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Rationale for PARP inhibitors in combination therapy with camptothecins or temozolomide based on PARP trapping versus catalytic inhibition

Junko Murai, Yiping Zhang, Joel Morris, Jiuping Ji, Shunichi Takeda, James H. Doroshow, and Yves Pommier

Developmental Therapeutics Branch, Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. (J.M., Y.P.)

Department of Radiation Genetics, Graduate School of Medicine, Kyoto University, Yoshidakonoe, Sakyo-ku, Kyoto 606-8501, Japan. (J.M., S.T.)

National Clinical Target Validation Laboratory, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. (Y.Z., J.J.)

Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA. (J.M., J.H.D.)
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Corresponding author: Yves Pommier, Developmental Therapeutics Branch, Laboratory of Molecular Pharmacology, Center for Cancer Research, NCI, 37 Convent Drive, Building 37, Room 5068, NIH, Bethesda, MD 20892-4255. Tel: 301-496-5944, Fax: 301-402-0752, email: pommier@nih.gov

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The number of words in Abstract is 233.

The number of words in Introduction is 542.

The number of words in Discussion is 858.

Abbreviations: AP, abasic; APE1, AP endonuclease 1; BRCA, breast cancer susceptibility gene; CI, combination index; CPT, camptothecin; DMSO, dimethyl sulfoxide; dRP, deoxyribose phosphate; DSB, double strand break; MMS, methyl methanesulfonate; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; SSB, single-strand break; Tdp1, tyrosyl-DNA phosphodiesterase 1; TMZ, temozolomide

A recommended section: Chemotherapy, Antibiotics, and Gene Therapy
Abstract

We recently showed that poly(ADP-ribose) polymerase (PARP) inhibitors exert their cytotoxicity primarily by trapping PARP-DNA complexes in addition to their NAD⁺-competitive catalytic inhibitory mechanism. PARP trapping is drug-specific with olaparib exhibiting a greater ability than veliparib, while both compounds are potent catalytic PARP inhibitors. Here, we evaluated the combination of olaparib or veliparib with therapeutically relevant DNA targeted drugs including the topoisomerase I inhibitor camptothecin, the alkylating agents temozolomide, the cross-linking agents cisplatin and the topoisomerase II inhibitor etoposide at the cellular and molecular levels. We determined PARP-DNA trapping and catalytic PARP inhibition in genetically modified chicken lymphoma DT40, human prostate DU145 and glioblastoma SF295 cancer cells. For camptothecin, both PARP inhibitors showed highly synergistic effects due to catalytic PARP inhibition, indicating the value of combining either veliparib or olaparib with topoisomerase I inhibitors. On the other hand, for temozolomide, PARP trapping was critical in addition to catalytic inhibition, consistent with the fact that olaparib was more effective than veliparib in combination with temozolomide. For cisplatin and etoposide, olaparib only showed no or only weak combination effect, which is consistent with the lack of involvement of PARP in the repair of cisplatin- and etoposide-induced lesions. Hence, we conclude that catalytic PARP inhibitors are highly effective in combination with camptothecins, whereas PARP inhibitors capable of PARP trapping are more effective with temozolomide. Our study provides insights in combination treatment rationales for different PARP inhibitors.
Introduction

Since the discovery of the synthetic lethality of PARP inhibitors in BRCA-deficient cells (Bryant et al., 2005; Farmer et al., 2005; McCabe et al., 2006; Helleday, 2011; Lord and Ashworth, 2012), the mechanism by which PARP inhibitors exert their cytotoxicity has been dominantly interpreted by an accumulation of unrepaired single-strand breaks (SSBs) resulting from catalytic PARP inhibition. This interpretation has recently been revisited after the demonstration that PARP inhibitors also trap PARP1- and PARP2-DNA complexes at DNA damage sites that arise spontaneously and/or are produced by the classical alkylating agent, methyl methanesulfonate (MMS) (Murai et al., 2012b). The fact that PARP1-depleted cells become tolerant to PARP inhibitors also supports the cytotoxic mechanisms of PARP-trapping (Liu et al., 2009; Pettitt et al., 2013). PARP trapping is not merely interpreted as resulting from catalytic PARP inhibition, which prevents dissociation of PARP from DNA and is required for repair completion (Satoh and Lindahl, 1992). Indeed, BMN 673, olaparib (AZD-2281) and niraparib (MK-4827) are much more effective than veliparib (ABT-888) for PARP trapping at concentration where BMN 673, olaparib, niraparib and veliparib fully inhibit PARPylation (Murai et al., 2012b; Murai et al., 2014). Based on the fact that olaparib and niraparib are much more cytotoxic than veliparib as single agents, it is plausible that PARP trapping is more cytotoxic than unrepaired SSBs caused by the absence of PARylation (Murai et al., 2012b; Murai et al., 2014). Chemical differences in drug structures may cause different allosteric effects between the PARP catalytic and DNA-binding domains, and we have proposed to classify PARP inhibitors based on their dual
molecular mechanisms of action: catalytic inhibition and trapping of PARP (Murai et al., 2012b; Fojo and Bates, 2013; Murai et al., 2014).

