Priority Report

Targeting PARP-1 Allosteric Regulation Offers Therapeutic Potential against Cancer

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Abstract

PARP-1 is a nuclear protein that has important roles in maintenance of genomic integrity. During genotoxic stress, PARP-1 recruits to sites of DNA damage where PARP-1 domain architecture initiates catalytic activation and subsequent poly(ADP-ribose)–dependent DNA repair. PARP-1 inhibition is a promising new way to selectively target cancers harboring DNA repair deficiencies. However, current inhibitors target other PARPs, raising important questions about long-term off-target effects. Here, we propose a new strategy that targets PARP-1 allosteric regulation as a selective way of inhibiting PARP-1. We found that disruption of PARP-1 domain-domain contacts through mutagenesis held no cellular consequences on recruitment to DNA damage or a model system of transcriptional regulation, but prevented DNA-damage–dependent catalytic activation. Furthermore, PARP-1 mutant overexpression in a pancreatic cancer cell line (MIA PaCa-2) increased sensitivity to platinum-based anticancer agents. These results not only highlight the potential of a synergistic drug combination of allosteric PARP inhibitors with DNA-damaging agents in genomically unstable cancer cells (regardless of homologous recombination status), but also signify important applications of selective PARP-1 inhibition. Finally, the development of a high-throughput PARP-1 assay is described as a tool to promote discovery of novel PARP-1 selective inhibitors. Cancer Res; 74(1); 31–37. ©2013 AACR.

Introduction

PARP-1 is a multidomain protein related to 16 other members of a family possessing ADP-ribose transferase catalytic domains with similar enzymatic and structural features (1). PARP-1 has multiple cellular functions, including transcriptional regulation, cell death signaling, and DNA damage repair. PARP-1 has recently garnered attention as a therapeutic target in lethal malignancies such as pancreatic cancer (2).

PARP-1 has six domains (Fig. 1). The N-terminus contains three zinc-binding domains: Zn1, Zn2, and Zn3. Zn1 and Zn2 bind to altered DNA structures (3), whereas Zn3 contains a structurally unique “zinc ribbon” fold that contributes to PARP-1 DNA damage–dependent activation through domain-domain contacts (4, 5). The central automodification domain contains a BRCA1 C-terminus (BRCT) fold, and serves as a major site of poly(ADP-ribose) (PAR) modification. Toward the C-terminus, the Tryptophan-Glycine-Arginine (WGR) domain makes DNA and protein contacts that are important for catalytic activation (6, 7). The C-terminal catalytic region contains the active site where NAD+ is consumed to synthesize PAR covalently modified onto nuclear targets such as PARP-1 itself (8). Under genotoxic stress, PARP-1 domains assemble on DNA damage and form a network of domain–domain contacts that imposes a structural distortion on the catalytic domain (CAT; ref. 7), leading to a dramatic increase in catalytic activity (9). This hyperactivation accounts for the majority of PAR accumulation at sites of DNA damage, leading to recruitment of repair proteins to facilitate DNA repair (10). PAR formation facilitates DNA repair under low to moderate levels of genomic stress, but can trigger cell death mechanisms upon excessive damage. These important roles of PARP-1 ensure maintenance of genomic integrity and provide protection from carcinogenesis of badly damaged cells.

Therapeutic strategies targeting PARP-1 have promising applications for the treatment of some cancers, drawing considerable attention in recent years (11). PARP-1 inhibitors have been optimized to low nanomolar potency and show clinical utility when used as a single agent or in combination therapy to treat cancers with DNA repair deficiencies (12). Although the clinical potential of PARP-1 inhibitors looks promising, therapeutic benefit versus adverse effects stemming from targeting other PARPs is not well understood. For instance, a study profiled the binding of
185 current PARP inhibitors to the active sites of 13 PARP family members and observed varying degrees of cross-selectivity (13). With such a high degree of conservation in the catalytic active sites of PARPs, it is not surprising that cross-selectivity exists among small molecules that target the substrate-binding site.

Toward achieving selective PARP-1 inhibition, we focused on the PARP-1 domains unique to DNA damage–dependent activation. The Zn1, Zn3, and WGR domains are essential for catalytic activation. A recent crystal structure of PARP-1 revealed that these domains assemble into a complex in the presence of damaged DNA (7). This structure has opened possibilities of selectively targeting damage-dependent activation through disruption of PARP-1 domain–domain contacts.

Here, we mutated key residues located at the interfaces between essential PARP-1 domains and observed no major changes in binding to DNA damage, but severe deficiencies in catalytic activation. A pancreatic cancer cell line carrying these deficiencies in PARP-1 showed an increase in sensitivity to platinum-based anticancer agents, suggesting a dominant negative effect that interferes with DNA damage processing. With prospective therapeutic applications of allosteric PARP-1 inhibition, we present a PARP-1 interdomain communication assay designed to identify new classes of selective PARP-1 inhibitors.

Figure 1. PARP-1 domain–domain contacts are necessary for DNA-dependent activity, but not high-affinity DNA binding. A, structural representation of activated PARP-1 highlighting the interdomain interfaces. B, schematic of full-length PARP-1. C, DNA-dependent PARP-1 catalytic activity. D, DNA-binding affinities of full-length PARP-1 mutants.
Materials and Methods

**Gene cloning and mutagenesis**
Full-length wild-type (WT) PARP-1 (1-1014) and ΔZn2 PARP-1 (97-206 deleted) were expressed with an N-terminal hexahistidine tag (pET-28). Zn1–Zn3 (1-366 with 97-206 deleted) and WGR-CAT (518-1014) were expressed with a C-terminal hexahistidine tag (pET-24). QuickChange mutations (Stratagene) were verified by automated sequencing. PARP-1 pcDNA constructs were cloned as described (3). EGFP-PARP-1 was generated by subcloning the Nhe1/Xho1 fragment from pcDNA-PARP-1 into pEGFP-N1.

