Mechanistic Dissection of PARP1 Trapping and the Impact on In Vivo Tolerability and Efficacy of PARP Inhibitors


Abstract

Poly(ADP-ribose) polymerases (PARP1, -2, and -3) play important roles in DNA damage repair. As such, a number of PARP inhibitors are undergoing clinical development as anticancer therapies, particularly in tumors with DNA repair deficits and in combination with DNA-damaging agents. Preclinical evidence indicates that PARP inhibitors potentiate the cytotoxicity of DNA alkylating agents. It has been proposed that a major mechanism underlying this activity is the allosteric trapping of PARP1 at DNA single-strand breaks during base excision repair; however, direct evidence of allosteroy has not been reported. Here we reveal that veliparib, olaparib, niraparib, and talazoparib (BMN-673) potentiate the cytotoxicity of alkylating agents. Consistent with this, all four drugs possess PARP1 trapping activity. Using biochemical and cellular approaches, we directly probe the trapping mechanism for an allosteric component. These studies indicate that trapping is due to catalytic inhibition and not allosteroy. The potency of PARP inhibitors with respect to trapping and catalytic inhibition is linearly correlated in biochemical systems but is nonlinear in cells. High-content imaging of γH2Ax levels suggests that this is attributable to differential potentiation of DNA damage in cells. Trapping potency is inversely correlated with tolerability when PARP inhibitors are combined with temozolomide in mouse xenograft studies. As a result, PARP inhibitors with dramatically different trapping potencies elicit comparable in vivo efficacy at maximum tolerated doses. Finally, the impact of trapping on tolerability and efficacy is likely to be context specific.

Implications: Understanding the context-specific relationships of trapping and catalytic inhibition with both tolerability and efficacy will aid in determining the suitability of a PARP inhibitor for inclusion in a particular clinical regimen.

Introduction

Poly(ADP-ribose) polymerase I (PARP1) is an abundant nuclear enzyme that catalyzes the formation of ADP-ribose polymers (PAR) on a host of protein substrates, including its own auto-modification domain (1). Extensive research has revealed numerous PARP1 functions spanning a diverse array of nuclear processes, including DNA damage repair (2–5), chromatin remodeling (6), transcriptional regulation (6), telomere maintenance (7), and cell death (8). Owing primarily to the role of PARP1 (and the related but less abundant PARP2 and PARP3) in DNA damage repair, a number of PARP inhibitors are undergoing clinical development as anticancer agents with a focus on tumors with impaired homologous recombination (HR) capability and on combination regimens with DNA-damaging chemotherapy or radiation (9). The function of PARP1 in DNA damage repair is complex and multifaceted, and includes contributions to multiple repair pathways including HR, base excision repair (BER), nucleotide excision repair (NER), and both classical and alternative nonhomologous end-joining (C-NHEJ and A-NHEJ; refs. 4, 10, 11). Preclinical studies have revealed combination activity of PARP inhibitors with DNA-alkylating agents, platinum, topoisomerase I inhibitors, and ionizing radiation (9). As the primary repair pathways for the lesions caused by each of these classes of agents differ, the mechanisms underlying their potentiation by PARP inhibitors is likely to be class specific.

An early step in BER is the high-affinity binding of PARP1 to a single-strand break, which causes a 500-fold increase in its catalytic activity (12). This stimulates the production of PAR, the nucleotide 2′-O-ribose (13). PAR synthesis recruits repair factors to the lesion and also electrostatically destabilizes the PARP1–DNA interaction, leading to rapid dissociation and allowing BER machinery access to the DNA break (14). Recent evidence indicates that PARP inhibitors potentiate the cytotoxicity of DNA alkylating agents such as methyl methanesulfonate (MMS) and temozolomide (TMZ) at least in part by preventing this destabilization, thereby trapping PARP1 at sites of DNA damage (15–20). A comparison of different PARP inhibitors revealed a lack of correlation between catalytic inhibition and trapping potency leading to the proposal of two nonmutually exclusive trapping mechanisms (17). The first is related to the inhibition of catalytic activity, wherein prevention of auto-modification leads to complex stabilization. In the second mechanism,
inhibitors allosterically enhance the affinity of PARP1 for damaged DNA independent of catalytic inhibition.

It has been proposed that veliparib is mechanistically distinct from olaparib, niraparib, and talazoparib in that it is unable to engage an allosteric trapping mechanism; however, direct evidence for this mechanism has not been reported for any PARP inhibitor. In addition, the impact of potent trapping activity on in vitro efficacy and tolerability has not been explored. In this study we probe the mechanism of PARP1 trapping by veliparib, olaparib, niraparib, and talazoparib and find no evidence of allosteric trapping activity. Moreover, our data reveal an inverse relationship between in vitro tolerability and trapping potency. As such, potent trapping activity is not associated with superior efficacy when PARP inhibitors are combined with TMZ at maximum tolerated doses (MTD) in HeyA8 xenografts. These observations have important implications for the translational relevance of differences in trapping activity observed in vitro.