Combinations of different PARP inhibitors with a broad spectrum of genotoxic drugs are in clinical trials. These combinations include alkylating agents (temozolomide), topoisomerase I inhibitors (the camptothecin derivatives topotecan and irinotecan), topoisomerase II inhibitors (etoposide) and crosslinking agents (cisplatin) (Rouleau et al., 2010; Kummar et al., 2012; Curtin and Szabo, 2013). However, based on the fact that not all PARP inhibitors act similarly (Murai et al., 2012b; Fojo and Bates, 2013; Murai et al., 2014), it is critical to rationalize the most relevant combinations by choosing which PARP inhibitor and which chemotherapeutic agent act most effectively. It is also important to elucidate which combinations induce PARP trapping. Under such circumstances, highly potent PARP trapping drugs should be more effective than simple catalytic PARP inhibitors (olaparib > veliparib). On the other hand, if the synergistic effect is caused by catalytic PARP inhibition, veliparib should be comparable to olaparib.

In this study, we compared olaparib and veliparib in combination with 4 drugs from different therapeutically relevant classes (temozolomide, camptothecin, cisplatin and etoposide) to evaluate the potential and rationale for each combination. To determine whether potentiation was related to PARP catalytic inhibition or trapping, we used genetically modified chicken lymphoma DT40 cells (Buerstedde and Takeda, 1991; Maede et al., 2014), as well as human cancer cell lines, and measured olaparib- and veliparib-induced PARP-DNA complexes (PARP trapping). We chose human prostate cancer cells (DU145) and human glioblastoma cells (SF295) from the NCI60 cell line panel, because in our previous studies these cell lines showed differential responses to
veliparib and olaparib with respect to drug sensitivity and PARP trapping (Murai et al., 2012b; Murai et al., 2014).

Materials and Methods

Cell lines and drugs. DT40 cell lines were obtained from the Laboratory of Radiation Genetics Graduate School of Medicine in Kyoto University, Japan. Human prostate cancer cells (DU145, sex: male) and human glioblastoma cells (SF295, sex: female) were obtained from the National Cancer Institute Developmental Therapeutics Program (Frederick, USA). Olaparib, veliparib and camptothecin were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, and Division of Cancer Treatment and Diagnosis, NCI (Bethesda, MD). Temozolomide (T2577) and cisplatin (P4394) were purchased from Sigma Aldrich, USA. Drug stock solutions were made in DMSO at 10 mM for olaparib and veliparib, 10 μM for camptothecin, 100 mM for temozolomide, and in 0.75 M NaCl (pH 5) at 5 mM for cisplatin. Drug stock solutions were stored at -20°C in the dark and diluted in culture medium immediately before use. MMS (10%) was prepared fresh from 99% MMS (129925, Sigma-Aldrich, USA) in PBS, and diluted in culture medium immediately.

Immunoblotting. Ten million DT40 cells with 10 ml medium in 15 ml tube or semi-confluent human cells with 5 ml medium in 6-well dish were treated as indicated for 30 min or 4 hours, respectively, and then cells were collected. To prepare subcellular chromatin fractions, we used a Subcellular Protein Fractionation Kit from Thermo Scientific (78840, Rockford, IL, USA) following the manufacturer’s instructions.
Immunoblotting was carried out using standard procedures (Murai et al., 2012b). Densitometric analyses of immunoblots were carried out using Image J software (NIH). Each band blotted against PARP1 or histone H3 was selected with the rectangular selection tool, measured the intensity (area*mean), and the intensity of background was subtracted from the intensity of each band. The intensity of the PARP1 band was divided by the intensity of the correspondent histone H3.

**Antibodies.** Rabbit polyclonal anti-PARP1 antibody (sc-7150) and mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (sc-56) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-histone H3 antibody (07-690) was from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal anti-PAR polymer antibody (#4336-BPC-100) was from Trevigen. Secondary antibodies were horseradish peroxidase (HRP)-conjugated antibodies to rabbit or mouse IgG (GE Healthcare, UK).

