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Targeting Plk1 to enhance efficacy of Olaparib in castration-resistant prostate cancer

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Abstract

Olaparib is a FDA-approved PARP inhibitor (PARPi) that has shown promise as a synthetic lethal treatment approach for BRCA-mutant castration-resistant prostate cancer (CRPC) in clinical use. However, emerging data has also shown that even BRCA-mutant cells may be resistant to PARPi. The mechanistic basis for these drug resistances is poorly understood. Polo-like kinase 1 (Plk1), a critical regulator of many cell cycle events, is significantly elevated upon castration of mice carrying xenograft prostate tumors. Herein, by combination with Plk1 inhibitor BI2536, we show a robust sensitization of Olaparib in 22RV1, a BRCA1 deficient CRPC cell line, as well as in CRPC xenograft tumors. Mechanistically, monotherapy with Olaparib results in an override of the G1/S checkpoint, leading to high expression of Plk1, which attenuates Olaparib's overall efficacy. In BRCA1 wild type C4-2 cells, Plk1 inhibition also significantly increases the efficacy of Olaparib in the presence of p53 inhibitor. Collectively, our findings not only implicate the critical role of Plk1 in PARPi resistance in BRCA-mutant CRPC cells, but also shed new light on the treatment of non-BRCA-mutant patient subgroups who might also respond favorably to PARPi.

Keywords

Plk1; Olaparib; combination therapy; Castration-resistant prostate cancer

Introduction

Prostate Cancer (PCa) is the second leading cause of cancer death in males in the United States, with 180,890 new cases and 26,120 deaths estimated in 2016 (1). The androgen receptor (AR) signaling pathway, which controls the growth of PCa cells, including those in CRPC, is a valid target for treatment (2). Consequently, current approaches to treat CRPC are to delay or replace treatment with cytotoxic agents (e.g., docetaxel) with androgen signaling inhibitors (ASIs), such as abiraterone and enzalutamide, instead (3,4). Despite success in targeting AR signaling pathway, resistance to these ASIs invariably develops (5).

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Therefore, it is urgent to identify novel therapeutic targets and strategies to manage this neoplasm.

Poly (ADP-ribose) polymerase 1 (PARP1) is the most abundant and best-characterized member of the family of PARP enzymes (6,7). PARP1 is required for the efficient repair of DNA single strand breaks (SSBs) during base excision repair (BER), and its inhibition leads to persistent single-strand gaps in DNA. If these gaps are encountered by a replication fork, arrest would occur and the single-strand gaps may be converted to double strand breaks (DSBs) (8–10). Normally these DSBs can be repaired by RAD51-dependent homologous recombination (HR), a process in which both BRCA1 and BRCA2 are involved. In the absence of BRCA1 or BRCA2, the replication fork cannot be restarted and collapses, resulting in persistent chromatid breaks (11). The repair of these breaks by alternative error-prone DSB repair mechanisms can cause large numbers of chromatid breaks and aberrations, leading to loss of viability. Thus, PARPi are strikingly toxic to cells with defects in HR (12).

Olaparib, a PARP1 inhibitor, is recently developed and used to target cancers with defects in DNA repair, such as BRCA mutations. It has already been approved by the FDA for treating advanced ovarian cancer associate with defective BRCA genes (13,14) and also granted Breakthrough Therapy designation for the treatment of BRCA-mutant CRPC. Results from the TOPARP-A Phase II clinical trial showed that treatment with Olaparib in patients whose prostate cancers were no longer responding to standard treatments and who had defects in DNA repair genes led to a high response rate (15). However, many groups have also shown that some BRCA-mutant cells may be resistant to PARPi (11,16–18). Thus, the major clinical challenges in terms of Olaparib usage are the acquisition of drug resistance, and defining non-BRCA-mutant patient subgroups who might also response favorably to PARPi.