**Materials and Methods**

**Cell culture**

HeyA8 cells purchased from M.D. Anderson were maintained in RPMI 1640 with 10% FBS at 5% CO2, 37°C. Cells were authenticated in October of 2013 using the Promega GenePrint 10 system immediately prior to freezing aliquots. Cells were used within 40 passages after thawing. Isogenic DLD1 and DLD1-BRCA2−/− cells with homozygous deletion of exon 11 of BRCA2 were licensed from Horizon Discovery, Ltd. and maintained in RPMI 1640 with 10% FBS (Gibco #10082-147) at 5% CO2, 37°C. HeyA8 cells for in vitro studies were grown to passage 3 in vitro in DMEM (Life Technologies Corp.) containing 10% FBS (Hyclone) and harvested while in log phase.

**Cellular trapping assays**

Cellular trapping assays were performed essentially as described (17), with minor modifications. Chromatin fractions were prepared using Thermo Scientific subcellular protein fractionation kits (cat. no. 78840) per manufacturer protocol. Inhibitors were included throughout fractionation to minimize dissociation. Samples were normalized for protein concentration and analyzed by immunoblotting (anti-PARP1 cat. no. 9542 and anti-H3, cat. no. 3638; Cell Signaling).

**Cell viability assays**

Cell viability was determined using CellTiter-Glo reagent (Promega, Inc.) per manufacturer protocol. For synergism experiments, excess over Bliss additivity was determined by standard methods (21).

**DNA duplexes for biochemical studies**

For time-resolved Förster resonance energy transfer (TR-FRET) experiments, a model single-strand break was generated by enzymatic digestion of a fluorescently labeled synthetic DNA duplex (5’-Alexa488-ACCCCTGTGCTGGCCCGGCGCAGACAGGTTCTCCCTCTCCTG-3’) with APE1 and UDG as described (17).

For biolayer interferometry (BLI) experiments, two synthetic oligonucleotides (dRP-GGAGAACAAGGTGAT and biotin-TEG-ATCACCTTGTCTACCACCGCCACCGGTTCTCTACCTCCCTGTGGCC) were annealed to generate a biotinylated hairpin with a single-strand break and a 5’-deoxyribose-5-phosphate at the break site.

**Biochemical trapping assays**

PARP1–DNA complexes were assembled by incubation of 1 nmol/L terbium-labeled anti-His antibody (Invitrogen), 2 nmol/L full-length His-tagged PARP1 and 0.4 nmol/L digested Alexa488-duplex in 10 mmol/L KPO4, pH 7.8, 50 mmol/L NaCl, 1 mmol/L EDTA, 0.05% pluronic F-68, and 1 mmol/L DTT for 1 hour at room temperature (RT). Where indicated, inhibitors were included during complex assembly. For kinetic experiments, NAD+ (10 nmol/mL final) was added to complexes and TR-FRET was measured using an Envision plate reader (Perkin Elmer). For dose–response experiments, TR-FRET was measured after 11 minutes. TR-FRET ratios were transformed into % dissociated by normalization to controls. Kinetic and dose–response data were fit with a single exponential and a four-parameter logistic equation, respectively.

**Equilibrium binding and kinetic studies of PARP1–DNA complexes**

PARP1–DNA complexes were evaluated in a TR-FRET-based system similar to that described above with the exception that NAD+ was omitted. For kinetic experiments, complexes were assembled at a concentration of 0.8 nmol/L fluorescent duplex until equilibrium was reached, at which point unlabeled duplex was added (167 nmol/mL final) and TR-FRET was measured as a function of time.

Alternatively, binding kinetics of PARP1–DNA complexes were analyzed via BLI (Octet Red 384; ForteBio) at 25°C. The assay used a 16 channel, 96-well plate format at a shake speed of 1000 rpm. Prior to the assay, sensors were pretreated in Buffer A (10 mmol/L HEPES, pH 7.5, 250 mmol/L NaCl, 3 mmol/L EDTA, 0.05% Tween 20, 5 mmol/mL DTT) for 15 minutes. Assay steps consisted of (i) 60-second equilibration in buffer A; (ii) 120-second immobilization of DNA (0.5 μg/mL in buffer A); (iii) 120 seconds of buffer A for baseline determination; (iv) 300-second association of PARP1 in buffer A as a six-point, three-fold concentration series (100–0.4 nmol/L); (v) 600-second dissociation. Where indicated, buffer A was formulated with 2 μmol/L inhibitor for steps 2–5. Responses were normalized to baseline and fit with a mass transport binding model to determine Kd (on-rate), koff (off-rate), and equilibrium dissociation constant (Kd).
incubated to equilibrium at RT with 10 mM L-KPRO₄, pH 7.8, 50 mM/L NaCl, 1 mM/L EDTA, 0.05% Pluronic F-68, 1 mM/L DTT, 1 mM/L terbium-anti-His, 2 mM/L His-PARP1, and 20 mM/L OG488-labeled NAD⁺ binding site probe. TR-FRET ratios were normalized to controls and fit with a four-parameter logistic equation. Where indicated, DNA was included at 10 mM/L (20-fold above Kₐ).