**Immunoassay for PAR substrate.** The validated chemiluminescent immunoassay for PAR using commercially available reagents was performed; for detailed lab procedures, go to [http://dctd.cancer.gov/ResearchResources/biomarkers/PolyAdenosylRibose.htm](http://dctd.cancer.gov/ResearchResources/biomarkers/PolyAdenosylRibose.htm)

**Measurement of cellular sensitivity to drug treatment.** Cells were continuously exposed in triplicate to the indicated drug concentrations for 72 hours. For DT40 cells, 200 cells were seeded into 384-well white plates (#6007680 Perkin Elmer Life Sciences, Waltham, MA) in 40 μl of medium per well (Murai et al., 2012a; Maede et al., 2014). For
human cells, 1,500 cells of DU145 or 500 cells of SF295 cells were seeded in 96-well while plates (#6005680 Perkin Elmer Life Sciences) in 100 μl of medium per well. Cell viability was determined using the ATPlite 1-step kit (PerkinElmer). Briefly, 20 μl or 50 μl ATPlite solution for 384-well or 96-well plates respectively was added to each well. After 5 min, luminescence was measured by EnVision 2104 Multilabel Reader (PerkinElmer). The ATP concentration in untreated cells was defined as 100%. Viability (%) of treated cells was defined as ATP concentration in treated cells/ ATP concentration in untreated cells x 100.

**Flow cytometric analysis of cell cycle progression.** Cells were pulse labeled with 50 μM BrdU during the last 20 min of drug treatment. Cells were harvested, fixed in 70% ethanol, and stored at -20°C for 10 min. Cells were incubated for 30 min at room temperature in 2 N HCL-0.5% Triton X-100 to allow DNA denaturation. The cells were washed twice with PBS containing 0.5% Tween 20 and 0.5% bovine serum albumin. Cells were incubated for 1 h at room temperature with a FITC-conjugated anti-BrdU antibody (BD) and were treated with 0.5 mg/ml RNase A and 50 mg/ml propidium idodide. Samples were analyzed with a flow cytometer (FACScan; BD) using the CellQuest software (BD).

**Analysis of combination effects.** The synergism analysis for the combination effects was analyzed using the Chou-Talalay method (Chou, 2010). Combination Index (CI) of each combination treatment was calculated using CalcuSyn software (Biosoft, Inc.,
Cambridge, United Kingdom), and CI: 0.3-0.7, CI: 0.1-0.3 and CI: <0.1 were defined as
synergism, strong synergism and very strong synergism, respectively (Chou, 2006).

**Statistical analyses.** Results are presented as means ± standard deviation (SD) (n ≥ 3) or
as means (n=2). Differences between samples were assessed using the unpaired t test. All
analyses were conducted with prism 5.0 (GraphPad Software). P-values were two sided
and considered statistically significant when p<0.05.

**Results**

**Olaparib potentiates temozolomide better than veliparib**

Temozolomide is a commonly used anticancer drug, which like MMS induces
base damage resulting in abasic sites and single-strand breaks. Synergy between
temozolomide and PARP inhibitors has been reported by independent studies (Boulton et
al., 1995; Horton and Wilson, 2012; Kedar et al., 2012). To compare olaparib and
veliparib, we exposed wild-type and PARP1-deficient (\textit{PARP1-/-}) DT40 cells to
temozolomide with or without the PARP inhibitors (Figure 1A and B). Note that \textit{PARP1-/-}
DT40 cells are equivalent to PARP1 and PARP2 dual-knockout cells due to genetic
lack of PARP2 in avian cells (Hochegger et al., 2006; Murai et al., 2012b). Consistent
with the widely accepted concept that PARP1 is necessary for SSB repair, we found that
\textit{PARP1-/-} cells were hypersensitive to temozolomide (compare upper and lower panels of
either Figure 1A or B). The addition of 10 \textmu M olaparib or veliparib had no impact on
\textit{PARP1-/-} cells, confirming no off-target effect of either drug (Murai et al., 2012b).
Olaparib synergistically sensitized wild-type cells in a concentration-dependent manner
with low combination index (CI<0.3, supplementary table 1) at several points, and the sensitization with 0.1 μM of olaparib went beyond that of PARP1-/− cells (compare the panels of Figure 1A). Veliparib also synergized the cytotoxicity of temozolomide with low CI (<0.3, supplementary table 1), but at higher concentration than olaparib. Moreover, high concentration of veliparib (10 μM; >100-folds higher than olaparib) was required to go beyond the sensitivity of PARP1-/− cell.

