Low Levels of Circulating Estrogen Sensitize PTEN-Null Endometrial Tumors to PARP Inhibition In Vivo

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Abstract

Earlier in vitro work demonstrated that PARP inhibition induces cell death in PTEN-null endometrial cancer cell lines, but the in vivo therapeutic efficacy of these agents against endometrial cancer remains unknown. Here, we test the efficacy of AZD2281 (olaparib), an oral PARP inhibitor, in the therapy of PTEN-null endometrial tumors in a preclinical endometrial cancer mouse model. Primary endometrial tumors were generated by epithelial loss of PTEN using an in vivo model. This model recapitulates epithelial-specific loss of PTEN seen in human tumors, and histologically resembles endometrioid carcinomas, the predominant subtype of human endometrial cancers. Olaparib was administered orally to tumor-bearing mice in two hormonal extremes: high or low estrogen. Olaparib treatment achieved a significant reduction in tumor size in a low estrogenic milieu. In striking contrast, no response to olaparib was seen in tumors exposed to high levels of estrogen. Two key observations were made when estrogen levels were dropped: (i) the serum concentration of olaparib was significantly increased, resulting in sustained PARP inhibition at the tumor bed; and (ii) the homologous recombination pathway was compromised, as evidenced by decreased Rad51 protein expression and function. These two mechanisms may account for the sensitization of PTEN-null tumors to olaparib with estrogen deprivation. Results of this preclinical trial suggest that orally administered PARP inhibitors in a low estrogenic hormonal milieu can effectively target PTEN-null endometrial tumors. Extension of this work to clinical trials could personalize the therapy of women afflicted with advanced endometrial cancer using well-tolerated orally administered therapeutic agents. Mol Cancer Ther; 12(12); 2917–28. ©2013 AACR.
pathway. However in cells with compromised HR, death ensues with PARP inhibition (8). As an example, PARP inhibitors exhibit antitumor activity in patients with BRCA-mutant cancers (9, 10). Because BRCA proteins are essential for homologous recombination, BRCA-deficient tumors regress because of their inability to repair double-strand DNA breaks induced by PARP inhibition (12, 13).

To extend the use of PARP inhibitors, nontraditional pathways that may interfere with HR have been investigated. Inactivation of PTEN in mouse embryonic fibroblasts induced chromosome instability due to defective Rad51-mediated HR DNA repair (14). Rad51 is a critical component of the HR pathway, functioning in the homology search and strand-exchange phases of DNA repair (15).

PTEN-null tumor cells were hypothesized to be sensitive to PARP inhibition as a result of Rad51 downregulation and or loss of function. Two in vitro studies reported cytotoxic effects of PARP inhibition in PTEN-null cancer cell lines associated with altered Rad51 function (7, 16), but the in vivo capacity of these drugs to resolve PTEN-null tumors, particularly endometrial cancer, has not been fully investigated. The existence of Rad51 defects in PTEN-null tumors has been controversial as recent studies did not demonstrate defective Rad51 function in PTEN-null prostate cancer cell lines (17). Although PTEN status may not predict response to PARP inhibition, Rad51 function may define sensitivity to this therapy (ref. 18 and reviewed in ref. 19). Decreased expression of Rad51 promoted sensitivity to PARP inhibition in vitro (17). Conversely, increased Rad51 levels have been implicated in the resistance to PARP inhibitors (20, 21). Overall, mechanisms accounting for sensitivity or resistance to PARP inhibitors remain debated.

To test the efficacy of PARP inhibitors in the therapy of endometrioid carcinomas driven by loss of PTEN, an in vivo mouse model established by our group was utilized (22). In this model, combinations of PTEN-null epithelia and wild-type (WT) stroma regenerate, giving rise to tumors that closely resemble human endometrial cancers based on two major criteria. In human endometrioid tumors, PTEN expression is lost in the tumor epithelia but preserved in the stroma (23). Unlike existing PTEN-null mouse models (24–26) but similar to human endometrioid cancers, tumors in our model harbor epithelial-specific loss of PTEN but maintain its expression in the tumor stroma. The histology of tumors established in this model recapitulates the histopathology of endometrioid carcinomas, the major subtype of human endometrial cancers. In this preclinical study, olaparib (AZD2281), an orally bioavailable PARP inhibitor tested in human trials (http://clinicaltrials.gov/show/NCT01237067), was administered to mice bearing PTEN-null endometrial tumors. The efficiency of this therapeutic agent was tested under two hormonal conditions, high or low estrogen. These hormonal milieus were examined as many patients with endometrial cancer have a hyperestrogenic state due to obesity (27) that can be reduced pharmacologically.

