New Insights into PARP Inhibitors' Effect on Cell Cycle and Homology-Directed DNA Damage Repair

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Abstract

In preclinical and clinical studies, olaparib and veliparib are the most represented PARP inhibitors (PARPi), which mainly target homologous DNA damage repair pathway-deficient cancer cells. Their off-target effects are not fully understood, especially with regard to cell cycle and homology-directed DNA damage repair. Our objective was to comparatively evaluate olaparib and veliparib in this context and correlate our findings with their therapeutic potential. We used a well-established direct repeat GFP (DR-GFP) reporter assay in U2OSDR-GFP and H1299DR-GFP cells and measured DNA damage repair activity upon drug treatment. Olaparib-treated U2OSDR-GFP cells showed a dramatic decrease in DNA damage repair versus veliparib irrespective of inhibitory potency. We demonstrate that this effect was a result of olaparib's strong effect on the cell cycle. Unlike in veliparib-treated U2OSDR-GFP cells, in olaparib-treated cells S-phase decreased and G2-phase increased sharply, indicating a G2-phase arrest-like state and replicative stress. This was further confirmed by upregulation of p53 and p21 and accumulation of cyclin A. Lack of the same effect in p53-null H1299DR-GFP and p53-null HCT116 cells. Importantly, we also demonstrate that olaparib, but not veliparib, induced a robust phosphorylation of Chk1, a crucial component of the replicative stress response pathway. Our data show olaparib and veliparib differ in their off-target effects; olaparib, unlike veliparib, mitigates DNA damage repair activity via G2 cell-cycle arrest-like effect in a p53-dependent manner. These off-target effects may add to PARPis' anticancer properties.

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Introduction

PARP inhibitors (PARPi) target PARPs by suppressing their enzymatic activity PARylation—the attachment of poly (ADP)-ribose polymers onto themselves and other acceptor proteins (1, 2). PARP-1, the most abundant among all PARPs, is a stress-sensing protein that acts mainly via the base excision repair (BER) pathway. One of the proposed mechanisms is that PARP-1 binds to single-strand break (SSB) intermediates. After binding and self-PARylating, PARP-1 dissociates from the SSB, which subsequently triggers the downstream BER pathway to repair the lesion (3). It has been suggested that PARP inhibition impairs the BER pathway by trapping PARP-1 at the SSBs and blocking further repair (4–6). When the replication fork encounters these SSBs, they are converted into DNA double-strand breaks (DSB), which are repaired predominantly by the homology-directed DNA damage repair (HDR) pathway. If the HDR pathway is impaired, cells rely on more error-prone pathways, resulting in genomic instability and loss of viability (7, 8). PARP inhibition in HDR-compromised cells was the first demonstration of the concept of synthetic lethality working in cancer cells. This provided a strong rationale for the development of PARPis as a treatment for patients with cancer with an impaired HDR pathway (9, 10).

Olaparib and veliparib are the most extensively studied PARPis in preclinical and clinical studies, showing greatest success in ovarian and breast cancers (11–14). Although data from early clinical trials looking at PARPis were promising, more recent results have failed to meet expectations, prompting a call for a better understanding of these drugs’ mode of action (15). PARPis are nicotinamide (NAM) mimetics that compete with NAD\(^+\) for the NAM pocket within PARPs' catalytic domain (16, 17). Although specifically developed as PARP-1 inhibitors, all PARPis exhibit a polypharmacology targeting other members of the PARP family (16, 18). Olaparib and veliparib fall within the same group of PARPis with the similar polypharmacology (16). It is well documented that olaparib and veliparib have similar inhibitory effects on PARP-1’s enzymatic activity (19). However, their off-target effects, which may contribute to their differential therapeutic properties, are not fully understood. It has...
been demonstrated that some PARPs cause cell-cycle abrogation, which has been recognized as an off-target effect (20, 21). Only a few PARPs share this characteristic, suggesting that it is not related to their enzymatic activity-targeted inhibitory properties. Considering that the HDR pathway is active predominately during the replicative phase of the cell cycle, the concept of synthetic lethality caused by PARP inhibition works only in HDR-impaired cells in which cell cycle is not arrested. Therefore, it is essential to evaluate PARP's therapeutic properties in the context of the cell cycle and HDR, which thus far has not been addressed. The important question remains whether or not olaparib and/or veliparib, the 2 most common PARPs in clinical trials, have an impact on the cell cycle that could compromise HDR activity and render these inhibitors less effective in the context of synthetic lethality.

