Vulnerabilities of PTEN-TP53-Deficient Prostate Cancers to Compound PARP-PI3K Inhibition

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ABSTRACT
Prostate cancer is the most prevalent cancer in males, and treatment options are limited for advanced forms of the disease. Loss of the PTEN and TP53 tumor suppressor genes is commonly observed in prostate cancer, whereas their compound loss is often observed in advanced prostate cancer. Here, we show that PARP inhibition triggers a p53-dependent cellular senescence in a PTEN-deficient setting in the prostate. Surprisingly, we also find that PARP-induced cellular senescence is morphed into an apoptotic response upon compound loss of PTEN and p53. We further show that superactivation of the prosurvival PI3K-AKT signaling pathway limits the efficacy of a PARP single-agent treatment, and that PARP and PI3K inhibitors effectively synergize to suppress tumorigenesis in human prostate cancer cell lines and in a Pten/Trp53-deficient mouse model of advanced prostate cancer. Our findings, therefore, identify a combinatorial treatment with PARP and PI3K inhibitors as an effective option for PTEN-deficient prostate cancer.

SIGNIFICANCE: The paucity of therapeutic options in advanced prostate cancer displays an urgent need for the preclinical assessment of novel therapeutic strategies. We identified differential therapeutic vulnerabilities that emerge upon the loss of both PTEN and p53, and observed that combined inhibition of PARP and PI3K provides increased efficacy in hormone-insensitive advanced prostate cancer. Cancer Discov; 4(8); 896–904. © 2014 AACR.

INTRODUCTION
There is an urgent need for the development of novel successful strategies for advanced prostate cancer to improve patient outcomes (1, 2).

The loss of at least one allele of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is observed in more than 40% of prostate cancers (3, 4). As a lipid phosphatase, PTEN suppresses the activation of the PI3K-AKT signaling cascade that is a central proto-oncogenic signaling pathway.
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**RESULTS**

**Common Genetic Alterations in Prostate Cancer Morph Senescence into Apoptosis in Response to PARP Inhibition**

We first aimed to determine whether common genetic alterations in prostate cancer respond toward olaparib. To mimic different stages of prostate cancer progression, we selected Pten+/− (early stage), Pten/Lxl/Lxl (advanced hormone sensitive), and Pten/Lxl/Lxl;Trp53/Lxl/Lxl mouse embryonic fibroblasts (MEF; advanced hormone insensitive; Supplementary Fig. S1A) to perform a cell proliferation assay upon treatment with 10 μmol/L olaparib. Interestingly, MEFs of all genotypes (Fig. 1A–C) showed a significant growth inhibition upon exposure to olaparib. Surprisingly, in contrast with Pten/Lxl/Lxl;Trp53/Lxl/Lxl MEFs, Pten+/− and Pten/Lxl/Lxl MEFs showed a robust senescence response (Supplementary Fig. S1B and S1C) that was significant and dose dependent in Pten+/− (Fig. 1D) as well as Pten/Lxl/Lxl;Trp53/Lxl/Lxl MEFs compared with wild-type MEFs. Strikingly and surprisingly, further analysis revealed that in contrast to Pten/Lxl/Lxl MEFs, Pten/Lxl/Lxl;Trp53/Lxl/Lxl MEFs showed a significant increase in apoptosis rather than senescence (Supplementary Fig. S1D and S1E) as analyzed by Annexin V staining (Supplementary Fig. S1F and S1G) and detection of cleaved caspase-3/7 (Fig. 1G).

Western blot analysis of Pten/Lxl/Lxl and Pten/Lxl/Lxl;Trp53/Lxl/Lxl MEFs treated with increasing concentrations of olaparib revealed that whereas Pten/Lxl/Lxl MEFs showed a further increase in p53 protein levels, both Pten/Lxl/Lxl and Pten/Lxl/Lxl;Trp53/Lxl/Lxl MEFs showed increased DNA damage as visualized by γH2AX staining (Fig. 1H). This analysis demonstrates that the senescence response in Pten+/− is likely driven by the induction of p53, as previously described (13). However, the concomitant loss of p53 induces increased DNA damage that in turn morphs this phenotype into an apoptotic response.