To further document the marked difference between olaparib and veliparib in the cell viability assays, we examined cell cycle after drug treatments (Figure 1C-F). While 1 μM olaparib or veliparib, or 100 μM temozolomide alone had no significant impact, the combination of temozolomide with olaparib induced substantial G2 accumulation in wild-type cells (Figure 1C and D, supplementary table 2). Temozolomide with veliparib (Figure 1C and D), and temozolomide in PARP1-/− cells (Figure 1E and F) also induced significant G2 accumulation. However these effects were not as pronounced as for temozolomide with olaparib. Together, these results suggest that the combination of temozolomide with olaparib (≥ 0.1 μM) or veliparib (≥ 10 μM) induces additional cytotoxicity to PARP catalytic inhibition.

Olaparib and veliparib potentiate camptothecin comparably

Synergism between camptothecin and PARP inhibitors is well established (Smith et al., 2005; Daniel et al., 2009; Rouleau et al., 2010; Zhang et al., 2011; Brenner et al., 2012). As expected, PARP1-/− cells were hypersensitive to camptothecin (Hochegger et al., 2006) (lower panels of Figure 2A and B) indicating the involvement of PARP1 for the repair of camptothecin-induced lesions. The combination of camptothecin with
veliparib or olaparib synergistically sensitized wild-type DT40 cells with low CI (<0.3, supplementary table 1) at several points (upper panels of Figure 2A and B). Olaparib was more potent than veliparib. Yet, its concentration-dependent effect was not as pronounced as for the combination of temozolomide and PARP inhibitors (compare Figure 2A and B with Figure 1A and B). Furthermore, the sensitivity curves of both combinations never went beyond those of PARP1/- cells (Figure 2A and B). Cell cycle analyses showed that the combination of 20 nM camptothecin with 1 μM olaparib or veliparib induced substantial G2 accumulation (Figure 2C and D, supplementary Table 2). Notably, this pattern was similar to that of PARP1/- cells treated with 20 nM camptothecin (Figure 2E and F), suggesting that catalytic PARP inhibition causes the synergistic effect in case of camptothecin.

We also examined the different synergistic patterns in human prostate DU145 and glioblastoma SF295 cell lines. We compared the effect of olaparib and veliparib at 1 μM where PAR levels are indistinguishably low (Supplementary figure S1 right) and viability is not affected (see the starting points at y-axis of each curve, Figure 3). The synergistic effect was markedly greater for olaparib with temozolomide than for veliparib with temozolomide in both cells (Figure 3A). For camptothecin, olaparib was still more potent than veliparib in DU145, but the difference was not as pronounced as for temozolomide (Figure 3B upper panel). Furthermore, olaparib and veliparib were comparably potent for camptothecin in SF295 cells (Figure 3B lower panel). These results are consistent to those obtained with DT40 cells (Figure 1 and 2).
PARP trapping by temozolomide but not camptothecin in combination with PARP inhibitors

We examined PARP-DNA complexes by Western blotting in different conditions (Figure 4). Ten-micromolar veliparib with temozolomide induced PARP-DNA complexes less efficiently than 1 μM olaparib in DT40 and DU145 cells (compare lanes 5 versus 8, and 13 versus 16 of Figure 4A, and Figure 4B), suggesting that 10-fold higher concentration of veliparib than olaparib is not enough to induce the same amount of PARP-DNA complexes induced by olaparib. On the other hand, PAR levels were quite low under conditions where the differential PARP-DNA complexes were detected (Figure 4A, lanes 5-8, 13-16). We also confirmed that PAR levels at 1 μM olaparib and 1 μM veliparib were 1.5% and 6.6%, respectively, for DT40 cells, and 7.4% and 8.1%, respectively, for DU145 cells (Supplementary figure S1). Thus, olaparib and veliparib had similar PARP catalytic inhibition potencies at ≥ 1 μM. These results suggest that PARP inhibitors synergize temozolomide primarily by trapping PARP rather than by inhibiting the catalytic activity of PARP. Therefore, we conclude that highly potent PARP trapping agents potentiate temozolomide more efficiently than simple catalytic PARP inhibitors, and that olaparib is superior to veliparib in combination with temozolomide.