Plk1, overexpressed in PCa, is linked to higher grade tumors, suggesting that Plk1 is involved in tumorigenesis and progression in this tumor entity (19). Of note, Plk1 is one of the top five upregulated pathways following castration (20). Several potent and selective ATP competitive inhibitors of Plk1, including BI2536 (21) and GSK461364, are in phase I or II clinical studies for patients with various cancers (22,23). In addition, we recently reported that inhibition of Plk1 enhances the efficacy of ASI in CRPC (24). In this study, we discovered that Plk1 and PARP1 interact with each other, and that Plk1 inhibition significantly enhances the efficacy of Olaparib in 22RV1 cells. We also demonstrate that combinatorial inhibition of Plk1 and PARP1 is a novel and therapeutically effective approach to treat PARPi-resistant CRPC in both cultured cells and 22RV1-derived xenograft tumors. Mechanistically, we propose that the G1/S checkpoint override triggered by Olaparib in some BRCA-mutant tumors may result in high expression of Plk1, which greatly reduces the efficacy of Olaparib. Thus, inhibition of Plk1 activity enhances the efficacy of Olaparib.

Materials and Methods

Cell culture and drugs

22RV1, PC3, and MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC). C4-2 cells were obtained from Dr. Leland Chung at the University of

Texas M. D. Anderson Cancer Center in 2010. Refer to Table 1 for information on the genetic backgrounds present in these cell lines. Cells were grown and aliquots were stored in liquid nitrogen for future use. Cells were purchased more than 6 months ago and were not further tested or authenticated by authors. 22RV1 and C4-2 cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 100 units/ml penicillin, 100 units/ml streptomycin at 37°C in 5% CO₂. PC3 cells were cultured in ATCC-formulated F-12K medium supplemented with 10% (vol/vol) FBS and 100 units/ml penicillin, 100 units/ml streptomycin at 37°C in 5% CO₂. MDA-MB-231 cells were cultured in ATCC-formulated Leibovitz's L-15 medium supplemented with 10% (vol/vol) FBS and 100 units/ml penicillin, 100 units/ml streptomycin at 37°C in 5% CO₂. BI2536 and Olaparib were purchased from Symansis and Santa Cruz, respectively. 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) was purchased from Tokyo Chemistry Industry. Pifithrin-1α (PFT1-α) was obtained from Calbiochem-Novabiochem.

Antibodies

Antibody against Plk1 (sc-17783) and PARP1 (sc-8007) were purchased from Santa Cruz Biotech. Antibodies against β-Actin (A-5441), cleaved-PARP (AB6535), and γH2AX (ab26350) were obtained from Sigma, EMD Millipore, and abcam, respectively. All other antibodies were purchased from Cell Signaling.

Immunoblotting (IB)

Upon harvest, cells were re-suspended with TBSN buffer with protease inhibitors and phosphatase inhibitors and sonicated. After lysates were collected, protein concentrations were measured using Protein Assay Dye Reagent from Bio-Rad. Equal amounts of proteins from each sample were mixed with SDS loading buffer and resolved by SDS-PAGE. Upon transferring to PVDF membranes, proteins were probed with indicated antibodies (25).

Immunofluorescent staining (IF)

IF was performed as described previously (20,24). Antibodies were incubated for 1 h at room temperature, followed by incubation with secondary antibody and 4,6-diamidino-2-phenylindole (DAPI, Sigma) for 1 h.

Colony formation assay

Cells (0.5×10^3) were seeded in 6-well plates and cultured in medium alone or containing different drugs for 20 days, with medium refreshment every 2 days. After culturing, cells were fixed in 10% formalin and stained with 0.5% crystal violet, and colony numbers were counted.

Cell apoptosis detected by Annexin V-fluorescein isothiocyanate (FITC)-Propidium Iodide (PI)

An annexin V apoptosis kit was purchased from BioVision (Catalog number: K101-25) and the annexin V assay was performed according to the manufacturer's instruction.

Fluorescence-activated cell sorting (FACS) analysis

Cells were harvested by trypsinization, fixed in 75% ethanol, stained with propidium iodide solution at a final concentration of 50 µg/ml, and subjected to FACS analysis.

Subcellular fractionation

Cytoplasmic and nuclear fractions of cells were prepared using Nuclear Extract Kit from Active Motif. Briefly, harvested cells were re-suspended in hypotonic buffer, vortexed for 10 s, and centrifuged for 30 s at 14,000 rpm at 4 °C. Supernatants were collected as cytoplasmic fractions. Nuclear pellets were re-suspended using complete lysis buffer, incubated on ice for 30 min, vortexed for 30 s, and centrifuged for 10 min at 14,000 rpm. Supernatants were collected as nuclear fractions.