To address this hypothesis, we took advantage of a well-established direct repeat GFP (DR-GFP) reporter assay to comparatively evaluate HDR activity in cells treated with olaparib or veliparib. We report a significant differential effect between the 2 PARPis on cell cycle and HDR activity irrespective of their catalytic inhibitory potency. This work provides a new insight into these 2 PARPis' mode of action, which may help define selection criteria when evaluating patients for treatment with olaparib, veliparib, or other PARPis.

Materials and Methods

Cell culture and drug preparation

U2OS\textsubscript{DR-GFP} human osteosarcoma cell line was cultured in high glucose (4.5 g/L) Dulbecco’s Modified Eagle Medium (DMEM-HG) supplemented with 10% fetal calf serum (FCS) and 2 mmol/L L-glutamine. H1299\textsubscript{DR-GFP} human non-small cell lung carcinoma and HCT116 [wild-type (WT) and p53\textsuperscript{-/-}] colorectal carcinoma cell lines were cultured in RPMI media supplemented with 10% FCS. U2OS\textsubscript{DR-GFP} and H1299\textsubscript{DR-GFP} cell lines were kindly donated by Dr. G. Schwartz (Department of Radiation Oncology, MSKCC). HCT116 (WT and p53\textsuperscript{-/-}) cell lines were kindly donated by Dr. S. Powell (Department of Radiation Oncology, MSKCC). HCT116 (WT and p53\textsuperscript{-/-}) cell lines were kindly donated by Dr. G. Schwartz (Department of Medicine, MSKCC). All cell lines were authenticated by the short tandem repeat DNA profiling method (Genetica Medicine, MSKCC). All cell lines were processed (1 × 10\textsuperscript{6}) cells and were then split into 2 wells of a 6-well plate. After 72 hours, cells were harvested and prepared for either HDR or PARP activity assessment. For HDR activity assessment, cells were harvested with 250 μL trypsin. An equal amount of media was added to neutralize trypsin, and percentages of GFP-positive cells were determined by flow cytometry (FACSCalibur; Becton Dickinson). For PARP activity assessment, we used Trevigen’s Universal Colorimetric PARP assay (#4677-096-K) according to the manufacturer’s directions, with minor modifications. Briefly, cells were washed 3 times in ice-cold PBS and lysed with PARP lysis buffer supplemented with 400 mmol/L NaCl, 1% Triton X-100, 0.4 mmol/L p- nethymethylsulfonylfluoride, and protease inhibitor cocktail (P8340; Sigma-Aldrich). After 30 minutes of incubation on ice, with occasional flicking, the lysates were centrifuged for 15 minutes at 4 °C at 10,000 × g. The supernatant was transferred to a fresh tube, and protein concentration was measured using a Bradford assay (#500-0006; Bio-Rad). Fifty micrograms of protein lysate was mixed with PARP Cocktail into a histone-coated 96-well dish. After 60 minutes of room temperature incubation, the plate was washed 4 times in PBS, 50 μL of Strep-HRP was added, and it was incubated for 20 minutes at room temperature. The plate was washed 4 times in PBS, and then 50 μL of TACS-Sapphire was added. After 15 minutes of incubation in the dark, the reaction was stopped by adding 50 μL of 0.2M HCl. The plate was read by a microplate reader (Synergy HT; BioTek) at 450 nm.

Cell-cycle analysis

After incubation in specific conditions, cells were harvested and counted to ensure equal numbers of cells were processed (1 × 10\textsuperscript{6}). Cells were then washed with ice-cold PBS, fixed in 80% ethanol at –20 °C for a minimum of 2 hours to overnight. Subsequently, cells were washed in ice-cold PBS and stained with propidium iodide DNA staining solution containing RNase A (200 μg/mL, Triton X-100 (0.1%, v/v), and propidium iodide (50 μg/mL) in PBS. After 20 minutes of incubation at 37 °C, the samples were stored at 4 °C and processed by flow cytometry (FACSCalibur; Becton Dickinson) the following day. Cell-cycle phase distributions were analyzed by the Flowjo software program.