We also checked PARP trapping by the olaparib-camptothecin combination, and found that olaparib did not produce detectable PARP-DNA complexes in combination with camptothecin (Figure 4C, lanes 5-7, and 12-13). These results indicate that the synergistic effect of camptothecin with PARP inhibitors is due to catalytic PARP inhibition rather than PARP trapping (Das et al., 2014). Therefore, we conclude that
catalytic PARP inhibition is critical to potentiate camptothecin, and that veliparib and olaparib are both potent in combination with camptothecins.

Neither PARP inactivation nor PARP trapping appears relevant for the combination of olaparib with cisplatin or etoposide

Next, we examined whether PARP inactivation or trapping affected the cytotoxicity of cisplatin or etoposide (Figure 5). Whereas PARP1-/- DT40 cells were hypersensitive to temozolomide and camptothecin (Figure 1A and 2A), PARP1-/- cells were neither sensitive to cisplatin nor etoposide compared to wild-type (Figure 5A and C, upper panels), indicating that PARP1 is not involved in the repair of cisplatin- and etoposide-induced lesions in cellular models. Addition of olaparib in wild-type DT40 cells reduced the viability in a concentration-dependent manner (Figure 5A and C, lower panels). However, the curves were almost parallel, indicating that olaparib added its own cytotoxicity to those of cisplatin or etoposide. Furthermore, the CI of each point was never below 0.3 (Supplementary table 1), indicating lack of strong synergy observed with temozolomide and camptothecin. Olaparib did not potentiate the activity of cisplatin and etoposide in DU145 cells (Figure 5B and D) to the extent observed for temozolomide and camptothecin (see Figure 3). Furthermore, the combination of cisplatin and olaparib did not induce detectable PARP-DNA complexes (Figure. S2). Hence, the rationale for combining PARP inhibitors with cisplatin or etoposide is based on neither PARP-DNA trapping nor PARP catalytic inhibition.

Finally, to compare the drug combination results better, we prepared Fraction affected (Fa)-CI plots from the wild-type DT40 data with olaparib and/or veliparib
(Figure 6) (Chou, 2010). In these plots, we fixed the concentration of PARP inhibitors at 1 μM, because this concentration was common to all the combination studies, and because 1 μM of either olaparib or veliparib reduced PAR levels below 10%. Full information of CI is shown in Supplementary Table 1. Fa=1.0 means 100% reduction of viability, and the CI at the highest Fa is the most meaningful for combination therapy. At the highest Fa, the combination of temozolomide with olaparib showed the lowest CI (CI=0.046), followed by camptothecin with olaparib (CI=0.211), camptothecin with veliparib (CI=0.296), temozolomide with veliparib (CI=0.357), etoposide with olaparib (CI=0.536), and cisplatin with olaparib (CI=0.562). All together, the combination demonstrates the highest synergisms for olaparib with temozolomide and for veliparib or olaparib with camptothecin.

**Discussion**

Although combining PARP inhibitors with a wide range of drugs is actively being pursued, it is critical to elucidate whether different PARP inhibitors should be considered equal and how such combinations work because some PARP inhibitors selectively induce PAPR-DNA trapping in addition to catalytic PARP inhibition (Murai et al., 2012b; Murai et al., 2014). Our study demonstrates marked differences among combinations of four established chemotherapeutic agent classes (temozolomide, camptothecin, cisplatin and etoposide) with two PARP inhibitors in advanced clinical development, olaparib and veliparib, which differ by their PARP trapping efficiencies [(Murai et al., 2012b) and present study].

Temozolomide induces base damage (Newlands et al., 1997), generating abasic (AP) sites that are cleaved by AP endonuclease 1 (APE1), producing a 1-nt gap with 3’-
OH and 5’-dRP (5’-deoxyribose phosphate) groups at ends of the breaks (Hazra et al., 2007). Camptothecin also induces single strand breaks, but their 3’-DNA ends are covalently attached to topoisomerase I, while the 5’-DNA ends bear a sugar hydroxyl (Pommier, 2012; Pommier, 2013). The binding of PARP1 depends on the DNA substrate (Horton and Wilson, 2012). PARP1 preferentially binds directly to BER-intermediates with a 5’-dRP rather than to 5’-phosphate ends (Cistulli et al., 2004). This can explain the formation of PARP-DNA complexes at SSBs induced by temozolomide and suggest that combining temozolomide with a potent PARP trapping agents, such as olaparib is more rational than with a potent catalytic inhibitor with lower PARP trapping potency such as veliparib (Table 1).