RNA expression analysis

Total RNA was extracted by the RNeasy Mini kit (Qiagen, Germantown, MD). For quantitative RT-PCR analysis, reverse transcription using 1 µg RNA was performed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), followed by gene amplification using FastStart Universal SYBR Green (Roche Applied Science, Indianapolis, IN) and a Roche LightCycler 96 thermocycler (Roche Diagnostics Corporation, Indianapolis, IN). All individual reactions were performed in duplicate and all genes were normalized to 18S ribosomal RNA.

Combination index

The combination index (CI) was calculated using the following equation (26): combination index = $(Am)_{50} / (As)_{50} + (Bm)_{50} / (Bs)_{50}$, where $(Am)_{50}$ is the concentration of BI2536 necessary to achieve a 50% inhibitory effect in the combination with half of the concentration of the Olaparib IC_{50} ; $(As)_{50}$ is the concentration of BI2536 that will produce the identical level of effect by itself; $(Bm)_{50}$ is the concentration of Olaparib that will produce a 50% inhibitory effect in the combination with half of the concentration of BI2536 IC_{50} and $(Bs)_{50}$ is the concentration of Olaparib that will produce the same level of effect by itself. Antagonism is indicated when $CI > 1$, $CI = 1$ indicates an additive effect and $CI < 1$ indicates synergy (27).

22RV1-derived mouse xenograft model

22RV1 cells (1×10^6 cells per mouse) were mixed with an equal volume of Matrigel (Collaborative Biomedical Products) and inoculated into the right flank of nude mice (Harlan Laboratories). Two weeks later, animals were randomized into treatment and control groups with 4 mice each. Both Olaparib and BI2536 were injected into the tail vein twice weekly. Tumor volumes, estimated from the formula: $V = L \times W^2 / 2$ (V , mm³; L , mm; W , mm), were measured with digital calipers.

Histology and immunohistochemistry

Xenograft tumors were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned to 5 µM, and stained using conventional hematoxylin and eosin (H&E) staining.

Immunohistochemistry (IHC) and immunofluorescent chemistry (IFC) staining was accomplished with Elite Vectastain ABC kit.

Statistical analysis

The statistical significance of the results was analyzed using an unpaired Student *t* test (StatView I, Abacus Concepts Inc., Berkeley, CA). A *P* value of less than 0.05 indicates statistical significance.

Results

Inhibition of Plk1 enhances the efficacy of Olaparib

The use of PARPi is an active area of clinical investigation in oncology, as it exploits synthetic lethality in tumors with defective HR and potentiates the cytotoxic effect of chemotherapy and radiation (28). However, emerging data has shown that some tumors with HR defects demonstrate apparent resistance to Olaparib treatment (17,18,29). Plk1, overexpressed in PCa and involved in PCa tumorigenesis and progression, is one of the top upregulated pathways following castration in PCa xenograft models (30). Whether Plk1 inhibition can enhance the antineoplastic activity of Olaparib is currently not known.

We investigated whether BI2536 and the PARPi Olaparib act synergistically to inhibit the growth of CRPC cells. First, 22RV1 cells were treated with BI2536, Olaparib or BI2536 in combination with Olaparib, and harvested for analysis of cleaved-PARP, a marker of apoptosis (Fig. 1A, Fig. S1A). As indicated, low dose Olaparib treatment (0.1 μ M, lane 4) showed very weak cellular apoptotic response in 22RV1 cells. The combination treatment of BI2536 and Olaparib led to a significantly increased cellular apoptotic response (lane 5) when compared to BI2536 or Olaparib alone (lane 2 and 4). Next, we performed fluorescence-activated cell sorter (FACS) analysis to monitor any cell cycle defects upon drug treatment. As indicated, the presence of BI2536 potentiated Olaparib-associated cell death in 22RV1 cells (Fig. S1C). Cell apoptosis, detected by Annexin V- FITC/Propidium iodide (PI), also show that combination treatment with BI2536 and Olaparib significantly increased the apoptotic cell population (Fig. 1D and 1E). In agreement, treatment of 22RV1 cells with the BI2536 and Olaparib combination showed a much stronger inhibitory effect on colony formation compared to BI2536 or Olaparib alone (Fig. 1B and 1C). We then investigated the formation of γ H2AX foci, which normally form in response to DSBs in mammalian cells. As shown in Fig. 1F, treatment with BI2536 or Olaparib alone had a marginal effect on the γ H2AX foci formation. When 22RV1 cells were treated with BI2536 plus Olaparib for 24 h, γ H2AX foci formation was significantly affected. The number of foci per cell was increased, the foci were enlarged, and the fluorescence intensity of foci was enhanced (Fig. 1G).