Michaelis–Menten kinetics
Initial rates were determined at different concentrations of NAD⁺ using a PARP1 Assay Kit from BPS biosciences according to manufacturer protocol (cat. no. 80551).

Cellular PAR assays
Cellular PAR levels were measured by ELISA as described (22–24).

High-content imaging of γH2Axl levels
Cells were seeded on collagen-coated 96-well plates overnight, treated with compounds for 4 hours and fixed with formaldehyde (2%) for 10 minutes at room temperature (RT). Cells were washed twice with PBS, permeabilized with 0.1% Triton X-100 for 15 minutes at RT, and washed three times with PBS. Plates were blocked in 1% bovine serum albumin (BSA) in PBS for 30 minutes at RT. Anti-phospho-Histone H2AX (Ser139) antibody (EMD Millipore, cat. no. 05-636) diluted 1:1 in glyceroil and then further diluted 1:1,600 in antibody dilution buffer (0.3% BSA in PBS) was added overnight at 4°C. Cells were washed three times in PBS and incubated with Alexa Fluor 555–conjugated goat antimouse Ab (Life Technologies, cat. no. A21424) and Hoechst 33342 (Life Technologies, cat no. H5370) diluted 1:500 and 1:10,000, respectively in antibody dilution buffer for 1 hour at RT. Plates were washed three times with PBS and scanned within 24 hours. Images were acquired on a CellInsight (Thermo Scientific) by automated image acquisition (12 fields per well) using a 10× objective. Data analysis was performed using CellSens View Software (Thermo Scientific).

In vivo pharmacology
HeyA8 xenograft models. A total of 1 × 10⁶ HeyA8 cells in 0.1 mL of a 1:1 mixture of S-MEM (Life Technologies Corp.) and Matrigel were inoculated subcutaneously into the right flank of female C. B.-17 SCID mice (Charles Rivers Laboratories) on Day 0.

Efficacy studies. Two separate in vivo studies were conducted; in the first study mice were allocated into study groups with mean tumor volume 439 ± 17 mm³ on Day 13 and dosing was initiated. In the second study, mice were allocated into study groups with mean tumor size of 490 ± 27 mm³ on Day 15 and dosing initiated on Day 16. For single agent studies, PARP inhibitors were administered orally, once daily for 5 days. For combination studies TMZ was administered alone or in combination with PARP inhibitors administered orally, once daily for 5 days. Mice were observed daily and measured twice weekly. Mice were euthanized when tumor volumes reached a maximum of 2500 mm³ or when skin ulcerations occurred. Tumor length (L) and width (W) were measured via electronic caliper and the volume was calculated according to the following equation: \( V = L \times W^2 / 2 \) using Study Director version 3.1 (Studylog Systems, Inc.). Animal research was approved and overseen by the AbbVie Institutional Animal Care and Use Committee (AbbVie IACUC, in accordance with all ALAAC guidelines).

Compound formulation. TMZ and Veliparib were prepared as previously described (25). Olaparib, purchased from Selleck Chemicals or synthesized at AbbVie, was dissolved in a vehicle containing 1% DMSO, 10% Ethanol, 30% PEG 400, 59% Phosal 53 MCT using a probe sonifier (Branson 450). Talazoparib was synthesized at AbbVie and kept frozen as a 3 mg/mL stock solution in 100% DMSO; the stock solution was diluted to 0.0036 mg/mL in 0.5% HPMC prior to dosing.

Determination of MTDs. Tolerability studies were conducted in naïve CB-17 SCID female mice. PARP inhibitors were administered orally, once daily for 21 days as single agents; for combination studies compounds were dosed orally, once daily for 5 days. Doses resulting in <15% weight loss were considered tolerable.

Pharmacokinetics (PK) and pharmacodynamics (PD). HeyA8 tumor bearing mice were size matched and dosed orally, once daily for 5 days. Plasma and tumor samples were collected at 1, 2, 4, 8, and 24 hours following dosing. Blood samples were collected in microtainer tubes (BD Bioscience) containing K⁺–EDTA and centrifuged. The upper layer consisting of the plasma was collected and frozen. Tumor samples were flash frozen in liquid nitrogen. Inhibitor (26) and PAR levels (22–24) were measured as described.

Results
Potentiation of alkylating agents by PARP inhibitors in vitro
Recent studies have revealed that trapping of PARP1 onto damaged chromatin is central to the synergistic cytotoxicity observed when PARP inhibitors are combined with alkylating agents (15, 17). These same studies suggested that veliparib is a weak trapping agent. This was somewhat unexpected given the well-established potentiation of alkylating agents by veliparib in vitro and in vivo (25, 27–31) and the observation that loss of PARP1 expression is a resistance mechanism in cells chronically exposed to veliparib and TMZ (16).