On the other hand, in the case of camptothecin, the absence of 5’-dRP ends and the steric hindrance of the breaks by the covalently bound topoisomerase I at the 3’-ends of the broken DNA (Pommier, 2012; Pommier, 2013) probably explain the lack of detectable PARP-DNA complexes (Figure 4). Yet, PARP is critical for the repair of topoisomerase I cleavage complexes (Smith et al., 2005; Zhang et al., 2011; Brenner et al., 2012). PARylation, which can be readily detected in camptothecin-treated cells (Zhang et al., 2011; Brenner et al., 2012), can reverse topoisomerase I cleavage complexes (Malanga and Althaus, 2004), limit replication fork collisions (Ray Chaudhuri et al., 2012; Berti et al., 2013) and facilitate homologous recombination at replication forks stalled by topoisomerase I cleavage complexes (Sugimura et al., 2008). PARP also repairs transcription-mediated DNA damage by topoisomerase I cleavage complexes by acting in the same pathway as Tdp1 (tyrosyl-DNA phosphodiesterase 1), a critical repair enzyme that removes topoisomerase I-DNA covalent complexes (Zhang et al., 2011). We
recently revealed the coupling of TDP1 and PARP1 for the repair of topoisomerase I-DNA covalent complexes (Das et al., 2014). Considering that PARP-DNA complexes are undetectable even at high concentrations of camptothecin (1 μM) and that the combination of olaparib or veliparib never exceeds the hypersensitivity of PARP1-/- cells, we conclude that the contribution of PARP-DNA complexes is minimal, if any in the case of camptothecin. Ten-micromolar veliparib, which reduces PAR levels to 1% (Figure S1), still was not enough to reach the hypersensitivity of PARP1-/- cells (Figure 2B). The greater sensitivity of the PARP1-/- cell compare to the drug treated wild-type cells could be due to the low level residual PARylation, which might be sufficient to counter DNA damage. Nevertheless, since catalytic PARP inhibition explains the potentiation of camptothecins, highly potent PARP catalytic inhibitors devoid of cytotoxic effects, such as veliparib can be viewed as a rational combination with camptothecins (Table 1). Indeed, recent publications show synergy between topoisomerase I inhibitors and veliparib in chemo-resistant colon cancer cell lines (in vitro and/or in vivo studies) (Zhang et al., 2011; Davidson et al., 2013; Shelton et al., 2013).

Enhanced effects of PARP inhibitors with cisplatin have been reported (Rottenberg et al., 2008; Hastak et al., 2010; Chuang et al., 2012). Yet, the synergistic effects in these studies are relatively weak compared to those in the reports dealing with temozolomide (Brenner et al., 2012; Horton and Wilson, 2012; Kedar et al., 2012) and camptothecin (Smith et al., 2005; Daniel et al., 2009; Zhang et al., 2011). The weaker synergistic effect of PARP inhibitors with cisplatin than with temozolomide or camptothecin would be caused by the lack of involvement of PARP in cisplatin
sensitivity (Figure 5A upper). The same holds true for the topoisomerase II-targeted drug etoposide. Accordingly, drug-induced topoisomerase II-DNA complexes fail to activate PARP (Zwelling et al., 1982). The lack of synergy between etoposide and PARP inhibitors has also been previously reported (Bowman et al., 2001).

In summary, we propose that combination strategies with PARP inhibitors and the choice of PARP inhibitor should be based not only on whether PARP is involved in the repair of the DNA lesions produced by the agent with which the PARP inhibitor will be combined, but also on whether the lesions produced by the combination trap PARP-DNA complexes. Accordingly, PARP inhibitors that most efficiently trap PARP-DNA complexes, such as olaparib, niraparib and BMN 673 (Murai et al., 2012b; Murai et al., 2014) would be preferable to veliparib in the case of temozolomide combinations. On the other hand, veliparib should remain a valuable agent in combination with camptothecins as synergism for topoisomerase I inhibitors involves catalytic inhibition but not PARP trapping (Das et al., 2014). Table 1 summarizes these differences.
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Authorship Contributions

Participated in research design: Murai, Ji, and Pommier

Conducted experiments: Murai, Zhang, and Ji

Contributed new reagents or analytic tools: Zhang, Ji, Morris, Takeda and Doroshow

Performed data analysis: Murai, Zhang, Ji, and Pommier

Wrote or contributed to the writing of the manuscript: Murai and Pommier
References


Footnotes

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Figure Legends

**Fig. 1.** Olaparib augments the cytotoxicity of temozolomide (TMZ) better than veliparib and PARP deficiency.  

**A and B:** Viability curves of the indicated cell lines after continuous treatment for 72 hours with the indicated drugs. Cellular ATP concentration was used to measure cell viability. The viability of untreated cells was set as 100%. Error bars represent standard deviation (SD) (n=3). *Combination Index (CI) = 0.1-0.3 and **CI < 0.1 are indicated as strong and very strong synergy between the two treatments, respectively (see Fig. 6 and Supplemental table 1). Viability curves of wild-type (upper panels) and *PARP1*−/− cells (lower panels) treated with temozolomide alone or with the indicated concentrations of olaparib (A) or veliparib (B). The concentrations of PARP inhibitors are shown beside each curve in micro molar unit.  