**PARP Inhibition Induces a Differential Response In Vivo with a Modest Effect on Overall Tumor Response**

To validate our findings in vivo, we next studied the sensitivity of genetically engineered mouse models (GEMM) of

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Figure 1. Common genetic alterations in prostate cancer morph senescence into apoptosis in response to PARP inhibition. Growth of Pten+/− (A), Pten/Lxl/Lxl (B), and Pten/Lxl/Lxl;Trp53/Lxl/Lxl (C) MEFs in the presence of 10 μmol/L olaparib (**, P < 0.0032; ***, P < 0.0001). D, quantification of SA-β-gal positivity in Pten+/− and Pten/Lxl/Lxl MEFs. (continued on following page)
tumors of MEFs, pharmacologic inhibition of PARP induced a strong response in vitro. Analysis of cytokeratin 14 showed a reduction of the vehicle control (Fig. 2D and Supplementary S3A). In addition, more glands were lined by a single layer compared with the luminal cells of olaparib-treated prostates revealed that only approximately 2 μmol/L olaparib for 48 hours (*, P < 0.01, **, P < 0.001). Western blot (WB) analysis of Pten+/− and Pten+/−;Trp53−/− MEFs after 3 days of olaparib treatment. WT, wild-type; fc, fold change.

Figure 1. (Continued) E, MEFs upon increasing doses of olaparib at day 4 (*, P < 0.05). F, quantification of SA-β-gal positivity in Pten+/− compared with Pten+/−;Trp53−/− MEFs upon increasing doses of olaparib at day 4 (*, P < 0.05). G, quantification of caspase-3/7 activity after treatment with 10 μmol/L olaparib for 48 hours (**, P < 0.01; ***, P < 0.001). H, Western blot (WB) analysis of Pten+/− and Pten+/−;Trp53−/− MEFs after 3 days of olaparib treatment. WT, wild-type; fc, fold change.

Next, we tested whether Pten+/−;Trp53−/− mice showed a similar apoptotic response upon treatment with olaparib as observed in vitro. In line with the in vitro data, olaparib treatment increased γH2AX in dorsolateral prostate (DLP) tumors of Pten+/−;Trp53−/− mice (Supplementary Fig. S2C). Surprisingly, macroscopic analysis and cytokeratin 8 staining (luminal cells) of olaparib-treated prostates revealed that more glands were lined by a single layer compared with the vehicle control (Fig. 2D and Supplementary S3A). In addition, analysis of cytokeratin 14 showed a reduction of the intermediate basal cell population in single-layered glands, suggesting a certain degree of normalization after treatment (Supplementary Fig. S3B). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and caspase-3 staining further revealed a significant increase in apoptotic cells upon olaparib treatment (Fig. 2E and F and Supplementary Fig. S3D). However, similar to Pten+/− mice, histologic analysis of Pten+/−;Trp53−/− tumor reduction after drug treatment did not reach statistical significance (Fig. 2G), suggesting that single-agent olaparib treatment is not sufficient to induce a robust antitumor response in these models. Interestingly, mass spectrometry analysis of olaparib in prostates revealed that only approximately 2 μmol/L of the drug is delivered into the individual lobes, an amount that is significantly lower when compared with the dose used in our in vitro studies (Supplementary Fig. S3C). This marked difference in drug concentration may in turn provide one possible explanation for the limited overall tumor response in vivo.

Hyperactivation of the PI3K–AKT Pathway Affects Sensitivity toward Olaparib

On the basis of our data in mice, we sought to determine whether olaparib could trigger a similar response in human prostate cancer cell lines. To this end, we engineered LNCaP prostate cancer toward PARP inhibition. Similar to our in vitro analysis, we enrolled Pten+/−, Pten+/−;Probasin-Cre (referred to as Pten+/−) and Pten+/−;Trp53+/−;Probasin-Cre (referred to as Pten+/−;Trp53++−) mice. In line with the data observed in MEFs, pharmacologic inhibition of PARP induced a strong and significant induction of senescence in Pten+/− (Supplementary Fig. S2A) and Pten+/− (Fig. 2A and B) models compared with vehicle-treated controls. In Pten+/− mice, the senescence response was accompanied by increased DNA damage as analyzed by γH2AX staining of treated prostate tumors (Supplementary Fig. S2B). Histologic analysis of Pten+/− tumors treated with olaparib revealed a modest decrease in high-grade prostatic intraepithelial neoplasia (HGPIN; Fig. 2C). However, this trend did not reach statistical significance.