Using this model, we demonstrate that PTEN-null endometrial tumors are sensitive to synthetic lethal effects of PARP inhibitors, but this response hinges on the hormonal milieu. When estrogen levels were high, a measurable response to PARP inhibition was not detected. In striking contrast, low levels of circulating estrogen sensitized these same tumors to PARP inhibition demonstrated by a reduction in tumor burden. Here, we dissect cellular mechanisms that account for this dichotomous response. Our results show that the endogenous estrogen levels impact: (i) levels of circulating olaparib modulating PARP inhibition at the tumor bed and (ii) levels and function of Rad51, a critical protein involved in homologous recombination. These results suggest that PARP inhibition should be tested in conjunction with hormonal ablation in clinical trials treating women with PTEN-null endometrial tumors.

Materials and Methods

Animals

Mouse strains B6 (C57BL/6), Pten\textsuperscript{loxP/loxP} (C;129S4-Pten\textsuperscript{tm1Hwu/J}), and CB17Scid/Scid were from Jackson Laboratories. Mice were maintained in accordance with University of California Los Angeles (UCLA) Division of Laboratory Animal Medicine guidelines. All animal experiments were approved by the UCLA Animal Research Committee.

Isolation of PTEN-null endometrial epithelia and assays

(i) Preparation of PTEN-null epithelia: Trop1\textsuperscript{+} CD90\textsuperscript{-} CD45\textsuperscript{-} Ter119\textsuperscript{-} epithelia were isolated by fluorescence-activated cell sorting (FACS) from the uteri of PTEN\textsuperscript{loxP/loxP} adult female mice (22). Epithelia were infected with Cre-RFP (22) to delete PTEN. (ii) In vivo tumor generation: PTEN-null tumors were prepared (22), and details are available in Supplementary Materials. Cellular combinations were grown for 8 weeks to establish tumors prior to initiation of therapy. (iii) In vitro generation of PTEN-null spheres: Cre-infected PTEN\textsuperscript{loxP/loxP} epithelia were plated in Matrigel (BD Bioscience) and PrEGM media (Lonza) as previously described (28). WT mouse uterine stroma was added to each well. Cells were cultured for 2 weeks. PTEN deletion was confirmed by PCR as described previously (29).

Tumor generation

Endometrial tumor generation was performed as previously described (22). Combinations of WT stroma and Cre-infected PTEN\textsuperscript{loxP/loxP} epithelia were suspended in collagen, implanted under the renal capsule of oophorectomized CB17Scid /Scid mice with a time-release estrogen pellet (60-day time release, 0.72-mg β-estradiol /pellet; SE-121 Innovative Research of America) and regenerated for 6 to 8 weeks. Details are outlined in Supplementary Methods.

Drug preparation and administration

(i) In vivo: Olaparib stocks were prepared as previously described (12, 13). Details are outlined in Supplementary Materials. Olaparib at a dose of 50 mg/kg or vehicle was...
orally administered to mice once daily for 3 weeks. Drug doses and schedules were selected on the basis of published data (12, 13). (ii) In vitro: Olaparib (10 μmol/L) or equal volume of vehicle were added to each well.

Measurement of drug levels and activity
The in vitro efficacy of olaparib was confirmed by examining drug-induced DNA damage measured by formation of γH2AX foci as previously described (7). Details are outlined in Supplementary Materials. PARP enzymatic activity in tumors was determined by measuring poly(ADP-ribose) levels using the PARP PDA II ELISA assay (cat no. 4520-096-K; Trevigen). Serum estrogen concentrations were determined using the estradiol EIA kit with associated controls according to the manufacturer’s instructions (Cayman Chemical). Serum olaparib concentrations were determined by high-performance liquid chromatography (HPLC)/mass spectrometry (MS). Details are given in Supplementary Materials. Three independent measurements were made for each time point.