Finally, the combination treatment of BI2536 and Olaparib also significantly increased the expression of γ H2AX at the protein level compared to the monotherapy with BI2536 or Olaparib (Fig. 1H). To further investigate the synergistic effect between BI2536 and Olaparib, we measured the combination index (CI). Measurements of IC₅₀ values revealed an IC₅₀ value of 70 μ M for Olaparib-treated 22RV1 cells. However, the IC₅₀ value of

Olaparib was reduced to 20 μ M when cells were treated in combination with 50 nM BI2536. The combination index of the two drugs was calculated to be 0.58, suggesting a strong synergistic effect between BI2536 and Olaparib (Table 2). Altogether, these results demonstrate that Plk1 inhibition can sensitize the antineoplastic activity of Olaparib, suggesting a strong synergistic effect between BI2536 and Olaparib.

Combination therapy with BI2536 and Olaparib synergistically blocks growth of CRPC cells-derived xenograft tumors

To assess this combinatorial activity, we next tested the effect of combination treatment in a 22RV1-derived xenograft mouse model. As shown in Fig. 2A, monotherapies with BI2536 or Olaparib did not significantly affect the tumor growth compare with untreated control group. In contrast, the combination of BI2536 and Olaparib led to a significantly greater tumor-inhibitory effect than did monotherapy with either BI2536 or Olaparib (Fig. 2A to 2C). Histologically, tumors from the control group were composed of sheets of neoplastic epithelial cells with marked nuclear pleomorphism, 1–2 prominent nucleoli and numerous mitotic figures within a scant fibrous stroma (Fig. 3A, H&E). These histopathologic features are similar to high-grade prostatic adenocarcinoma *in vivo*, which is typically associated with poor clinical outcomes. However, tumors treated with BI2536 and Olaparib combination therapy showed increased numbers of apoptotic bodies with condensed cytoplasm and pyknotic nuclei compared to BI2536 or Olaparib monotherapy-treated tumors or controls. Immunostaining for Ki67 and cleaved caspase 3 also confirmed that tumors from combination therapy had a significant reduction in overall proliferation and a significant increase in apoptosis (Fig. 3B to 3D), suggesting that the combination of Olaparib and BI2536 led to more effective prostate cancer cell killing compared to monotherapy with Olaparib.

In summary, these studies support the notion that the Plk1 inhibitor BI2536 and Olaparib act synergistically in human prostate cancer cells, providing a novel and promising therapeutic option to treat CRPC patients. These results are also consistent with our observation in the cell-based study, providing additional evidence of a strong synergistic effect between Olaparib and BI2536 *in vitro* and *in vivo*.

Interaction between PARP1 and Plk1

Because combined inhibition of Plk1 and PARP1 shows a strong synergistic effect in blocking human prostate tumor growth, we investigated whether PARP1 and Plk1 interact with each other in CRPC cells. Firstly, 22RV1 cells were treated with nocodazole for 12 h, and harvested for anti-Plk1 IP, followed by Western blotting. Co-immunoprecipitation assays showed an interaction between endogenous Plk1 and endogenous PARP1 (Fig. 4A). To further understand where and at what stage of the cell cycle the coassociation of Plk1 and PARP1 might occur, we synchronized cells at G1 (mimosine treated), G1/S (hydroxyurea treated), the G2/M boundary (RO-3306 treated) and G2/M phase (nocodazole treated). IF staining showed that the intensities of both Plk1 and PARP1 increased at the G2/M boundary and peaked in mitosis. The colocalization of Plk1 and PARP1 mainly occurs at the nucleus of the cells (Fig. 4C). Next, 22RV1 cells were treated with mimosine, hydroxyurea, or nocodazole to block at G1, G1/S, or G2/M phase, respectively, and incubated with or