In vivo
**Figure 2.** PARP inhibition induces a differential response in vivo with a modest effect on overall tumor response. A, SA-β-gal staining in prostates of 8-week-old *Pten<sup>pc−/−</sup>* mice upon olaparib (*n* = 3) or vehicle (*n* = 3) treatment for 2 weeks. B, quantification of SA-β-gal positivity from A (*P* = 0.0419). C, histopathologic analysis of HGPIN status from A. D, hematoxylin and eosin (H&E) staining of DLP tumors from 4-month-old *Pten<sup>pc−/−</sup>;Trp53<sup>pc−/−</sup>* mice upon olaparib (*n* = 3) or vehicle (*n* = 3) treatment for 1 week. E, TUNEL staining to visualize apoptosis induction in D and its quantification (*P* = 0.0006). F, histopathologic analysis of HGPIN status from D. ns, not significant.
cells that lack PTEN to additionally lose p53 function by overexpressing either a p53 short hairpin construct (Supplementary Fig. S4A) or dominant-negative p53 (p53-DN; Supplementary Fig. S4B). Similar to MEFs, olaparib induced senescence in a p53-proficient setting that was blunted upon loss of p53 (Fig. 3A). In contrast, LNCaP cells expressing p53-DN did not show differential sensitivity to olaparib treatment. Furthermore, only high olaparib concentrations (10 μmol/L) induced a strong growth inhibition overall (Fig. 3B), suggesting mechanisms limiting olaparib efficacy beyond the limited uptake observed in vivo.

We therefore investigated whether classical survival signaling such as the PI3K–AKT pathway might be superactivated upon olaparib exposure and therefore analyzed AKT activation upon olaparib treatment in vitro (7, 14–16). Indeed, Pten<sup>−/−</sup>;Trp53<sup>−/−</sup> MEFs (Fig. 3C) and LNCaP cells (Fig. 3D) hyperactivated AKT upon exposure to olaparib, suggesting that AKT could affect the response to olaparib. Indeed,
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knockdown of AKT1, the major AKT isoform, significantly increased growth inhibition upon PARP inhibition (Supplementary Fig. S4C and 3E) and induced apoptosis, as shown by increased PARP cleavage (Fig. 3F).

To investigate the mechanism of AKT hyperactivation, we made use of the dual PI3K inhibitor BKM120 that efficiently blocked AKT activation in LNCaP cells (Fig. 3F). Strikingly, PI3K inhibition was able to restore pAKT to DMSO control levels in olaparib-treated cells, suggesting that the major activator of AKT upon PARP inhibition is indeed PI3K (Fig. 3G). Importantly, analysis of cleaved PARP demonstrated that the combination of PARP and PI3K inhibition shows synergistic apoptosis induction, suggesting efficacy for a combinatorial treatment strategy in prostate cancer (Fig. 3G).

Combination of PARP and PI3K Inhibitors as a Novel Therapeutic Approach

On the basis of our findings, we next aimed to investigate whether a combination treatment including PARP and PI3K inhibition would be synergistic in vitro. To this end, we treated Pten+/−;Trp53+/− MEFs and human prostate cancer cells with either single or combination therapy (Supplementary Fig. S4D–S4G). Indeed, the combination significantly increased growth suppression compared with the single treatments in all tested cell lines.

These promising in vitro data prompted us to next evaluate the efficacy of the combinatorial inhibition of PARP and PI3K in our Pten−/−;Trp53+/− model of highly aggressive prostate cancer (Supplementary Fig. S5A). In vivo efficacy of control, single agent, and the combination arm was assessed by MRI analysis for the dorso-lateral prostate (DLP) and the anterior prostate (AP; Supplementary Fig. S5B and S5C), followed by the analysis of survival and histopathologic differences.