Measurement of Rad51 foci formation
In vitro cocultures of Cre-infected PTENloxP/loxP epithelia with WT stroma were treated with 10 μmol/L olaparib or vehicle for 3 days. Epithelial spheres were released from Matrigel as previously described (28). RFP-positive spheres were picked, dissociated to single cells (28), and transferred to slides by cytospin. Cells were fixed and costained for γH2AX and Rad51 (antibodies in Supplementary Table S1). Cells were visualized using a Zeiss Axiovert with Apotome, EXFO X-Cite series 120Q source, and vehicle-treated controls (Supplementary Fig. S1D). To validate our results, this experiment was performed on an additional cohort of mice confirming that olaparib treatment had no significant effect on the size of PTEN-null tumors in a high estrogenic hormonal milieu (Supplementary Fig S1E).

Statistical analysis
Results are expressed as the mean of three or more measurements with standard errors. Normal distributions were verified using normal quantile plots. To determine if differences between two groups were significant, P values were calculated using the unpaired Student t test. For comparisons between three or more groups, one-way ANOVA was performed where all pairwise mean comparisons were assessed using the Tukey HSD criterion. ANOVA analysis of serum estrogen and CYP3A41 transcript levels was performed on log-transformed values. All statistical calculations were performed using GraphPad Prism software.

Results
PTEN-deficient endometrial tumors were resistant to olaparib in a high estrogenic hormonal milieu
Many patients with endometrial cancer also have high serum estrogen. Based on in vitro studies (7, 16), we hypothesized that endometrial tumors initiated by epithelial loss of PTEN would respond to PARP inhibition in vivo. To mimic the high estrogen levels in patients with endometrial cancer, the response of PTEN-null tumors to PARP inhibition was initially examined in a high estrogenic milieu.

The effects of olaparib were tested in vivo using an endometrial cancer model developed by our group (22). Endometrial epithelia FACS isolated from PTENloxP/loxP uteri were infected with a Cre-expressing lentivirus, resulting in epithelial-specific deletion of PTEN. Combinations of PTEN-null epithelia and WT stroma were placed under the kidney capsule of immunocompromised mice to prevent rejection of grafted tissue (22). Concurrent with cell implantation, mice were oophorectomized and supplemented with a 60-day time-release estradiol pellet to establish high and steady circulating estrogen levels. Within 8 weeks, cell combinations gave rise to endometrial tumors in the context of cell-autonomous loss of PTEN evidenced by presence of tumor attached to the kidney (Fig. 1A and Supplementary Fig. S1A). This mouse tumor model is highly reproducible, resulting in the formation of PTEN-null tumors that share histologic features and molecular markers of human endometrioid endometrial cancer with 100% efficiency (Supplementary Fig S1A; ref. 22). Sixteen independent tumor-bearing mice were supplemented with fresh estrogen pellets to maintain a high estrogen milieu then separated into two groups. The first eight mice were treated daily with olaparib at 50 mg/kg, whereas the remaining eight mice were treated on the same schedule with 10% dimethyl sulfoxide (DMSO) vehicle for 3 weeks (Fig. 1B). All treatments were delivered by oral gavage to replicate the oral route of administration in patients. Prior to the initiation of therapy, drug efficacy was verified in vitro (Supplementary Fig. S1B). After 3 weeks of treatment, tumors were harvested and analyzed. Olaparib treatment had no effect on tumor size or mass in this hormonal milieu (Fig. 1C). Tumors treated with olaparib or vehicle had similar histology, characterized by infiltrating glands mostly with lumens containing atypical and prominent nucleoli (Fig. 1D, a and b vs. d and e). In these tumors, only the epithelia was PTEN-null whereas the stroma expressed PTEN (Fig. 1D, c and f and Supplementary Fig. S1C). The expression of estrogen receptor alpha (ERα) was similar in olaparib- and vehicle-treated controls (Supplementary Fig. S1D). To validate our results, this experiment was performed on an additional cohort of mice confirming that olaparib treatment had no significant effect on the size of PTEN-null tumors in a high estrogenic hormonal milieu (Supplementary Fig S1E).