**C-F:** Cell cycle analyses of wild-type (C and D) and *PARP1*−/− cells (E and F) 12 hours after the indicated drug treatments [TMZ (100 μM), olaparib (1 μM) and veliparib (1 μM)]. Representative data are shown from independent experiments with consistent results experiments (C and E). Percentages of cells in the subG1, G1, S, and G2/M phases are shown (D and F). Total counts within the outer frame of (C) or (E) are set as 100%. Results are average of three independent experiments. Statistical analyses were performed for G2/M population. *p<0.05 and **p < 0.01 are indicated. Data of average and SD for all phases are shown in Supplementary table 2.

**Fig. 2.** Olaparib and veliparib augment the cytotoxicity of camptothecin (CPT) comparably.  

**A and B:** Viability curves are shown as in Figure 1A. *Combination Index (CI) 0.1-0.3 and **CI < 0.1 are described as strong and very strong synergy between the
two treatments, respectively. C-F: Cell cycle analyses of wild-type (C and D) and PARP1-/− cells (E and F) 12 hours after the indicated drug treatments [CPT (20 nM), olaparib (1 μM) and veliparib (1 μM)]. Representative data are shown from multiple experiments (C and E). Percentages of cells in the subG1, G1, S, and G2/M phases are shown (D and F). Total counts within the outer frame of (C) or (E) are set as 100%. Results are average of three independent experiments. Statistical analyses were performed for G2/M population. *p<0.05 and **p < 0.01 are indicated. Data of average and SD for all phases are shown in Supplementary table 2.

**Fig. 3. Differential effect of olaparib and veliparib with temozolomide or camptothecin in human cell lines.** Viability curves of human prostate cancer DU145 cells (upper panels) and human glioblastoma SF295 cells (lower panels) after continuous treatment for 72 hours with the indicated drugs treatments [olaparib (1 μM) and veliparib (1 μM)]. Viability curves are shown as in Figure 1A. *Combination Index (CI) = 0.1-0.3 and **CI < 0.1 are described as strong and very strong synergy between the two treatments, respectively.

**Fig. 4. PARP inhibitors induce PARP-DNA complexes with temozolomide (TMZ) but not with camptothecin.** A and C: Western blot of chromatin-bound fractions against anti-PARP1, anti-histone H3, and anti-PAR (poly ADP-ribose) antibodies (A). Western blot of chromatin-bound fractions against anti-PARP1, and anti-PCNA
antibodies (C). Samples were prepared from wild-type DT40 cells (left) and DU145 cells (right) treated for 30 min and 4 hours, respectively, with the indicated drugs. Controls without drug are shown in lanes 1 and 9. Histone H3 (A) and PCNA (C) were used as positive markers for loading control. The blots are representatives of multiple experiments. **B:** Quantification of PARP-DNA complexes after the indicated treatments. Signal intensity was quantified using Image J software (NIH) from 4 independent Western blot analyses (2 blots of wild-type DT40 and 2 blots of DU145 cells). The intensity of PARP1 blot divided by the intensity of the corresponding histone H3 blot was measured for each treatment, and normalized to the sample of veliparib 1 μM + TMZ 1 μM treatment. Means ± SD (n = 4) are shown.

**Fig. 5. PARP1 is not involved for the repair of cisplatin- and etoposide-induced lesions.** Viability curves are shown as in Figure 1A. **A and C:** Viability curves of wild-type and *PARP1/-/-* DT40 cells treated with cisplatin (left) or etoposide (right) (upper panels). Lower panels: Viability curves of wild-type DT40 cells in combinations with the indicated concentrations of olaparib (micro molar units beside curves) with cisplatin (left) or etoposide (right). **B and D:** Viability curves of human prostate cancer DU145 cells in combinations with the indicated concentrations of olaparib (micromolar units beside curves) with cisplatin (left) or etoposide (right).