To assess the efficacy of PARP and PI3K inhibition in vivo, we first followed tumor progression by MRI (Fig. 4A). Interestingly, treatment with olaparib or BKM120 showed tumor stabilization but eventually tumors regrew due to potential resistance mechanisms. Strikingly, the combination of the two compounds potentiated tumor stabilization and induced robust tumor regression. These data indicate that the combinatorial treatment of PI3K and PARP inhibition is synergistic to the single-compound arms in vivo. In contrast with the DLP, only slight growth suppression was seen in APs that was potentiated in the combination, suggesting a similar cooperation as seen in the DLP albeit with a much lower efficacy (Supplementary Fig. S6A).

We further analyzed tumor specimens of all treatment arms after 1 to 4 weeks by histopathology (Fig. 4B and C and Supplementary S6B and S6C). Surprisingly, despite the initial response toward olaparib observed at 1 week, histopathologic analysis and cytokeratin 8 staining revealed no difference in the occurrence of HGPIN after 4 weeks compared with the vehicle control. In contrast, the BKM120 treatment arm triggered a clear decrease in HGPIN. Importantly, the combinatorial treatment significantly potentiated this effect, clearly showing cooperation between the two compounds. Furthermore, cytokeratin 14 staining in the DLPs treated with the combination showed a clear reduction in the intermediate basal cell population that is not apparent in the vehicle- or single-arm-treated tumors. These data indicate a trend toward normalization of the glands specifically in the combination treatment.

In line with these data, progression-free survival (PFS) was markedly improved in the combinatorial treatment arm compared with the single and control arms (Fig. 4D and Supplementary S7A and S7B). However, and coherent with the lack of response in the AP, overall survival (OS) analysis did not reveal a striking difference between the single and combination treatment arms (Supplementary Fig. S7C).

Finally, to determine whether the data obtained in our GEMMs could be confirmed in human cell lines, we performed xenograft studies using PC3 prostate cancer cell lines (Fig. 4E). Similar to our GEMMs, PC3 xenografts displayed a significant and synergistic response when treated with the combination compared with the single agents. Collectively, these data strongly support the notion of a marked cooperative effect between the two drugs for the treatment of prostate cancer.

DISCUSSION

This study allowed us to reach a number of important conclusions:

First, we demonstrated, in vitro as well as in vivo, that PTEN deficiency triggers sensitivity toward PARP inhibition with differential responses. In a p53-proficient setting, loss of PTEN triggers a senescence response upon PARP inhibition that is morphed into apoptosis upon loss of p53.

Second, a robust response toward PARP inhibition was observed only at very high concentrations of olaparib in cell lines, whereas mouse DLPs treated with olaparib displayed no significant difference in HGPIN grades. In line with these observations, we find that olaparib reaches only suboptimal concentration in vivo in the prostate.

Third, we found that olaparib triggers the activation of AKT, a classical cell survival mechanism in response to cellular stresses (7, 14–16). These data demonstrated that olaparib treatment on its own is insufficient to trigger a significant tumor response in the prostate due to a number of reasons.

Fourth, we showed that PI3K inhibition by BKM120 significantly increased sensitivity toward olaparib both in GEMMs and xenograft models. Importantly, PC3 xenografts showed a clear synergistic effect of growth inhibition in the combination arm in vivo, validating the efficacy of the combination in a human setting. Intriguingly, these data, especially in PC3 xenografts, are in clear contrast with the in vitro data that rather suggested an additive effect of the combination. These results could be explained by the profound differences in nutrients and growth factors in the two experimental settings, or by the fact that the tumor microenvironment/metabolism may also affect the efficacy of the treatments, as previously suggested (17–19).