These findings suggest that olaparib was ineffective in treating PTEN-null tumors exposed to high levels of estrogen. One possibility is that PARP inhibition is not effective against PTEN-null tumors in an in vivo setting. Alternatively, high levels of estrogen may induce resistance to PARP inhibition.
Low levels of circulating estrogen sensitized PTEN-deficient endometrial cancers to the antitumor effects of olaparib

Our results demonstrate that PTEN-null endometrial tumors are resistant to PARP inhibition in a high estrogenic milieu. Clinically, endogenous estrogen levels can be reduced by surgical removal of ovaries and coadministration of aromatase inhibitors. Aromatase inhibitors block the conversion of adrenal androgens to estrogen in adipose tissue (31). Given the feasibility of this clinical approach, we wanted to test the efficacy of PARP inhibitors in achieving tumor response in a low estrogen state. We hypothesized that reduced of estrogen levels might sensitize PTEN-null endometrial tumors to the synthetic lethal effects of PARP inhibitors.

PTEN-null endometrial tumors were established in 17 independent oophorectomized, estrogen-supplemented SCID (n = 8) or vehicle- (n = 8) treated tumors (P = 0.70). D, endometrioid tumors (a and d) containing crowded pankeratin-positive (b and e) PTEN-null (c and f) epithelial glands with lumens were observed in both vehicle- and olaparib-treated groups. Scale bars, 5 mm in A and C and 100 μm in D.

Figure 1. PTEN-deficient endometrial carcinomas were refractory to PARP inhibition with high levels of circulating estrogen. A, schema for in vivo generation and therapy of endometrial tumors driven by epithelial loss of PTEN in a high estrogen milieu. B, design for therapy with hormonal supplementation. C, no significant difference in tumor mass was detected between olaparib- (n = 8) or vehicle- (n = 8) treated tumors (P = 0.70). D, endometrioid tumors (a and d) containing crowded pankeratin-positive (b and e) PTEN-null (c and f) epithelial glands with lumens were observed in both vehicle- and olaparib-treated groups. Scale bars, 5 mm in A and C and 100 μm in D.
whereas remaining mice received vehicle (Fig. 2B). After 3 weeks of therapy, all tumors were harvested and analyzed. Estrogen deprivation was confirmed in this experimental cohort as compared with estrogen supplemented mice (Supplementary Fig. S2B). The concentration of estrogen in our hormone-depleted mice was within the range of plasma estradiol concentrations reported in postmenopausal women with or without aromatase therapy (32). Olaparib treatment resulted in a 6-fold decrease in tumor mass when administered under low levels of circulating estrogen (Fig. 2C). Despite the efficacy of this treatment in reducing tumor size, olaparib did not eradicate the PTEN-null tumors demonstrated by evidence of residual cancer in these tissues (Fig. 2D, a-d) that were pankeratin positive (b and e) but PTEN negative (c and f). Scale bars, 5 mm in A and C and 100 μm in D.

Figure 2. PARP inhibition decreased tumor mass when administered under low levels of circulating estrogen. A, strategy for therapy of PTEN-null tumors in a low estrogen milieu. B, treatment design. C, PARP inhibition with low circulating estrogen levels significantly decreased tumor mass compared with vehicle-treated controls (n = 8 per group, \( P < 0.0001 \)). D, both olaparib- and vehicle-treated tumors contained neoplastic epithelial glands with lumens (a and d) that were pankeratin positive (b and e) but PTEN negative (c and f). Scale bars, 5 mm in A and C and 100 μm in D.

Targeting PTEN-Null Endometrial Tumors with PARP Inhibition

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would impact response to this combined therapy (olaparib + estrogen depletion), the number of implanted epithelia and stroma were reduced approximately 2.5-fold. Results of this experiment confirmed previous findings by showing olaparib resistance in a high estrogenic milieu (Fig 3B). In this experiment, complete resolution of PTEN-null tumors treated with olaparib in a low estrogenic milieu was found (Fig. 3B), suggesting that the initial tumor burden may impact response to this dual therapy.