To better understand the relationship between trapping and potentiation of alkylating agents, we compared the abilities of veliparib, olaparib, niraparib, and talazoparib to potentiate the activity of MMS and TMZ in HeyA8, DLD1, and DLD1-BRCA2⁻/⁻ cells (Fig. 1). Bliss response surfaces (Supplementary Fig. S1) revealed robust synergism between MMS and each PARP inhibitor in all three cell lines, with peak synergism observed at a concentration of 20–100 μmol/L MMS in all cases. Similar results were observed with TMZ; however, the degree of synergism was more modest in some cases and required somewhat higher concentrations (60–300 μmol/L TMZ).

Examination of Bliss surface cross-sections (Fig. 1) allows for a comparison of the concentrations of PARP inhibitor required for comparable synergism. In HeyA8 cells at 20 μmol/L MMS, talazoparib elicited peak synergism at 5 mMol/L, while much higher concentrations of olaparib, niraparib and veliparib were required (1.5, 3, and 25 μmol/L, respectively; Fig. 1A). Similar profiles were observed at 300 μmol/L TMZ, albeit with a more modest degree of synergism (Fig. 1B). Similar results were observed in DLD1 cells; however, the degree of synergism with TMZ greater than that
In vitro synergism of PARP inhibitors with MMS or TMZ. Cells were treated with two-dimensional dose responses of PARP inhibitors and DNA alkylating agents for 5 days. Excess over Bliss additivity was determined for each condition. For full response surfaces, see Supplementary Fig. S1. To facilitate comparison of PARP inhibitors, cross-sections of response surfaces at the concentrations of alkylating agent eliciting peak synergism are overlaid. In this analysis, values of zero indicate no activity or additivity whereas higher values indicate stronger synergism. The decreases observed at higher PARP inhibitor concentrations are due to a loss of synergism to single-agent PARP inhibitor activity. A, HeyA8 cells with 20 \( \mu \text{mol/L} \) MMS. B, HeyA8 cells with 300 \( \mu \text{mol/L} \) TMZ. C, DLD1 cells with 100 \( \mu \text{mol/L} \) MMS. D, DLD1 cells with 300 \( \mu \text{mol/L} \) TMZ. E, DLD1-BRCA2\( ^{−/−} \) cells with 20 \( \mu \text{mol/L} \) MMS. F, DLD1-BRCA2\( ^{−/−} \) cells with 60 \( \mu \text{mol/L} \) TMZ. Data represent means with standard errors from at least two independent experiments run in duplicate.
in HeyA8 cells (Fig. 1C and D). BRCA2 deletion in these cells resulted in a decrease in the concentration of PARP inhibitor required for synergism (Fig. 1E and F). Although the rank order of potency with respect to potentiation of alkylating agents was concordant with catalytic inhibition potency in all three cell lines, the broad range of PARP inhibitor concentrations required to elicit synergism did not reflect the much smaller differences in cellular PAR IC50, enzyme inhibition IC50, Kd, or binding kinetics (Table 1). As these agents have been reported to vary widely in their PARP1 trapping activity (17, 18), these results further delineate the mechanism whereby trapped PARP1 leads to cell death.

Strikingly, talazoparib was unique in that the concentration required for half-maximal trapping was approximately 100-fold higher than plasma exposures in mouse models (17). This duplex bound to full-length PARP1 in vitro, and catalytic inhibition potency in all three cell lines, was concordant with catalytic inhibition potency in all three cell lines, but is consistent with its relative potency of PARP inhibitors used in this study

<table>
<thead>
<tr>
<th>Table 1. Summary of in vitro potency of PARP inhibitors used in this study</th>
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<td>PARP Trapping: Impact on in Vivo Activity of PARP Inhibitors</td>
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<tr>
<th>IC50 (PARP enzyme activity, nmol/L)</th>
<th>Veliparib</th>
<th>Olaparib</th>
<th>Niraparib</th>
<th>Talazoparib</th>
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<tr>
<td>PAR IC50 (HeyA8, nmol/L)</td>
<td>3.0 ± 0.2</td>
<td>0.42 ± 0.02</td>
<td>5.0 ± 1.0</td>
<td>0.60 ± 0.18</td>
</tr>
<tr>
<td>PAR IC50 (DLD1, nmol/L)</td>
<td>3.7 ± 0.2</td>
<td>1.3 ± 0.5</td>
<td>7.9 ± 12</td>
<td>0.5 ± 0.16</td>
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<tr>
<td>PAR IC50 (MMS, nmol/L)</td>
<td>2.0 ± 0.2</td>
<td>0.86 ± 0.26</td>
<td>9.5 ± 0.09</td>
<td>0.47 ± 0.11</td>
</tr>
<tr>
<td>PAR IC50 (TMZ, nmol/L)</td>
<td>3.3 ± 2.3</td>
<td>1.3 ± 0.7</td>
<td>1.0 ± 0.5</td>
<td>0.50 ± 0.26</td>
</tr>
<tr>
<td>PAR IC50 (PARP trapping, nmol/L)</td>
<td>1.8 ± 10^6</td>
<td>2.5 ± 10^6</td>
<td>4.1 ± 10^6</td>
<td>3.6 ± 10^6</td>
</tr>
<tr>
<td>PAR IC50 (SB DNA trapping, nmol/L)</td>
<td>7.0 ± 10^-4</td>
<td>3.2 ± 10^-4</td>
<td>5.6 ± 10^-4</td>
<td>6.3 ± 10^-5</td>
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<tr>
<td>PAR IC50 (DNA trapping, nmol/L)</td>
<td>4.4 ± 1</td>
<td>1.3 ± 1</td>
<td>1.4 ± 1</td>
<td>0.17 ± 1</td>
</tr>
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*Data are presented as means and standard errors from at least 3 independent experiments.
The concentration of this nicked duplex (10 nmol/L) did not stabilize inhibitor binding to PARP1.