without the PARP1 activating compound 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) before harvest. As indicated, upon treatment with MNNG, the overall levels of Plk1 at different phases of cell cycle were significantly lower when compared to cells without MNNG treatment (Fig. 4B). Moreover, the level of PARP1 protein expression was significantly decreased upon treatment with the Plk1 inhibitor BI2536 (Fig. 4D, lanes 1 and 2), and with a 2 h treatment of MNNG the protein level of Plk1 was almost completely abolished (Fig. 4D, lane 6). Because Plk1 is cell cycle regulated, the MNNG-induced suppression of Plk1 may be the result of some cell cycle effects caused by MNNG such as cell cycle arrest at G1 where Plk1 levels are low. To exclude that possibility, we performed FACS analysis to follow any defects upon drug treatment. We observed a decrease in cells at G1 after MNNG treatment, suggesting that MNNG-induced suppression of Plk1 is not due to a secondary effect of the G1 cell cycle arrest (Fig. 4E). Indeed, MNNG treatment led to a mild increase of G2/M population (Fig. 4E). Altogether, these results demonstrate that in 22RV1 cells PARP1 activation significantly suppresses the expression of Plk1 and that inhibition of Plk1 activity dramatically reduces the level of PARP1 protein expression.

Olaparib treatment elevates the Plk1 expression in 22RV1 cells but not in CRPC C4-2 cells

To further dissect the underlying mechanism that Plk1 inhibition enhances the efficacy of Olaparib, we treated 22RV1 and another CRPC cell line, C4-2, with Olaparib, followed by IB analysis. While C4-2 is a BRCA1 wild type CRPC cell line, 22RV1 is a BRCA1-deficient CRPC cell line (Fig. 5D, Table 1). As indicated in Figs. 5A and 5B, there is a substantial increase of Plk1 expression at both the protein and mRNA levels in 22RV1 cells in response to Olaparib treatment. In a striking contrast, Plk1 expression was decreased at both the protein and mRNA levels in Olaparib-treated C4-2 cells (Figs. 5E and 5F). Next, we compared the FACS profiles of 22RV1 and C4-2 cells following Olaparib treatment. Consistent with the changes of Plk1 mRNA and protein expression, 24 h after Olaparib treatment a large population of 22RV1 cells quickly accumulated at the G2/M phase, indicated by the apparent increase of cell population with 4n DNA content (Fig. 5C). In contrast, in response to the Olaparib treatment, a similar G2/M accumulation as in 22RV1 cells was not observed in C4-2 cells (Fig. 5G). Furthermore, results from colony formation assays (Fig. 5I) and several other synergistic effect tests (Fig. 5H, Fig. S2B and S2C) in C4-2 cells showed that there is no synergistic effect existed between Olaparib and BI2536. One possible explanation for this different response of the two CRPC cells lines to Olaparib plus BI2536 is the status of p53. The PARP1 inhibition with Olaparib induces SSBs, which are subsequently converted to DSBs by collisions with the replication machinery. The DSBs then activate the G1/S checkpoint, leading to cell cycle arrest in the p53 wild type C4-2 cells. In contrast, the p53 mutant 22RV1 cells likely override the Olaparib-induced checkpoint and continue to progress into the G2/M phase, resulting in the high expression of Plk1 in these cells (Fig. 5M). To better understand how p53 status might affect relative response, we tested the drug response of additional cancer cell lines with various p53 and BRCA backgrounds (Table 1). As the data shows in Figs. 1A and 5K, Plk1 inhibition enhances the efficacy of Olaparib in BRCA1-null 22RV1 and PC3 cells. For the BRCA1 wildtype C4-2 and MDA-MB-231 cell lines, this strong synergistic effect was observed in p53 mutant MDA-MB-231 but not in the p53 wildtype C4-2 cells (Fig. 5H and 5L). Furthermore, inhibition of p53 activity in C4-2 cells synergizes the combination treatment

with Olaparib plus BI2536, and greatly enhancing the efficacy of Olaparib (Fig. 5J). These results suggest that BRCA-mutant CRPC tumors with mutant p53 might have the best response to the combination therapy of Olaparib plus Plk1 inhibitor, as the Plk1 levels are elevated in these cells upon Olaparib treatment due to the lack of p53-mediated cell cycle arrest (Fig. 5M), and the status of p53 in cancer cells might be a key factor for determining how effective this combination therapy will be, regardless of BRCA1 status.