Our results suggest that modulation of the hormonal milieu can sensitize PTEN-deficient endometrial carcinomas to olaparib therapy. No response to olaparib was noted when estrogen levels were high. The same dose of olaparib, in an estrogen-deprived milieu, resulted in significant decreases in tumor size. A number of factors could underpin this dichotomous response, including effects of estrogen on drug metabolism or components of the HR DNA repair pathway.

Figure 3. Levels of estrogen modulate the sensitivity of PTEN-null endometrial tumors to PARP inhibition in vivo. A, schema for generation and treatment of tumors. B, with estrogen supplementation tumors were resistant to PARP inhibition based on histology and tumor weight. Estrogen deprivation sensitized tumors to PARP inhibition, as demonstrated by reduction in graft size and resolution of tumor tissue.
Estrogen depletion maximized PARP inhibition at the tumor bed concomitant with an increase in circulating levels of olaparib

To assess the bioavailability and activity of olaparib in a high and low estrogenic milieu, we measured serum levels of olaparib in experimental mice and its efficacy in inhibiting PARP activity in the tumors. Effects of olaparib on tumor cell proliferation and apoptosis-induced cell death were also examined in each hormonal condition.

To measure the kinetics of drug response, serum and tumors were collected from tumor-bearing mice sacrificed at 30 minutes, and 2, 6, and 24 hours after olaparib administration. Serum olaparib concentrations were measured using mass spectrometry. To assess inhibition of PARP enzyme, poly-ADP ribose levels, a surrogate marker for PARP activity (33), were measured in tumor extracts. In mice supplemented with estrogen, serum olaparib concentrations peaked at 2 hours coincident with maximal PARP inhibition in the tumor (Fig. 4A, a). No differences were detected in the epithelial tumor proliferation (Fig. 4A, b) or apoptosis (Fig. 4A, c). After 2 hours, serum levels of olaparib steadily declined, concomitant with decreased PARP inhibition in the tumor (Fig. 4A, a). The serum olaparib concentration achieved with the dose in our study (50 mg/kg daily) was 5.2 µg/mL at peak concentration. This is comparable with serum concentrations of 5.9 µg/mL in patients treated with a commonly administered dose of olaparib (400 mg twice/d; ref. 34). In tumor-bearing mice subjected to low estrogen levels, serum olaparib concentrations peaked earlier at 30 minutes and were 20-fold higher than in estrogen-supplemented mice (Fig. 4B, a vs. 4A, a). An olaparib-induced increase in tumor apoptotic cell death (Fig. 4B, c) was detected in this hormonal milieu without a change in the proliferation (Fig. 4B, b). In this low estrogenic milieu, a profound and sustained inhibition of PARP enzyme activity was detected in the tumor bed (Fig. 4B, a), likely resulting in increased cell death and decreased tumor size.

CYP3A4, the primary enzyme that metabolizes olaparib in humans (35, 36), is reported to be regulated by estrogen (37). To account for difference in circulating levels of olaparib in each hormonal milieu, we examined the expression of CYP3A41, a mouse homolog of CYP3A4, in the livers of experimental mice. Significantly higher levels of CYP3A41 transcript was detected in estrogen-treated compared with estrogen-depleted mice (Supplementary Fig S3A). These findings were confirmed on Western blot analysis (Supplementary Fig S3B). This may explain why concentrations of olaparib were lower in estradiol-supplemented mice. Definitive testing of this hypothesis would require measurement of PARP metabolites in each hormonal condition, an approach that was not feasible as specific metabolites for olaparib are not published or disclosed.

Results here demonstrate that the bioavailability and activity of olaparib were enhanced in a low estrogenic hormonal milieu in our *in vivo* model. This may be one mechanism sensitizing estrogen-deprived PTEN-null endometrial tumors to olaparib therapy. However, olaparib resistance was noted despite measurable inhibition of PARP enzyme activity in estrogen-supplemented mice. Failure of olaparib therapy in this cohort may be due to an intact HR pathway repairing olaparib-induced DNA damage.