Immunoblotting and antibodies

To prepare whole cell lysates, cells were washed twice in ice-cold PBS, and the pellets were thoroughly mixed with RIPA lysis buffer (sc-24948; Santa Cruz Biotechnology) supplemented with protease and phosphatase inhibitor cocktail (#88668; Pierce) and incubated on ice for 20 minutes with occasional flicking. After centrifugation at 17,000 × g at 4 °C for 15 minutes, supernatants were collected.
Protein concentration was measured by Bradford assay (BS-0006; Bio-Rad) to ensure equal loading. Proteins were boiled at 95°C for 5 minutes in Laemmli sample buffer (S3401; Sigma-Aldrich). Proteins were then resolved by SDS-PAGE electrophoresis, transferred on nitrocellulose, and blotted with corresponding antibodies. Anti-p53 (sc-98), anti-CASPASE3 (sc-7148), anti-NOXA (sc-7148), and anti-β-Actin (sc-69879) were obtained from Santa Cruz Biotechnology. Anti-p21 (#2947), anti-cyclin A2 (#4656), anti-cyclin B1 (#4138), anti-Chk1 S345 (#2348), anti-Chk1 (#2360), anti-Chk2 T68 (#2661), and anti-Chk2 (#2662) were obtained from Cell Signaling Technology. Anti-PARP (#MA3-950) was obtained from Thermo Scientific. Secondary antibodies were horseradish peroxidase conjugated to mouse or rabbit immunoglobulin G (IgG; GE Healthcare).

siRNA transfection
Nontargeting siRNA (siNT) was used as a negative control (#D-001810-10-05; Dharmacon). Gene-specific siRNA pools of 4 sequences targeted p53 (sip53; L-003329-00-0005; Dharmacon). A Nucleofector (Amara) cell electroporator was used to transfect 2 x 10^6 U2OSDR-GFP cells with either siNT or sip53 (10 μL of 20 μmol/L siRNA stock per 100 μL electroporation solution/cell mix). Cells were then split into 3 100-mm-diameter dishes in the media with corresponding drug treatment (Mock/DMSO, 6 μmol/L olaparib, or 15 μmol/L veliparib). After 72 hours, cells were collected for further analysis. Knockdown was confirmed by Real-Time PCR using a p53-specific TaqMan probe (#Hs0103429_m1; Applied Biosystems). Cell-cycle analysis, HDR, and PARP activity analyses were performed as indicated above.

Irradiation and RAD51 foci formation assessment by immunocytochemistry
The experiment’s treatment protocol is shown in Fig. 5A. U2OSDR-GFP cells were grown in serum-depleted DMEM-HG media on 100-mm-diameter dishes for 72 hours and then trypsinized and seeded at 2 x 10^5/well on an 8-chamber tissue culture slide (Millipore) for an additional 16 hours in serum-depleted media supplemented with drugs (Mock/DMSO, 10 μmol/L olaparib, or 30 μmol/L veliparib). Subsequently, cells were irradiated with 10 Gy using a Mark1 generator. After 8 hours of incubation in DMEM-HG media supplemented with 15% FCS, cells were fixed and permeabilized for 15 minutes at room temperature with a 4% paraformaldehyde PBS solution supplemented with 0.1% Triton X-100. Cells were then washed in PBS and blocked in PBS containing 1% bovine serum albumin and 0.1% Triton X-100. Cells were stained overnight at 4°C with rabbit anti-RAD51 (sc-8349; Santa Cruz Biotechnologies) antibody at 1:100 dilution.
Cells were then stained with secondary antibody Alexa Fluor 488–conjugated chicken anti-rabbit IgG (#A-21441; Invitrogen) at 1:1,000 dilution for 1 hour at room temperature. After primary and secondary antibodies were applied, cells were washed 3 times with PBS containing 0.1% Triton X-100. Slides were prepared using vectorshield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories), and images were obtained using a Leica-Upright SP-5 TCS confocal microscope with objective ×60 1.4NA. Maximum-intensity images were generated by the Z-stack protocol to display foci in all sections.

Results

Olaparib, but not veliparib, significantly decreases HDR activity in U2OSDR-GFP cells

To evaluate whether or not olaparib and/or veliparib compromise HDR activity, we used 2 HDR-proficient cell lines that have been extensively used in HDR-related studies—U2OS human osteosarcoma and H1299 human non–small-cell lung carcinoma. These cell lines differ in p53 status (U2OS are p53 wild type, H1299 are p53-null), which allowed us to also explore the importance of p53, particularly in the context of cell cycle. Both cell lines also have a limited number of mutations, ensuring maximal integrity of the HDR pathway, which rendered them suitable as models for our experiments.