Discussion

Olaparib is an innovative, first-in-class PARP inhibitor that exploits tumor DNA repair pathway deficiencies to preferentially kill cancer cells. The DNA repair defect present in BRCA-mutant cancers has been used to develop ‘synthetic lethal’ treatment strategies that have been clinically validated (13,15, 25, 27). Although it has been reported that BRCA-mutant cancers respond very well to Olaparib monotherapy, a substantial fraction of advanced BRCA-mutant cancers are resistant to Olaparib (11,16–18,29). Therefore, identifying additional targets whose inhibition might enhance the efficacy of Olaparib to overcome the drug resistance will have a major clinical impact. On the other hand, BRCA mutations are not common for many types of cancers, and BRCA wildtype patients do not respond very well to the Olaparib treatment. Therefore, co-targeting PARP1 and additional target(s) might be an effective approach to overcome drug resistance by enhancing the efficacy of Olaparib in BRCA-mutated patient and/or make the non-BRCA-mutant patients also respond well to PARPi.

Increasing evidence supports the notion that Plk1-associated activity might be involved in acquisition of resistance for various cancer therapies. For example, Plk1 phosphorylation of Orc2 drives continued DNA replication even in the presence of gemcitabine, the major approach for treatment of pancreatic cancer (31). Plk1-associated kinase activity is a predictable marker for the efficacy of radiotherapy in rectal cancer (32). Plk1 also plays a critical role for therapy resistance in prostate cancer. Plk1 phosphorylation of CLIP-170 and p150^{Glued}, two microtubule plus-end binding proteins, enhances microtubule dynamic which results in resistance to docetaxel, the first line treatment for CRPC (33). Furthermore, Plk1-mediated activation of the PI3K/AKT/mTOR pathway leads to activation of the AR signaling, thus antagonizing the effect of ASIs, such as abiraterone and enzalutamide (24). Recently, we also demonstrated that inhibition of Plk1 enhances the anti-neoplastic activity of metformin and β -catenin inhibitor in CRPC (20,34). Therefore, Plk1 elevation/activation is likely a general mechanism for therapy resistance in CRPC, albeit via multiple mechanisms. For DNA damage-based therapy, the underlying mechanism for the Plk1 involvement is clearly the p53 pathway. Enough evidence supports the link between Plk1 and the p53 pathway, as Plk1 is inhibited upon DNA damage and p53 activation. During the subsequent checkpoint recovery, Plk1 phosphorylation of GTSE1 drives p53 translocation from the nucleus to the cytoplasm, resulting in p53 degradation and eventually cell cycle re-entry (35).

In this study, we investigated the sensitivity of several different cell lines with various genetic backgrounds to the Olaparib monotherapy or combination therapy with Olaparib plus a Plk1 inhibitor BI2536. Among the cell lines used in this study (Table 1), both 22RV1