Rad51 function in a high estrogen milieu may promote resistance of PTEN-null endometrial tumors to PARP inhibition

Homologous recombination requires the action of a large complement of proteins that normally safeguard the genome (15). If the HR is defective, cells become susceptible to synthetic lethal effects of PARP inhibitors. Previous work suggests that estrogen may regulate the expression of key components of the HR pathway (38–40). Given that the expression and function of Rad51 has been implicated as a potential biomarker of response to PARP inhibition (17, 18, 41), we first examined the expression of Rad51 protein in PTEN-null tumors propagated in low and high estrogen environments.

Western blotting was performed to measure levels of Rad51 in tumor lysates from three independent mice treated with olaparib or vehicle in low or high estrogenic milieu. In the presence of estrogen, significant amounts of tumor Rad51 were detected (Fig. 5A). Conversely, when estrogen was withdrawn a dramatic reduction in tumor Rad51 expression was noted (Fig. 5A). To test whether estrogen deprivation altered the expression of other HR proteins, levels of BRCA1 were also measured in tumor lysates (Fig. 5A). Similar to Rad51, higher levels of BRCA1 were detected in estrogen-supplemented versus estrogen-deprived PTEN-null tumors (Fig. 5A). Our findings demonstrate that the estrogenic hormonal milieu may affect expression of HR proteins in PTEN-null endometrial tumors.

To test the functional implications of reduced Rad51 levels, we examined recruitment of Rad51 to sites of DNA damage in olaparib- or vehicle-treated PTEN-null endometrial epithelial cells exposed to high and low levels of estrogen. Addressing this question requires clear visualization of Rad51 and γH2AX colocalization in nuclei of olaparib-treated cells. Given that nuclei are tightly packed in the tumor, we chose an alternative *in vitro* experimental approach to measure this colocalization. Primary endometrial epithelium harvested from *PTENloxP/loxP* mice were infected with a Cre-red fluorescent protein (RFP)-tagged lentivirus and plated in a three-dimensional (3D) sphere assay (28), supplemented with estrogen or placebo, and cocultured with WT endometrial stroma to model the paracrine interactions between epithelium and stroma seen in tumors (Supplementary Fig. S4). Within 2 weeks, spheroid epithelial structures outgrew from single cells, many of which were RFP positive, indicating expression of Cre and deletion of PTEN. Established spheres were treated with olaparib or vehicle for 36 hours. Following treatment, RFP-positive spheres were handpicked and...
dissociated into single cells (Supplementary Fig. S4). Loss of PTEN was validated in this cell population (Supplementary Fig S5A). A 6-fold reduction in the Rad51 transcript was detected in PTEN-null epithelia exposed to low levels of estrogen, suggesting estrogen modulation of Rad51 expression in vitro (Supplementary Fig. S5B) as well as in vivo (Fig. 5A). To assess if reduced Rad51 levels led to compromised HR, colocalization of Rad51 to γH2AX foci was examined in all experimental groups. Robust recruitment of Rad51 to sites of DNA damage was detected in the nuclei of olaparib-treated, estrogen-exposed PTEN-null endometrial epithelia (Fig. 5B and Supplementary Fig. SSC). In contrast, in the absence of estrogen, a significant reduction in Rad51 recruitment was

Figure 4. Pharmacokinetics and pharmacodynamics of olaparib varied with circulating estrogen levels. A, olaparib was detected in the serum of estrogen-supplemented mice with measurable inhibition of PARP enzyme activity but with no apparent effects on tumor proliferation or apoptosis. a, olaparib serum concentrations were undetectable in vehicle-treated mice, peaked at 2 hours after drug ingestion, and then slowly returned to baseline levels. Inhibition of PARP enzymatic activity at the tumor bed correlated with the olaparib serum concentration. b, epithelial proliferation (pankeratin and Ki67 dual-positive cells; i vs ii, $P = 0.16$), c, increased apoptosis was observed in tumors subjected to PARP inhibition with estrogen deprivation (i vs. ii, $P < 0.0001$). Scale bars, 100 μm.