Next, we compared the trapping activity of the inhibitors in a biochemical system similar to that reported previously (17, 18, 33). PARP1–DNA complexes were preincubated in the presence or absence of PARP inhibitors after which NAD\(^+\) was added to initiate automodification. In the absence of a PARP inhibitor, automodification led to the rapid dissociation of PARP1 from the DNA with dissociation complete in approximately 10 minutes (Fig. 3A). As expected, high concentrations (10 \mu mol/L) of veliparib, olaparib, niraparib, or talazoparib revealed PARP1 trapping activity, with significant decreases in the rate of PARP1 dissociation from DNA. Dose responses in this system (Fig. 3B) revealed EC\(_{50}\)s that were well correlated with PARP1 IC\(_{50}\)s (Table 1). Notably, dramatic differences in cellular trapping potency were not recapitulated in this system.

Although these experiments allow for a quantitative analysis of PARP1 trapping, the inclusion of NAD\(^+\) precludes determination

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Figure 2.
PARP1 trapping in cells cotreated with alkylating agents and PARP inhibitors. Unless otherwise indicated, cells were treated for four hours prior to chromatin fractionation and immunoblotting. A, MMS dose response (three-fold serial dilutions, 1 mmol/L top dose) in HeyA8 cells. B, TMZ dose response (three-fold serial dilutions, 1 mmol/L top dose) in HeyA8 cells. C, time-course of PARP1 trapping in HeyA8 cells treated with 1 mmol/L MMS and five-fold serial dilutions of PARP inhibitors (1 \mu mol/L top dose for talazoparib, 100 \mu mol/L top dose for all others). E and F, quantification of PARP1 trapping in human cancer cells. Densitometry was performed on immunoblots such as those in D. PARP1 levels were normalized to histone H3 levels. Data represent means with standard errors from at least three independent experiments. E, HeyA8; F, DLD1; G, DLD1-BRCA2\(^{+/−}\).
of the relative contributions of allostery and catalytic inhibition. To directly assess the ability of PARP inhibitors to allosterically trap PARP1 onto DNA, we utilized TR-FRET to explore the dissociation kinetics of PARP1–DNA complexes in the absence of NAD⁺. In these studies, apparent PARP1 dissociation was initiated by addition of a large excess of unlabeled nicked duplex. In these experiments, PARP inhibitors failed to stabilize the complex (Fig. 3C). Similarly, PARP inhibitors did not enhance the equilibrium binding affinity of PARP1 for DNA (Fig. 3D). These results agree with our observation that nicked DNA does not enhance the affinity of PARP inhibitors for PARP1 and strongly suggest that there is no allostERIC component to the PARP trapping mechanism.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Mechanistic analysis of PARP1 trapping in biochemical systems. A–D, analysis of PARP1 trapping using TR-FRET. A, dissociation curves of PARP1 from DNA in the presence of DMSO or 10 μM veliparib, olaparib, niraparib, or talazoparib. B, PARP1 trapping dose responses 10 minutes after addition of NAD⁺ to preassembled PARP1-DNA complexes. Higher TR-FRET ratios indicate that complex dissociation has been inhibited. C, dissociation of PARP1–DNA complexes in the absence of NAD⁺ following addition of a large excess of unlabeled DNA in the presence of DMSO or PARP inhibitors (10 μM). D, effects of PARP inhibitors (10 μM) on binding of PARP1 to nicked DNA in the absence of NAD⁺. E, analysis of PARP1 trapping using BLI.
To further test this hypothesis in an orthogonal biochemical system, we evaluated the binding kinetics of the PARP1–DNA interaction by bio-layer interferometry. These studies employed a synthetic hairpin containing a single-strand break with a 5'-dRP. The purpose of the hairpin was to prevent PARP1 from binding to the blunt duplex ends. As we observed in the TR-FRET experiments, saturating concentrations (2 μmol/L) of PARP inhibitors had no impact on the kinetics or equilibrium binding affinity of the PARP1–DNA complex (Fig. 3E and Supplementary Fig. S3).