Finally, despite these striking findings, combinatorial PARP and PI3K inhibition prolonged PFS and OS significantly only when compared to vehicle, but not to single-agent–treated animals. However, these data are potentially influenced by the general characteristics of Pten−/−;Trp53+/− prostate tumors (13). Cancers in the APs in this model are intrinsically resistant to therapy, including androgen deprivation, and showed only a slight growth inhibition in all of the treatment arms.
Figure 4. Combination of PARP and PI3K inhibitors as a novel therapeutic approach. A, DLP tumor volume change of Pten<sup>−/−</sup>;Trp53<sup>−/−</sup> mice upon vehicle (n = 7), olaparib (n = 8), BKM120 (n = 6), or combination (n = 9) treatment. B, histopathologic analysis of HGPIN status from vehicle (n = 3, 1 and 4 weeks pooled), olaparib (n = 3), BKM120 (n = 3), or combination (n = 3) treatment for 4 weeks (*, P < 0.03). C, H&E staining from A. D, PFS of Pten<sup>−/−</sup>;Trp53<sup>−/−</sup> mice upon vehicle (n = 7), olaparib (n = 8), BKM120 (n = 6), or combination (n = 9) treatment. ns, not significant. E, tumor growth of PC3 xenografts upon vehicle (n = 5), olaparib (n = 5), BKM120 (n = 4), or combination (n = 5; **, P < 0.009) treatment; olaparib, 50 mg/kg; BKM120, 30 mg/kg.
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(20). However, the rapid growth of the APs strongly influences the survival of Pten−/−;Trp53−/− mice and, thus, strongly affects the assessment and interpretation of OS.

In conclusion, we show that PTEN-deficient prostate cancer is sensitive to PARP inhibition. Because of AKT hyperactivation and poor pharmacokinetics, the in vivo efficacy of olaparib is modest, but concomitant suppression of PARP and PI3K results in a cooperative antitumor effect. Thus, the combination of PI3K and PARP inhibitors represents a promising therapeutic approach for the treatment of PTEN-deficient prostate tumors.

METHODS

Cells

Pten−/−, Pten−/−, and Pten−/−;Trp53−/− MEFs were prepared as previously described. For the excision of target genes, MEFS were retrovirally infected with pMSCV–Cre–PURO–IRES–GFP (13).

Human cell lines were obtained from the ATCC and cultured according to the manufacturer’s instructions. Cell lines were tested for mycoplasma (MycoAlert; Lonza), but not further authenticated.

Cell Proliferation, Senescence, and Apoptosis Assays

To study proliferation, cells were plated in a 12-well plate (MEFs at 1×10⁴ cells/well and prostate cancer cells at 1×10⁵ cells/well). After 2 to 9 days, cells were washed with PBS, fixed with 10% paraformaldehyde, and stained with crystal violet. Crystal violet was extracted with 10% acetic acid and absorbance was detected at 595 nm.

To determine senescence, cells were plated in a 6-well plate (MEFs at 3.4×10⁴ cells/well and LNCAp at 1.7×10⁴ cells/well). SA-β-Gal was detected after 4 days with the Senescence Detection Kit (Calbiochem) following the manufacturer’s instructions. For quantification, more than 200 cells per sample were counted.

For prostate tissue, frozen sections (6 mm) were stained for mycoplasma (MycoAlert; Lonza), but not further authenticated.

Western Blot Analysis and Immunohistochemistry

For Western blotting, cell lysates were prepared with RIPA buffer (Promega) and additionally analyzed by a dual Annexin V–APC/7-AAD (BD Biosciences) staining using flow cytometry following the manufacturer’s instructions.

For in vivo apoptosis, samples were analyzed with the In Situ Cell Death Detection Kit (Roche) following the manufacturer’s instructions. For the quantification of positive TUNEL staining, a total of 500 cells were counted from five different fields.

Statistical and Histological Analyses

For all statistical analyses, GraphPad Prism 6 software was used, and values of P < 0.05 were considered statistically significant. Survival was determined by Kaplan–Meier curves. The Mantel-Cox test was used to determine significance between survival curves. All other statistical analyses were done by an unpaired Student t test. Scoring of HGPIN status was performed as previously described (23). At least three samples were analyzed at the accorded time points. For the preclinical assessment of the combinational treatment in Pten−/−;Trp53−/− mice, at least three mice were analyzed after 4 weeks of treatment except the vehicle control, in which mice were pooled from 1- and 4-week treatment.

Disclosure of Potential Conflicts of Interest

L.C. Cantley has received a commercial research grant from GlaxoSmithKline and is a consultant/advisory board member for Novartis. No potential conflicts of interest were disclosed by the other authors. The Editor-in-Chief of Cancer Discovery (L.C. Cantley) is an author of this article. In keeping with the AACR’s Editorial Policy, the paper was peer reviewed and an AACR journal editor not affiliated with Cancer Discovery rendered the decision concerning acceptability.

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