To assess HDR activity, we used a well-established DR-GFP reporter assay that measures HDR of an I-SceI endonuclease-induced DNA DSB in which GFP-positive cells represent readout for HDR activity (22). We used U2OSDR-GFP and H1299DR-GFP cells that carry a stably integrated DR-GFP reporter. Previous biologic evaluation of olaparib and veliparib has demonstrated a similar inhibitory effect on PARP’s enzymatic activity, with olaparib being slightly more potent (19, 23). To correlate the 2 inhibitors’ effect on HDR activity according to their enzyme-targeted inhibitory potency, we used a range of drug concentrations: 0 to 8 μmol/L for olaparib and 0 to 40 μmol/L for veliparib. As illustrated in Fig. 1A, before electroporation with the I-SceI plasmid, cells were pretreated with olaparib and veliparib for 48 hours. An equal number of cells were then electroporated with I-SceI plasmid to introduce DNA DSBs, subsequently reseeded in media containing olaparib or veliparib and incubated for an additional 72 hours, allowing enough time for the DNA damage to be repaired. GFP-positive cells were then quantified by flow cytometry. To correlate the inhibitory potency of olaparib and veliparib to HDR activity, each sample was simultaneously assessed for PARP activity using a Universal Colorimetric PARP assay.

In U2OSDR-GFP cells, HDR activity was significantly decreased when treated with olaparib (Fig. 1B, left). Interestingly, HDR activity was not markedly suppressed with veliparib treatment despite PARP activity being less in veliparib-treated cells: 7.2% versus 24% at the maximum doses of veliparib- and olaparib-treated cells, respectively (Fig. 1B, right). This suggests that the decrease in HDR activity in olaparib-treated U2OSDR-GFP cells is not related to catalytic inhibitory potency. Similar levels of PARP enzymatic inhibition were seen in H1299DR-GFP cells (Fig. 1C). However, levels of HDR are unaffected by olaparib treatment in contrast to U2OSDR-GFP cells, and there was no significant difference when compared with veliparib-treated cells. One of the notable differences between U2OS and H1299 cells is their p53 status, with wild type and null, respectively, which suggests a role of p53 in olaparib’s inhibitory effect on HDR activity in U2OSDR-GFP cells.

A robust decrease in S-phase is detected only in olaparib-treated U2OSDR-GFP cells

Because olaparib seems to compromise HDR activity only in p53 wild-type cells, and p53 has a major role in cell-cycle control, we expanded our experiments to test if olaparib’s effect on HDR activity in U2OSDR-GFP cells was cell cycle related. U2OSDR-GFP and H1299DR-GFP cells were treated with either olaparib or veliparib, as shown in Fig. 1A, with the exception of the I-SceI electroporation. After 48 hours of pretreatment, cells were trypsinized and reseeded to allow for continued PARP1 treatment in log phase for another 72 hours. Subsequently, cells were stained with propidium iodide, and cell-cycle progression was measured by flow cytometry.

We observed a significant decrease in S-phase in U2OSDR-GFP cells treated with olaparib, but not in the cells treated with veliparib (Fig. 2A). The highest dose of olaparib (8 μmol/L) reduced the S-phase cell subpopulation by almost 45% when compared with untreated cells, whereas veliparib-treated cells (40 μmol/L) showed only a slight decrease of less than 4%. However, treatment of H1299DR-GFP cells with either olaparib or veliparib did not result in any significant reduction in S-phase (Fig. 2B). Given that the significant decrease in HDR activity and S-phase has been seen only in olaparib-treated U2OSDR-GFP cells, this suggests a correlation between the 2 effects.

