA2 that restores an open reading frame and reverses the BRCA1/2 defect. It is possible that the inherent genomic instability associated with loss of BRCA1/2 function could give rise to such spontaneous reversions, which may account for the PARP inhibitor resistance seen in the CAPAN-1 cell lines (18). Indeed, it seems likely that spontaneous reversion of BRCA2 mutations in CAPAN-1 cells may be common because single-strand break repair dysfunction from reduced ligase III activity has been reported, which would severely compromise the viability of a HRR dysfunctional cell (39).

We found that AG014699 treatment caused increased γH2AX foci formation in all cells studied but caused increased RAD51 foci formation only cells with functional HRR. These results support the proposed mechanism of selective AG014699 toxicity (Figure 5), in which inhibition of PARP-1 leads to failure of single-strand break repair, which then leads to a double-strand break and γH2AX focus formation. In HRR competent, BRCA1/2-proficient cells, these double-strand breaks will eventually be repaired by error-free HRR, accompanied by RAD51 focus formation, without deleterious effects; however, in HRR-deficient cells, repair does not occur resulting in the accumulation of double-strand breaks and cell death.

Concerns about potential toxicity in normal tissue of patients receiving PARP inhibitors is not unfounded on the basis of our observation that a 24-hour exposure to 10 μM AG014699 induces a level of DNA double-strand breaks that is similar to that caused by 2 Gy of irradiation. However, it is encouraging that we found no increase in cytotoxicity in BRCA1/2 heterozygous cell lines after short-term AG014699 exposure and no decrease in survival of BRCA2 heterozygous mice after long-term AG014699 treatment that was equivalent to approximately 10 times the maximum tolerated patient dose in the phase I trial (27). The finding of multiple liver tumors in one of the heterozygous mice was not unexpected because sporadic tumor formation in mice of that age is not uncommon (40). In addition, the phase I–II clinical trials of AG014699 in combination with temozolomide in cancer patients with nonmutated BRCA1/2 did not report any single-agent AG014699 toxicity (27). However, dose-limiting toxicities of myelosuppression and central nervous system side effects were observed in the phase I trial of the PARP inhibitor olaparib (previously AZD2281 or KU-0059436) in patients with advanced solid tumors with a population enriched for BRCA1/2 mutation carriers (41).

Clinical trials of PARP inhibitors are ongoing in BRCA1/2-mutated cancers (http://www.clinicaltrials.gov/ct2/results?term =PARP). The first to report was the pivotal phase I study of the oral PARP inhibitor, olaparib, in which a confirmed partial response rate of 39% (nine of the 23 patients) was observed (41). After this initial report (41), phase II studies of olaparib in BRCA1/2-associated breast and ovarian cancers have reported objective response rates in the 400-mg twice daily dosing cohorts of 33% (11 of the 33 patients) in ovarian cancer patients (42) and 41% (11 of the 27 patients) in breast cancer patients (43). With different modes of administration, scheduling, specificity, and potency of PARP inhibition, it may be that not all PARP inhibitors are equal and it will be interesting to see if the response observed with olaparib can be validated with other PARP inhibitors. Further studies will hopefully address the many unanswered questions about the safety and efficacy of PARP inhibitors in this patient population.

In summary, we have reported, to our knowledge, the first efficacy results of a PARP inhibitor in both in vitro and in vivo models.
of human cancer that is associated with epigenetically silenced BRCA1 as well as mutated BRCA1/2. Antitumor activity was observed at nontoxic doses of AG014699 monotherapy, and the combination of AG014699 with carboplatin resulted in 40% complete tumor regression and prolonged tumor growth delay without statistically significant toxicity. Furthermore, AG014699 was not toxic to BRCA2 heterozygous mice. Our data demonstrating the superiority of a more continuous AG014699 dosing schedule (5 days per week for 6 weeks) in comparison to a 10-day schedule have implications for the clinical scheduling of PARP inhibitor monotherapy. Encouragingly, although the mechanisms of PARP inhibitors and carboplatin cytotoxicity and resistance somewhat overlap, combination therapy with the two drugs was particularly effective in human BRCA1/2-defective xenograft tumors, which provide robust preclinical support to the conduct of clinical trials of combination therapy with carboplatin and PARP inhibitors. The most important and, to our knowledge, novel observation—that epigenetically silenced BRCA1 also confers sensitivity to PARP inhibitors monotherapy in vitro and in vivo—indicates that screening for BRCA1/2 mutations will only identify a subset of patients who may respond to PARP inhibitors monotherapy and that PARP inhibitors may have a broader therapeutic potential. These data provide robust evidence to support the use of PARP inhibitors in the treatment of both sporadic and hereditary BRCA1/2-related cancers. How to identify which tumors will respond to treatment with PARP inhibitors is the next challenge.

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**Notes**
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The authors had full responsibility for the design of the study; the collection, the analysis, and interpretation of the data; the decision to submit the article for publication; and the writing of the article, all of which were approved by Pfizer. Z. Hostomsky and G. Los are employees of Pfizer GRD. N. J. Curtin, E. R. Plummer, and R. J. Edmondson are recipients of funding from Pfizer GRD to support E. A. Mulligan. N. J. Curtin and E. R. Plummer are named inventors on patents of PARP inhibitors, including that for AG014699.

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