B, higher serum olaparib levels concomitant with sustained PARP inhibition was detected in estrogen-deprived tumor-bearing mice. a, in estrogen-deprived mice, peak concentrations of serum olaparib were detected 30 minutes after dosing, then dropped to baseline within 24 hours. PARP activity at the tumor bed was sharply decreased with compared with vehicle-treated mice, and PARP inhibition was sustained throughout a 24-hour period. b, PARP inhibition did not diminish epithelial proliferation in estrogen-depleted mice (i vs. ii, $P = 0.57$). c, increased apoptosis was observed in tumors subjected to PARP inhibition with estrogen deprivation (i vs. ii, $P < 0.0001$).
Targeting PTEN-Null Endometrial Tumors with PARP Inhibition

Figure 5. Decreased levels and function of Rad51 may drive the sensitivity of PTEN-null tumors to PARP inhibition in estrogen-depleted conditions. A, Western blot reveals diminished expression of both Rad51 and BRCA1 in lysates of tumors from estrogen-deprived compared with estrogen-supplemented mice regardless of treatment with olaparib or vehicle. 3T3 epithelial cells supplemented with estrogen (a) or no (g–i) levels of Rad51 incorporation into γH2AX foci were observed compared with that in estrogen-supplemented mice (P < 0.0001). Olaparib-induced γH2AX foci formation was unaffected by estrogen deprivation (P = 0.29). Scale bars, 10 μm.

Noted despite olaparib treatment (Fig. 5B and Supplementary Fig. S5C). In the absence of olaparib, γH2AX foci formation was minimal (Supplementary Fig. S5D). Findings demonstrate that the recruitment of Rad51 is compromised in estrogen-deprived conditions.

Based on our results, PTEN-null endometrial tumors are sensitized to PARP inhibition in a low estrogenic milieu. When estrogen is depleted, increased amounts of DNA damage may occur due to sustained PARP enzyme inhibition at the tumor bed as well as impaired Rad51-mediated HR. High levels of estrogen may induce PARP resistance in PTEN-null endometrial tumors by lowering levels of circulating olaparib in vivo, resulting in less PARP inhibition in the tumor. Simultaneously, robust expression and recruitment of Rad51 in this hormonal milieu may induce efficient tumor DNA repair. The net result is resistance to olaparib therapy when estrogen levels are high (Fig. 6).

Discussion

PARP inhibitors have been tested in clinical trials as single-agent therapies against hormonally regulated solid tumors (reviewed in ref. 20). Although administration of this targeted therapy has been coupled with chemotherapeutic agents, no studies have examined the efficacy of PARP inhibition in conjunction with hormonal blockade (reviewed in ref. 20). Results of our experiments demonstrate that estrogen levels affect both the availability of olaparib and efficiency of cell death induced by PARP inhibition. When estrogen levels were high, resistance to olaparib was noted in PTEN-null endometrial tumors. In contrast, a measurable tumor response to PARP inhibition was detected with the same dose of olaparib in a low estrogenic milieu.

In clinical trials of patients with breast (42) or ovarian cancer (10), improved outcomes were reported with administration of higher olaparib doses. An unexpected finding in our study was that estrogen significantly impacted serum concentrations of olaparib. Despite the administration of a fixed dose of olaparib in our experiments, higher levels of circulating olaparib coupled with greater and sustained PARP inhibition at the tumor bed was detected upon estrogen depletion. The cytochrome P450 superfamily of enzymes play a role in hepatic drug metabolism (43). A member of this family, CYP3A4, is thought to be the enzyme responsible for olaparib metabolism in humans (35, 36). In our experiments, an upregulation of CYP3A4 was detected with the same dose of olaparib in a low estrogenic milieu. When estrogen is depleted, increased amounts of DNA damage may occur due to sustained PARP enzyme inhibition at the tumor bed as well as impaired Rad51-mediated HR. High levels of estrogen may induce PARP resistance in PTEN-null endometrial tumors by lowering levels of circulating olaparib in vivo, resulting in less PARP inhibition in the tumor. Simultaneously, robust expression and recruitment of Rad51 in this hormonal milieu may induce efficient tumor DNA repair. The net result is resistance to olaparib therapy when estrogen levels are high (Fig. 6).