and C4-2 are considered CRPC cells, as their growth is androgen independent (36,37). Herein, we showed that Plk1 inhibition significantly potentiates Olaparib-associated cell death in BRCA1-null 22RV1 cells as well as the 22RV1-derived xenograft tumors, supporting an immediate clinical trial by combining BI2536 and Olaparib in treatment of BRCA-defective, PARPi resistant CRPC patients. As expected, the BRCA1-wildtype C4-2 cells do not respond well to the Olaparib monotherapy, and a synergistic effect similar to that in 22RV1 cells was not observed (Fig. 5J, left panel). Surprisingly, inhibition of p53 activity significantly resensitized the BRCA1-wildtype C4-2 cells to Olaparib monotherapy and combination with Plk1 inhibitor further enhanced the efficacy of Olaparib to kill these cells, as indicated by the substantial increase in the level of cleaved-PARP (Fig. 5J, right panel). A similar synergistic effect as in BRCA1-null p53mutant 22RV1 cells was observed in BRCA1-wildtype but p53 mutant MDA-MB-231 cells. These results implicate that p53 status might be an important factor for the proposed combination therapy with Olaparib and BI2536. In case of p53-mutant CRPC cells, Olaparib treatment clearly causes accumulation of cells at G2/M, thus elevation of Plk1, which eventually counteract the efficacy of Olaparib. It provides a strong rationale for combining BI2536 with Olaparib in these patients. This is an excellent example for personalized medicine in terms of Olaparib use in CRPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Plk1 inhibition enhances the efficacy of Olaparib in BRCA1-deficient 22RV1 cells

A, 22RV1 cells were treated with indicated concentrations of BI2536, Olaparib or both for 24 h and harvested for immunoblotting (IB) with antibodies against cleaved-poly (ADP-ribose) polymerase (PARP).

B, 22RV1 cells (0.5×10^3) were plated in 6-well plates for 24 h and then treated with BI2536 (2.5 nM), Olaparib (1 μ M) or both drugs. After changing fresh media containing drug(s) every 3 days for two weeks, cells were PFA fixed and colony formation was monitored by crystal violet staining. The experiments shown are representatives of 3 repeats.

C, Quantification of the colonies in (B). The numbers of colonies were quantified using ImageJ software (mean $\pm$ SD; n=3 independent experiment). p value = 1.04648E-07 (BI2536 vs BI2536+Olaparib); p value = 9.11184E-06 (Olaparib vs BI2536+Olaparib). **, p < 0.001.

D, Flow cytometry analysis of annexin-V and propidium iodide (PI) staining of apoptotic cells following BI2536 (5 nM) and Olaparib (10 μ M) treatment or combination of both for 24 h in 22RV1 cells.

E, Quantification of apoptotic cells (annexin-V positive + PI positive + annexin-V and PI double positive cells). p value = 0.000884 (BI2536 vs BI2536+Olaparib); p value = 0.000985 (Olaparib vs BI2536+Olaparib). **, p value < 0.001).

F, Representative images of immunofluorescence (IF) staining for γ H2AX in 22RV1 cells treated with of BI2536 (5 nM), Olaparib (10 μ M) or both for 24 h. 22RV1 cells (1×10^5) were plated in 6-well plates on day 0 and then treated with BI2536, Olaparib or both for 24 h.

G, Quantification of the γ H2AX signaling in (F). For quantification, at least 300 cells were scored for γ -H2AX foci and percentages of cells with 5 or more foci are shown. Values are mean $\pm$ SEM of three independent experiments. p value = 0.0013512 (BI2536 vs BI2536+Olaparib);

p value = 0.009928462 (Olaparib vs BI2536+Olaparib). *, p < 0.05 (two-tailed unpaired t-test).

H, 22RV1 cells were treated with BI2536 (5 nM), Olaparib (10 μ M) or combination of 2 drugs for 24 h and harvested for IB.

Figure 2. Combination therapy with BI2536 and Olaparib synergistically inhibits growth of 22RV1-derived xenograft tumors

A, Tumor growth curves of the 22RV1-derived xenograft mouse study. Nude mice were inoculated with 22RV1 cells (1×10^6) for two weeks, intravenously injected with BI2536 (12 mg/kg), Olaparib (50 mg/kg) by oral gavage or a combination of both drugs. The sizes of the tumors in each group were measured every 3 or 4 days (mean $\pm$ SEM; $n = 3$ mice from each experiment group). “NS” represents “no significant difference”; *, $p < 0.05$ compared with

the BI2536 or Olaparib treated monotherapy group or the untreated group at the end of the study.

B, Images of the 22RV1-derived xenograft tumors at the end of the study.

C, Tumor weight measurement after being freshly removed from the bodies. **, $p < 0.001$ compared with the BI2536 or Olaparib-treated monotherapy group at the end of the study.