All of the PARP inhibitors included in this study are based on nicotinamide-like pharmacophores and are thought to bind to the NAD$^+$ binding pocket within the catalytic domain of PARP1 and inhibit the enzyme competitively (34). However, most of the direct evidence supporting this mode of inhibition is from crystallographic studies, which have been limited to the catalytic domain. To further investigate the mode of inhibition, we conducted a Michaelis–Menten kinetic analysis using full-length PARP1 (Supplementary Fig. S4). All four inhibitors in this study displayed kinetic behavior consistent with a purely competitive mode of inhibition, eliciting significant increases in the apparent $K_m$ for NAD$^+$ with no significant changes in $V_{\text{max}}$. Moreover, the $K_s$ determined from this analysis were in very good agreement with cellular PAR IC$_{50}$s as well as the trapping EC$_{50}$s in the TR-FRET assays (Table 1). Consistent with the DNA binding data, these results did not reveal evidence of an allosteric interaction for any of the four PARP inhibitors.

Collectively, the data from purified biochemical systems support the conclusion that PARP inhibitors do not allosterically stabilize the PARP1–DNA complex and that PARP1 trapping is entirely attributable to inhibition of autodisplacement. To confirm that trapping is driven by inhibition of autodisplacement in cells, we utilized the potent, selective nicotinamide phosphoribosyltransferase (NAMPT) inhibitor FK866 to deplete cellular NAD. As FK866 is known to be broadly cytotoxic to cancer cells (35, 36), careful optimization of treatment conditions was necessary. Treatment of HeyA8 cells with 1 μmol/L FK866 for 24 hours was sufficient to deplete cellular total NAD (NAD$^+$ and NADH) and PAR by 90% and 95% respectively, while sparing cellular ATP and preserving cell viability (Fig. 4A). After depletion of cellular NAD, treatment with MMS resulted in PARP1 trapping to an extent comparable to cotreatment of cells with MMS and PARP inhibitors (Fig. 4B). Addition of the NAMPT reaction product, nicotinamide mononucleotide (NMN), prevented NAD depletion by FK866 (Fig. 4C) and reversed the trapping effect. This confirmed that FK866-induced trapping was due to NAD depletion and not off-target effects such as direct PARP inhibition or DNA damage. These results indicate that inhibition of PARP catalytic activity is sufficient to account for the maximum trapping activity associated with any of the four PARP inhibitors. Furthermore, if PARP inhibitors were capable of allosterically trapping PARP1, additive trapping activity between FK866 and PARP inhibitors may be expected. Even at high doses, PARP inhibitors were not capable of enhancing trapping after cellular NAD depletion (Fig. 4B). These results are in agreement with our observations in biochemical systems and indicate that PARP1 trapping in cells is not allosteric in nature and is instead mediated by inhibition of catalytic activity.

### Figure 4.

The effects of NAD depletion on PARP1 trapping in cancer cells. A, treatment of HeyA8 cells with FK866 for 24 hours depletes total NAD (NAD$^+$ + NADH) and PAR while sparing ATP and cell viability. For the PAR ELISA control, cells were treated with 3 μmol/L olaparib. B, PARP1 trapping in HeyA8 cells treated with MMS and FK866. Cells were treated with 1 μmol/L FK866 (where indicated) for 24 hours prior to treatment with 1 μmol/L MMS ± 50 μmol/L of PARP inhibitors for 4 hours. Treatments were also performed in the presence of 300 μmol/L NMN (the product of NAMPT). C, total NAD levels in cells treated as in B.

### Comparison of DNA damage induction by different PARP inhibitors

It has been reported that talazoparib has extremely potent DNA damaging activity as a single agent in HeLa cells and that it is at least 100-fold more potent than olaparib in this respect (37). Much like the stark differences in trapping activity, this difference is greater than expected based on comparisons of catalytic potency. Given that we were unable to demonstrate an allosteric PARP trapping mechanism, we considered the alternative hypothesis...
that differences in observed cellular trapping activity may be in part due to differences in the extent of DNA damage under the conditions of the PARP trapping experiments.

To test this, we determined the levels of γH2Ax in HeyA8 cells cotreated with PARP inhibitors and MMS for four hours to reflect the conditions of the PARP trapping experiments. All four PARP inhibitors significantly increased γH2Ax levels compared with MMS alone (Fig. 5A). Similar to PARP1 trapping activity, the compounds spanned a broad range of potency (Fig. 5B) with 3 nmol/L talazoparib eliciting γH2Ax signal comparable to 4 µmol/L veliparib, 2 µmol/L olaparib, and 4 µmol/L niraparib. Treatment of HeyA8 cells with PARP inhibitors alone for four hours resulted in minimal γH2Ax signal (Fig. 5C), indicating that γH2Ax observed after such short treatments is due entirely to potentiation of MMS-induced damage.