Both cell lines treated with either olaparib or veliparib showed, to a variable extent, an increase in G2-phase when compared with untreated cells. Notably, the most dramatic increase in G2-phase was observed in olaparib-treated U2OSDR-GFP cells, in which a 60% increase in 8 μmol/L olaparib-treated versus untreated cells was seen. Veliparib-treated U2OSDR-GFP cells and olaparib- and veliparib-treated H1299DR-GFP cells at highest doses manifested a lower increase in G2-phase (42%, 48%, and 39%, respectively) than olaparib-treated U2OSDR-GFP cells (Figs. 2A and B). This less robust increase in G2 and no significant change in S-phase may suggest that these cells manifest a G2 delay-like state reflecting only mild replicative stress, unlike olaparib-treated U2OSDR-GFP cells that feature a G2 arrest-like state and strong replicative stress. Also, cell-cycle analysis revealed that sub-G1 cell subpopulation remained low in all samples, suggesting that cells are not going through apoptosis, which was further supported by immunoblotting (Supplementary Fig. S1).
Accumulation of olaparib-treated U2OS<sup>DR-GFP</sup> cells in G<sub>2</sub>-phase correlates with p53/p21 upregulation and reduced cell growth

p21, one of the main p53 targets activated upon cellular stress, regulates G<sub>2</sub> checkpoint activation and maintenance of G<sub>2</sub>-phase arrest (24–26). To further understand the nature of olaparib and veliparib's impact on cell cycle, and particularly to test if a G<sub>2</sub>-phase accumulation of olaparib-treated U2OS<sup>DR-GFP</sup> cells correlates with p53 function, we examined p21 and p53 protein expression (Fig. 3A).

Olaparib-treated U2OS<sup>DR-GFP</sup> cells showed upregulation of p53 and robust expression of p21. We detected less upregulation of p53 and p21 in veliparib-treated U2OS<sup>DR-GFP</sup> cells. The response seen in veliparib-treated cells was even more modest compared with that of olaparib-treated cells considering PARP inhibition by veliparib is stronger overall (Fig. 1B, right). As expected from cells that are p53-null, H1299<sup>DR-GFP</sup> cells did not show any p21 upregulation in response to either olaparib or veliparib treatment.

Considering p21 plays a role in both G<sub>1</sub> and G<sub>2</sub> cell-cycle arrest (24–26), it is important to clarify that the p21 upregulation seen in U2OS<sup>DR-GFP</sup> cells is G<sub>2</sub>-phase specific. During cell-cycle arrest, p21 engages cyclin A2, which peaks during G<sub>2</sub>-phase of the cell cycle (25). Cyclin B1 is also highly expressed during G<sub>2</sub>-phase; but it peaks at the G<sub>2</sub>–M transition, and as such, is a marker for mitotic entry. In U2OS<sup>DR-GFP</sup> cells treated with olaparib, we observed a greater accumulation of cyclin A2 than with veliparib (Fig. 3A). Veliparib-treated U2OS<sup>DR-GFP</sup> cells showed a slight increase in cyclin
B1 (Fig. 3A), which suggests that G2 delay might be taking place during mitotic entry rather than within G2-phase itself. H1299DR-GFP cells treated with either olaparib or veliparib did not show change in cyclin B1 or cyclin A2 expression.

Given that cell-cycle analysis suggests that olaparib and veliparib drive cells into G2-arrest or delay-like state, we sought to correlate the cell-cycle data with cell growth (Fig. 3B). U2OSDR-GFP cells treated with maximum doses of olaparib (8 μmol/L) or veliparib (40 μmol/L) for 72 hours showed a cell number decrease of 64% and 43%, respectively, when compared with untreated cells (Fig. 3B, left). Olaparib and veliparib showed less impact on H1299DR-GFP cell growth, with a cell number decrease of 39% and 28%, respectively (Fig. 3B, right). The findings of greater growth retardation in olaparib-treated U2OSDR-GFP cells compared with veliparib-treated U2OSDR-GFP cells are consistent with and complementary to our cell-cycle results.

**Chk1 is strongly phosphorylated in cells treated with olaparib but not veliparib**

Given that PARPis may cause replication fork stalling, we wanted to confirm whether or not the cell-cycle alterations we observed in cells treated with PARPis were the consequence of replicative stress. ATR and its downstream phosphorylation target, Chk1, are major responders to DNA SSBs and replicative stress, and are activated predominantly during late S- and G2-phase of the cell cycle (27, 28). We examined Chk1 phosphorylation 24 hours after treatment with either 10 μmol/L olaparib or 30 μmol/L veliparib (Fig. 3C). We also measured Chk2, a kinase that is phosphorylated by ATM but not responsive to DNA SSB-triggered replicative stress. Both U2OSDR-GFP and H1299DR-GFP cells treated with olaparib, but not veliparib, showed Chk1 phosphorylation, confirming that olaparib caused strong replicative stress. Unmodified Chk1, used as a control, did not show any differences, validating that Chk1 activation but not upregulation results in phospho-Chk1 increase in olaparib-treated cells. We did not observe a clear increase of phospho-Chk2 in either olaparib-treated U2OSDR-GFP or H1299DR-GFP cells.