**Fig. 6. Quantitative analyses of synergistic effects in the different combinations. A:** Fraction affected (Fa) - Combination Index (CI) plots obtained from the data of Figure 1A-B for temozolomide in combination with 1 μM olaparib or 1 μM veliparib. **B:** Fa-CI
plots obtained from the data of Figure 2A-B for camptothecin in combination with 1 μM olaparib or 1 μM veliparib. **C and D:** Fa-CI plots obtained from the data of Figure 5A (lower panel) for cisplatin (C) and Figure 5C (lower panel) for etoposide (D) in combination with 1 μM olaparib. **A-D:** Shading reflects the level of synergism. CI between 0.3 and 0.7, CI between 0.1 and 0.3, and CI less than 0.1 indicate synergy, strong synergy, and very strong synergy, respectively. All data of CI are shown in Supplementary Table 1.
Table 1.: Summary of the differential effects of the PARP inhibitors, olaparib and veliparib in combination with temozolomide or camptothecin.

<table>
<thead>
<tr>
<th></th>
<th>Temozolomide</th>
<th>Camptothecins</th>
</tr>
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<tbody>
<tr>
<td>Trapping of PARP-DNA complex</td>
<td>++&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PARylation inhibition</td>
<td>++&lt;sup&gt;b&lt;/sup&gt;</td>
<td>++&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>=&gt; BER inhibition</td>
<td>=&gt; TDP1 inactivation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>=&gt; Persistent Top1cc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>=&gt; Replication fork stalling</td>
</tr>
<tr>
<td>Combination Index&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Olaparib &gt;&gt; Veliparib</td>
<td>Veliparib ≈ Olaparib</td>
</tr>
</tbody>
</table>

<sup>a</sup> Temozolomide in combination with PARP inhibitors (olaparib >> veliparib) induces the trapping of cytotoxic PARP-DNA complexes, whereas camptothecin does not induce detectable PARP-DNA complexes (see Figure 3).

<sup>b</sup> The effect of PARylation inhibiton for temozolomide and camptothecin are functionally different (see discussion), and both olaparib and veliparib effectively inhibit PARylation.

<sup>c</sup> Fraction affected (Fa) - Combination Index (CI) plots (see Figure 6) revealed that olaparib is more effective than veliparib for temozolomide, whereas veliparib is at least as effective as olaparib.
Figure 1

A

Wild-type
+ Olaparib (µM)

(% Viability)

Wild-type
+ Veliparib (µM)

(% Viability)

PARP1-/-
+ Olaparib (µM)

(% Viability)

PARP1-/-
+ Veliparib (µM)

(% Viability)

B

Temozolomide (µM)

(% Viability)

Temozolomide (µM)

(% Viability)

C

No drug

Propidium iodide

BrdU FITC

Temozolomide (µM)

(% Viability)

D

Propotion of cell cycle phase (%)

No drug

Temozolomide

Olaparib

TMZ+Olaparib

Veliparib

TMZ+Veliparib

E

PARP1-/-

No drug

Propidium iodide

BrdU FITC

F

Propotion of cell cycle phase (%)

No drug

Temozolomide

Olaparib

TMZ+Olaparib

Veliparib

TMZ+Veliparib
Figure 2

Wild-type

+ Olaparib (µM)

PARP1-/−

+ Olaparib (µM)

Camptothecin (nM)

Camptothecin (nM)

Wild-type

+ Veliparib (µM)

PARP1-/−

+ Veliparib (µM)

Camptothecin (nM)

Camptothecin (nM)

C

Wild-type

BrdU FITC

No drug

CPT

Olaparib

Veliparib

Propidium iodide

D

Propotion of cell cycle phase (%)

G2-M

S

G1

SubG1

E

PARP1-/−

BrdU FITC

No drug

CPT

Olaparib

Veliparib

Propidium iodide

F

Propotion of cell cycle phase (%)

G2-M

S

G1

SubG1
Figure 3

(A) DU145

(B) DU145

SF295

Camptothecin (nM)

Temozolomide (µM)

% Viability

% Viability

0 1 2 3 4 5

0 100 200 300

0 100 200 300

0 1 2 3 4 5

0 100 200 300

0 100 200 300

- no drug

- Veliparib

- Olaparib
Figure 5

A

B

C

D
Figure 6

A. Temozolomide + Olaparib

B. Camptothecin + Olaparib

C. Cisplatin + Olaparib

D. Etoposide + Olaparib

Fraction affected (Fa) vs. Combination Index (CI) for various drug combinations.