It is thought that PARP inhibition leads to double-strand breaks via replication fork collapse at sites of unrepaired single-strand breaks. Our observation that cotreatment of cells with MMS and PARP inhibitors led to significant induction of γH2AX in nearly 100% of cells in an asynchronous population within 4 hours suggested that the treatments used for PARP trapping assays cause double-strand breaks by a more direct mechanism. To investigate this, we extracted cell-cycle profiles from our high-content imaging data and determined the mean intensity of γH2AX staining as a function of cell-cycle phase (Supplementary Fig. S5). Strikingly, we observed robust γH2AX staining in G1, S, and G2–M after a four hour treatment with 1 mmol/L MMS alone. This signal was potentiated by all four PARP inhibitors in all phases of the cell cycle to a similar extent. These data indicate that the high concentrations of MMS required to detect PARP trapping cause double-strand breaks in the absence of PARP inhibition and that replication fork collapse cannot alone account for the potentiation of this damage by PARP inhibitors.

**In vivo efficacy of different PARP inhibitors in combination with TMZ**

To establish the relationship between potent PARP trapping activity and in vivo efficacy, we first determined the MTDs of veliparib, olaparib, and talazoparib in naïve C.B-17 SCID mice alone or in combination with 50 mg/kg/day of TMZ. This dose of TMZ was selected because it is well-tolerated and comparable to the clinically recommended dose of 150 mg/m². The monotherapy MTDs for veliparib, olaparib, and talazoparib were 200, 200, and 0.33 mg/kg/day, respectively (once daily, 21 days). When
combined with TMZ (once daily, 5 days) in HeyA8 xenograft tumor-bearing mice, MTDs were reduced to 75, 25, and 0.033 mg/kg/day for veliparib, olaparib, and talazoparib, respectively (Supplementary Table S1). These data demonstrate a strong inverse relationship between PARP trapping activity and tolerability that results in significant differences in achievable plasma and tumor exposures (Supplementary Table S2).

We next designed a comparative efficacy study in a HeyA8 xenograft model (Fig. 6). Although all three PARP inhibitors significantly reduced tumor PAR levels at monotherapy doses (Supplementary Fig. S6A–S6C), they did not slow tumor growth in this setting (Fig. 6A). In contrast, 50 mg/kg/day of TMZ demonstrated significant monotherapy activity, with tumor outgrowth beginning on day 43. The combination of 75 mg/kg/day of veliparib with 50 mg/kg/day of TMZ resulted in significant combination efficacy and delayed tumor outgrowth for 20 days (Fig. 6B). Similarly, the combination of 25 mg/kg/day of olaparib or 0.033 mg/kg/day of talazoparib with TMZ delayed tumor outgrowth by 13 and 9 days, respectively. All three PARP inhibitors resulted in a highly significant advantage in survival to 1 cm² relative to TMZ alone (P < 0.0001; Fig. 6C and D). The veliparib/TMZ group displayed a statistically significant survival advantage relative to both the olaparib/TMZ and talazoparib/TMZ groups (P < 0.045 and 0.02, respectively) despite veliparib being the least potent trapping agent of the three. These results were correlated with differences in the durability of the reduction in tumor PAR levels elicited by these inhibitors at these doses (Supplementary Fig. S6D–S6F). The Cmax for all three PARP inhibitors significantly exceeded the respective PAR IC50s in HeyA8 cells consistent with the robust tumor PAR inhibition observed at Cmax. At Cmin (24 hours after dosing), veliparib, olaparib, and talazoparib were at concentrations approximately equal to 15 ×, 4 ×, and 2 × their respective PAR IC50s. The pharmacokinetic data are thus well correlated with the rate of recovery of tumor PAR levels after dosing.

**Discussion**

Several recent reports have established PARP trapping as an important mechanism whereby PARP inhibitors potentiate the cytotoxicity of DNA alkylating agents (15–18). Observations that trapping activity is not linearly correlated with enzymatic...
inhibition potency led to the proposal of a novel allosteric trapping mechanism (17). In this study, we have thoroughly investigated the mechanism of PARP trapping by veliparib, olaparib, niraparib, and talazoparib and found no evidence of allostery. This is consistent with previous observations in a fluorescence polarization assay where omission of NAD⁺ stabilized PARP1 binding to DNA as effectively as talazoparib (18). These results demonstrate that PARP trapping is attributable to the well-established mechanism whereby inhibition of automodification stabilizes DNA binding (14). Recently, arguments in support of an allosteric trapping mechanism have been made on the basis of molecular dynamics simulations (33) and structural studies of the PARP1 catalytic domain in complex with talazoparib (36). However, crystal structures of PARP1 bound to PARP inhibitors have been restricted to the catalytic domain and reveal that conformational differences induced by different PARP inhibitors are rather minor. Currently, there is no direct evidence that PARP inhibitor binding alters the conformation of the DNA binding domain. If subtle alterations in the conformation of the catalytic domain were indeed accountable for the dramatic variance in cellular PARP trapping activity, this would be expected to manifest in the biochemical trapping experiments in Fig. 3. Instead, we observe much smaller differences that are well correlated with catalytic potency.