**Olaparib’s effect on cell cycle is p53 dependent**

To further confirm that olaparib but not veliparib affects cells in a p53-dependent manner, we took advantage of isogenic p53-null and wild-type HCT116 colorectal carcinoma cell lines (29). In addition, we depleted U2OSDR-GFP cells of p53 using a transient siRNA approach. The cells were electroporated with corresponding siRNAs and incubated for 72 hours in media with or without PARPis. RT-PCR showed successful knockdown through p53 mRNA...
depletion in cells treated with sip53 in comparison to non-targeting siRNA (siNT)-treated cells (Fig. 4A). U2OSDR-GFP cells depleted of p53 compared with the siNT showed characteristics of reduced p53 levels, similar to HCT116 p53-null cells when compared with wild type, such as decrease in G1-phase and increase in cell growth (Figs. 4B and C). All cells also showed an increase in S-phase; however, wild-type/p53-depleted ratio (Fig. 4B, right) revealed the greatest increase in olaparib-treated cells (46% and 63% in U2OSDR-GFP and HCT116, respectively). We also saw a decrease in G2-phase only in olaparib-treated p53-depleted versus wild-type cells—8.5% in U2OSDR-GFP and 29% in HCT116 cells. When the cell growth of p53-depleted cells was compared with wild-type cells (Fig. 4C), the relative growth increase was greater in olaparib-treated compared with either mock- or veliparib-treated cells. Finally, we confirmed in HCT116 wild-type cells that olaparib unlike veliparib triggers p53 and p21 activation (Fig. 4D). In addition, we observed Chk1 activation only in olaparib-treated cells. These results further support the conclusion that olaparib’s effect on cell cycle and growth retardation is p53 dependent, whereas veliparib is independent of p53 status.

Olaparib and veliparib do not compromise RAD51 foci formation

Our data suggest that olaparib-treated U2OSDR-GFP cells have reduced HDR activity as a result of olaparib’s strong effect on the cell cycle. To exclude the possibility that this reduced activity could be an effect of olaparib on the HDR pathway itself, we assessed RAD51 foci formation in γ-irradiated U2OSDR-GFP cells with or without PARPis treatment. The formation of RAD51 foci after DNA damage reflects the assembly of protein complexes necessary for the HDR pathway, and it is commonly used to evaluate the pathway activity (30). The experimental procedure is illustrated in Fig. 5A. Before induction of DNA DSBs by irradiation, the cells were serum-deprived to enrich for G1-phase and minimize the G2-subpopulation to bypass the G2-arrest/delay effects of the PARPis. After irradiation, the cells were incubated in serum-enriched media for 8 hours to allow S-phase entry and repair of the damaged DNA.
DNA. Subsequently, cells were fixed and prepared for RAD51 immunohistochemistry. PARP activity was efficiently inhibited at the time of RAD51 immunohistochemistry (Fig. 5B). Cells with 5 or more RAD51 foci were considered positive for HDR pathway activity. In comparison to mock-treated cells, the percentage of RAD51 foci-positive cells in olaparib- and veliparib-treated cells was not decreased (Fig. 5C). This confirms that the HDR pathway is not compromised upon PARP inhibition and that the decrease in HDR activity in olaparib-treated U2OSDR-GFP cells is indeed a result of olaparib’s effect on the cell cycle.

Discussion

We comparatively evaluated olaparib and veliparib’s off-target effects on the cell cycle and HDR. Our data suggest that olaparib and veliparib, irrespective of their catalytic inhibitory potency, significantly differ in how they impact cell cycle and consequently HDR activity. Olaparib, unlike veliparib, reduces a replicative S-phase in U2OSDR-GFP cells, resulting in a dramatic decrease in HDR activity. Moreover, olaparib, but not veliparib, triggers a Chk1 phosphorylation indicative of strong replicative stress in both p53 wild-type and mutant cell lines. Finally, we also demonstrated that olaparib’s effect on the cell cycle is more prominent in a p53 wild-type background, whereas veliparib seems more ambivalent towards p53 status.