Figure 3. Histological analysis of 22RV1-derived xenograft tumors

A, Representative images of H&E on formaldehyde-fixed, paraffin-embedded 22RV1-derived xenograft tumors sections from different treatment groups.

B, Representative images of immunofluorescence staining for Ki67 and cleaved caspase 3 with tumors as in (A).

C & D, Microscopic quantification of Ki67 or cleaved caspase 3 as percentages of Ki67- or cleaved caspase 3-positive cells in total numbers of cells. For quantification, at least 300 cells were scored on each field (20× fields, more than 3 sections at different tumor depths

per mouse) as the percentages of Ki67 or cleaved-caspase 3 positive cells compared to the total numbers of cells counted. *: $p < 0.05$, **: $p < 0.001$ (two-tailed unpaired t-test).

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Figure 4. Plk1 inhibition profoundly suppresses the level of PARP1 protein expression and PARP1 activation inhibits the Plk1 expression

A, PARP1 binds to Plk1. 22RV1 cells were treated with nocodazole and harvested for immunoprecipitation (IP) with antibodies against Plk1, followed by IB.

B, 22RV1 cells were treated with 0.3 mM mimosine (Mimo) for 20 h, 4 mM hydroxyurea (HU) for 24 h, or 200 ng/ml nocodazole (Noc) for 12 h to block cells at G1, G1/S or G2/M phase, respectively. Cell were harvested after 30 minutes treatment with 50 μ M MNNG.

C, Representative images of IF staining for PARP1 and Plk1 in 22RV1 cells treated with 0.3 mM mimosine for 20 h, 4 mM hydroxyurea for 24 h, 10 μ M RO-3306 for 7 h or 200 ng/ml nocodazole (Noc) for 12 h.

D, 22RV1 cells were treated with BI2536 (5 nM, 24 h) or in combination with MNNG (50 μ M), and harvested at indicated time points for IB.

E, 22RV1 cells were treated with BI2536 (5 nM, 24 h), MNNG (50 μ M, 30 minutes), or both, and subjected to FACS analysis.

Figure 5. p53 inhibition restores the synergistic effect between BI2536 and Olaparib in BRCA1 wild type C4-2 cells

A to C, 22RV1 cells were treated with or without Olaparib (10 μ M) for 24 h, and harvested for IB (A), quantitative RT-PCR (B) or FACS analysis (C).

D, BRCA1 expression was determined by IB in 22RV1 and C4-2.

E to G, C4-2 cells were treated with or without Olaparib (10 μ M) for 24 h and harvested for IB (D), quantitative RT-PCR (E) or FACS analysis (F).

H, C4-2 cells were treated with indicated concentrations of BI2536, Olaparib or both for 24 h and harvested for IB with antibodies against cleaved-PARP.

I, C4-2 cells (0.5×10^3) were plated in 6-well plates for 24 h and treated with BI2536, Olaparib or both drugs. After changing fresh media containing drug(s) every 3 days for two weeks, cells were PFA fixed and colony formation was monitored by crystal violet staining. The experiments shown are representatives of 3 repeats.

J, C4-2 cells were treated with BI2536 (5 nM), Olaparib (10 μ M) or both drugs in the presence/absence of p53 inhibitor Pifithrin-1 α (PFT1- α , 40 μ M) and harvested for IB.

K to L: PC3 or MDA-MB-231 cells were treated with indicated concentrations of BI2536, Olaparib or both for 24 h and harvested for IB.

M, Proposed working model based on the results of this study.

Table 1

Genetic backgrounds of several human cancer cell lines with various p53, BRCA1 status and their sensitivity to the combination treatment.

Cell line	PTEN	p53	AR	BRCA1	Sensitivity to Olaparib+BI2536
PC3	mutant	mutant	null	null	+
C4-2	null	mutant	wildtype	null	-
22RV-1	wildtype	null	wildtype	wildtype	++
MDA-MB-231	wildtype	wildtype		wildtype	++

Table 2

The IC₅₀ values of Olaparib and BI2536 in 22RV1 cells

Drugs	IC ₅₀	CI
BI2536	70 μ M	
Olaparib	100 nM	
Olaparib (in combination with 50nM BI2536)	20 μ M	combination index=0.58