Our data demonstrate that differential trapping activity is concordant with differences in the potentiation of MMS-induced DNA damage. Taken alone, these results cannot distinguish whether potent trapping activity leads to greater potentiation of DNA damage or differential binding of DNA damage. The data obtained accounts for apparent differences in trapping activity in cells. Our observation that trapping is not allosteric in nature and is very well correlated with catalytic potency in purified biochemical systems is consistent with the latter hypothesis. The precise role of trapping in the potentiation of DNA damage remains to be elucidated. We observe significant γH2AX induction in all phases of the cell cycle by MMS alone and this signal is potentiated by all four PARP inhibitors. These results indicate that double-strand breaks induced by the extreme treatment conditions required for PARP trapping assays are not simply the result of replication fork collapse at unrepaired single-strand breaks or trapped PARP complexes. As PARP1 is well established to bind to both single-strand and double-strand breaks, it remains possible that PARP1 binding to double-strand breaks contributes significantly to the trapping observed in cells treated with high concentrations of MMS.

Strikingly, talazoparib is unique in that it potentiates γH2AX at concentrations below its cellular PAR IC₅₀ in HeyA8 cells. In contrast, other inhibitors require concentrations greater than their respective PAR IC₅₀ to elicit a similar effect. Likewise, talazoparib is unique in that its trapping activity is concordant with cellular PAR inhibition, while other inhibitors in the class require concentrations significantly higher than those required to elicit reductions in cellular PAR levels. The reasons for these differences remain unclear, however talazoparib is the most potent PARP inhibitor reported and possesses a slow dissociation half-life (37). Previous studies have suggested positive correlations between longer drug residue time, extent of DNA damage and cytotoxic potency for the camptothecin family of topoisomerase I inhibitors, another class of drugs in which trapping of the target enzyme onto DNA is central to the cytotoxic mechanism (39). The relationship between these features and potent PARP trapping activity warrants further investigation.

Previous characterization of PARP trapping has been limited to comparisons of potency in vitro (15, 17–19). Understanding the relevance of trapping to potential clinical benefit will require a thorough understanding of the relationships between trapping activity, tolerability and efficacy in vivo. It has been argued that trapping is likely to be clinically relevant because the concentrations of PARP inhibitors required to detect trapping are within range of clinical exposures in a monotherapy setting (17). However, trapping has yet to be demonstrated with PARP inhibitors alone. It is most easily detected in combination with MMS, but this is not a clinically approved agent. To date, the only clinically approved agent with which trapping has been demonstrated is TMZ (Fig. 2B; ref. 19). The high concentrations of TMZ required (>300 μmol/L) significantly exceed the maximum exposures observed in the clinic (32). This suggests that either clinically tolerated regimens containing PARP inhibitors and TMZ cannot engage the trapping mechanism or currently available assays are not sensitive enough to detect physiologically relevant levels of trapping. In support of the latter, genetic evidence supports a role for trapping in the potentiation of MMS activity by PARP inhibitors in vitro (17), yet we observe robust synergism at MMS concentrations below those required to detect trapping (20 μmol/L vs. 100 μmol/L; compare Figs. 1A to 2A).

Our data reveal that trapping activity and tolerability are inversely correlated in mouse models when PARP inhibitors are combined with TMZ. As a result, the nature of these agents is not fully understood, recent evidence indicates that PARP trapping may be less relevant in some contexts such as combination regimens including platinums or topoisomerase I inhibitors (19, 40). Interestingly, however, clinical evidence reveals that the maximum tolerated exposures for different PARP inhibitors in the monotherapy setting are greater than expected based on relatively small differences in catalytic potency. Specifically, weaker trapping agents such as olaparib, niraparib, and rucaparib achieve micromolar exposures whereas the potent trapping agent talazoparib has a C₉₀ of 50 nmol/L and a C₉₀ of 10 nmol/L at the recommended phase II dose (41–44). This suggests that trapping may play a role in dose-limiting toxicities observed in the clinic. Importantly, our results demonstrate that PARP inhibitors can differ in the resolution between the concentrations required to elicit PARP inhibition and PARP trapping. The rational selection of the most suitable PARP inhibitor to include with a particular regimen will require a thorough understanding of the relative roles of trapping and catalytic inhibition in both the efficacy and tolerability of that regimen.
Disclosure of Potential Conflicts of Interest

D. Maag reports receiving commercial research support from AbbVie. No potential conflicts of interest were disclosed by the other authors.

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PARP Trapping: Impact on In Vivo Activity of PARP Inhibitors


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Mechanistic Dissection of PARP1 Trapping and the Impact on In Vivo Tolerability and Efficacy of PARP Inhibitors


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