The assessment of PARP activity led us to the conclusion that olaparib’s effect on the cell cycle and HDR in U2OSDR-GFP cells is independent of its potency to inhibit PARPs’ enzymatic activity. This is supported by the notion that veliparib does not have the same effect as olaparib at the same levels of PARP inhibition (Fig. 1B; 1 and 2 μmol/L olaparib versus 5 and 10 μmol/L veliparib). In addition, veliparib reduces PARP activity more than olaparib, which further confirms that the effects we report here are specific to olaparib, independent of its catalytic inhibitory potency. In support of this, there have been recent studies reporting differences between olaparib and veliparib irrespective of their potency to inhibit PARPs’
enzymatic activity (5, 31). Because PARP’s indirect involvement in HDR has been suggested (32–35), we confirmed by the RAD51 foci formation assay that the HDR pathway itself is not compromised by olaparib.

Olaparib has been shown to have a significantly stronger potency to trap PARP-1 and -2 to DNA in comparison to veliparib (5). Proteins tightly bound to DNA and consequently pausing the replication fork can cause replicative stress, leading to accumulation of G2-phase cell subpopulation (27, 36). The increase in G2-phase that we see in U2OSDR-GFP and H1299DR-GFP cells treated with either olaparib or veliparib may reflect their PARP-trapping ability and induction of replicative stress response. Given the polypharmacology nature of PARPis, other off-target effects could contribute to olaparib and veliparib’s effect on the cell cycle.

Olaparib caused a greater G2-phase accumulation than veliparib. Chk1 phosphorylation was observed in all cell lines treated with olaparib but not veliparib, suggesting a stronger replicative stress response. Chk1 is also phosphorylated in response to SSBs. Although the differences we see in Chk1 phosphorylation may be because of variability in SSBs, this is unlikely given the similar extent of PARPis’ catalytic activity inhibition in olaparib and veliparib. Chk1 and p21 cooperate during replicative stress (37). In addition to Chk1 phosphorylation, we demonstrated a robust increase in p53 and p21 levels in U2OSDR-GFP and H1299DR-GFP cells treated with olaparib but not in the cells treated with veliparib, supporting the correlation with replicative stress severity. Our conclusion that olaparib’s effect on the cell cycle is p53 associated is supported by recent data showing that lymphoma cells treated with olaparib also feature increased p53 (38). Chk1 phosphorylation likely contributes to the increased p53 levels in olaparib-treated U2OSDR-GFP cells, given that phosphorylation and stabilization of p53 is one of the prime targets of Chk1.

Depletion of p53 in U2OSDR-GFP olaparib-treated cells had a greater impact on the cell cycle than veliparib-treated cells. This was even more pronounced in p53-null HCT116 cells. The cell growth upon p53 depletion was greater in olaparib-treated cells versus mock and veliparib-treated cells. This is in agreement with recent studies in HDR-proficient Cal-51 breast cancer cells depleted of p53 and treated with olaparib or veliparib (31).

Our data suggest that other PARPis similar to olaparib in their off-target effects cause HDR suppression in p53 wild-type cells. PJ34 is an earlier generation and less selective, it may target non-PARPis (e.g., enzymes that use NAD as a cofactor) contributing to observed off-target effects.

For PARPis to be effective in the context of synthetic lethality, it is important to have cells passing from G2-phase into mitosis and entering the replicative phase, considering that only then the HDR deficiency will result in accumulation of DSBs and ultimately cell death. The data suggest that veliparib unlike olaparib does not cause cell-cycle arrest in a p53 WT or null background. Olaparib treatment leads to G2 accumulation in p53 wild-type cell lines and, therefore, may be more effective in a p53 wild-type background when combined with the drugs that abrogate G2 checkpoint activation (e.g., Chk1 inhibitors).

In conclusion, monitoring the cell cycle, in combination with HDR activity assessment, is a useful tool in evaluating the effectiveness of PARPis. With more PARPis entering clinical trials, these data should help to better clarify which PARPis will be most effective for specific patient cohorts, resulting in improved treatments and combinatorial approaches.

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References