Mechanisms that induce resistance or sensitivity to PARP inhibition remain poorly understood. One challenge in interpreting human trials has been the variability of the biologic response (44), which should be carefully taken into account.

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account in the treatment. An advantage of our model is the use of genetically reproducible tissues from genetically identical backgrounds, which decrease the variability of olaparib response. A second challenge has been a dearth of data analyzing PARP-resistant tumor tissue from patients treated with these agents (20). An advantage of our model is the ability to harvest both PARP-sensitive and -resistant tumors generated from the same genetic background. Analysis of this tissue revealed that levels of Rad51, a key modulator of HR, were significantly lower in estrogen-deprived compared with estrogen-supplemented tumors. In addition, recruitment of Rad51 to sites of DNA damage was compromised in estrogen-deprived PTEN-null endometrial cells. Our results suggest that estrogen modulation of the HR pathway proteins, such as Rad51, may regulate sensitivity to PARP inhibition. In addition, this relationship may hold true for human endometrial tumors. Microarray analysis of control- and estrogen-treated normal human endometrial tissue demonstrated upregulation of both $Rad51$ (1.5-fold) and $BRCA1$ (2.2-fold) expression upon estrogen treatment (45).

Loss of PTEN function has been proposed as a potential biomarker for sensitivity to PARP inhibition (41). Prior studies examining the effects of PARP inhibition in a PTEN-null background have all utilized cancer cell lines (7, 16). In these cells lines, coexisting genetic defects in addition to PTEN loss may account for defective HR and sensitivity to PARP inhibition. An advantage of our model is that epithelial inactivation of PTEN is the sole genetic change, allowing for a definitive evaluation of PARP response in PTEN-null tumor cells. In this model, PTEN status was not an independent biomarker of sensitivity to PARP inhibition as endometrial tumors were resistant to olaparib therapy in a high estrogenic milieu. However, a significant response to therapy was observed in PTEN-null endometrial tumors when serum estrogen was decreased. PTEN-null cells are proposed to have compromised HR DNA repair that stems from decreased activity of Rad51 (14), but the association between PTEN deletion and Rad51 function has been controversial (7, 14, 16, 17). Here, we find that Rad51 expression and recruitment to sites of DNA damage was robust in PTEN-null epithelia exposed to high estrogen levels. With estrogen deprivation, Rad51 protein levels were diminished and its function was decreased in PTEN-null epithelia. We suspect that estrogen-mediated regulation of Rad51 in PTEN-null endometrial epithelia is a regulator of PARP sensitivity in our model.

Collectively, our findings suggest that the use of PARP inhibitors in combination with hormonal therapy could provide an exciting novel approach to the treatment of PTEN-null endometrial cancers. Preclinical studies have demonstrated the chemo- and radiosensitizing activity of PARP inhibitors, but despite these positive results, a major limiting factor in translation to clinical trials has been the untoward toxicities of chemo- and radiotherapies (46). Aromatase inhibitors and estrogen-receptor antagonists are well-tolerated medications that can decrease the
production and block the effects of estrogen (31, 47). Although they have been unsuccessful as monotherapy against endometrial cancer (48, 49), they could be used to sensitize estrogen-dependent tumors to PARP inhibition. Our preclinical findings support this combined approach and merit progression into clinical trials investigating the synergistic effects of PARP inhibitors and hormonal ablation in patients with PTEN-null endometrial cancers. We recognize that there are limitations in extrapolating the data from these experiments to human patients, as the hormonal manipulations in our mouse model do not completely represent the hormonal milieu of patients with endometrial cancer. However, some similarities do exist. Similar to our model, almost all endometrial cancer patients are oophorectomized as part of their treatment. A recent case report showed that the administration of olaparib was efficacious in the therapy of a patient with a PTEN-null endometrial tumor and a predicted low estrogen level due to removal of her ovaries (50). Based on our findings, the combined use of PARP inhibition and hormonal ablation should also be tested in breast and prostate cancer tumor models. We demonstrate that the hormonal milieu can profoundly affect sensitivity to PARP inhibition in this preclinical in vivo endometrial cancer model. Future work by our group will focus on testing this therapeutic strategy in endometrial tumors driven by alternative genetic pathways.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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