**Protein production**
PARP-1 proteins were expressed in *E. coli* and purified as described (14).

**Transient transfection and immunofluorescent staining**
PARP-1/−/− mouse embryonic fibroblasts (MEF) were treated as described (3). To induce DNA damage, *H*₂*O₃* was added to the cells for 10 minutes before fixation. Cells were transfected 24 hours later with 1 μg DNA and 3 μL Fugene (Promega) in serum-free media following the recommended protocol.

**Live-cell microscopy and laser irradiation**
HeLa cells were treated under the same conditions as the MEFs. Cells were sensitized posttransfection with 1 μmol/L bromodeoxyuridine (BrdUrd) in warm phenol-red free media (Ham’s F-12 with 25 mmol/L HEPES pH 8.0, 10% FBS) for 24 hours at 37°C, 5% CO₂ before addition of Hoechst stain (10 μmol/L). Experiments were performed using a Zeiss LSM-510 Meta Confocal laser scanning microscope equipped with a 405-nm diode laser (set to 100% power) to locally irradiate nuclear sites for 1 second. Images were recorded by excitation with a 488-nm argon laser (set to 10% power) focused through a ×63/1.4 numerical aperture oil-immersion lens.

**Drug sensitivity assays**
Stably transfected MIA PaCa-2 cells (Supplementary Fig. S3) were seeded at low confluence and incubated at 37°C overnight. Cells were treated with drug and then grown to confluence (5–6 days). Cell viability was assessed by quantification of double-stranded DNA using Quant-IT PicoGreen (Invitrogen). Gemcitabine was purchased from Lilly. All other drugs were purchased from Sigma-Aldrich.

**Fluorescent polarization DNA-binding assay**
Reactions were carried out as previously described using an 18-bp DNA duplex (5). For the PARP-1 release experiment, WT and mutant proteins (200 nmol/L) were first incubated with the DNA duplex (100 nmol/L total DNA, 5% fluorescein labeled) for 30 minutes before addition of NAD⁺ (5 mmol/L) or *H*₂*O₃*. Polarization was measured over time on a plate reader (PerkinElmer).

**Colorimetric PARP-1 automodification assay**
This assay measures incorporation of biotinylated-NAD⁺ into PAR (14).

**Androgen receptor reporter assay**
Androgen receptor (AR) ligand–induced transcriptional activity was measured by relative luciferase activity as described (15).

**Results and Discussion**

**Disruption of PARP-1 domain–domain contacts impairs catalytic activation without affecting high-affinity interaction with DNA damage**
The essential Zn1, Zn3, and WGR domains each have low binding affinity for DNA damage, but in combination their collective affinity increases nearly 100-fold (Supplementary Fig. S1). The activated PARP-1 structure indicated that each of these domains forms contacts with DNA that are mutually compatible and consistent with their high collective DNA-binding affinity. We tested whether the contacts at the interfaces between the domains contributed to the collective assembly on DNA, thus forming a high-affinity interaction with DNA. Key residues located at domain interfaces were mutated (Fig. 1A). Although the mutations had a severe impact on DNA damage–dependent catalytic activity (Fig. 1C), they did not affect the overall PARP-1 DNA-binding affinity (Fig. 1D). The Zn2 domain is not essential for DNA-dependent PARP-1 activity (7); however, Zn2 has high binding affinity that could potentially mask DNA-binding deficiencies of inter-domain mutants. Thus, several mutants were tested in a PARP-1 construct with Zn2 deleted (ΔZn2). All mutants tested retained a high DNA-binding affinity (Supplementary Fig. S2), indicating that high-affinity binding to DNA mediated by the assembly of Zn1, Zn3, and WGR is independent from the allosteric regulation that triggers activation.

**Allosteric mutant W318R localizes to sites of DNA damage, but is defective in damage-induced PAR synthesis and release from DNA**
Cellular tests of PARP-1 function assessed the effect of disrupting allosteric activation. PARP-1/W318R was compared with PARP-1/WT, as it showed major deficiencies in DNA-dependent activation biochemically (Fig. 1C, ref. 5). In the absence of DNA damage, both localized to the nucleus of PARP-1/−/− MEFs and did not produce a notable PAR signal using indirect immunohistochemistry (Fig. 2A). Upon treatment with *H*₂*O₃*, PAR production was significantly increased in cells positive for the presence of PARP-1/WT but not PARP-1/W318R, indicating that the cellular deficiency of PARP-1/W318R resembles the biochemical observation. We next tested the ability of PARP-1/W318R to localize to damage sites. GFP-PARP-1 was transfected into HeLa cells. GFP-PARP-1/WT and GFP-PARP-1/W318R both recruited to DNA damage sites in a similar manner (Fig. 2B), further supporting that recognition of damaged DNA is not affected by disruptions in PARP-1 allosteric communication.

DNA-activated PARP-1 consumes NAD⁺ in an automodification reaction that results in covalently attached PAR, which ultimatelyases PARP-1 from DNA. PARP-1 inhibitors cause deficiencies in PARP-1 release from DNA (16). Prolonged residency at DNA damage sites is proposed to contribute to the genotoxic effect of PARP-1 inhibition, and to the synergistic
effect of combining PARP-1 inhibitors with DNA-damaging agents. We tested whether interdomain mutants showed deficiencies in the mechanism of release from DNA damage. Upon addition of NAD⁺, the W318R mutant exhibited a drastic delay in release kinetics compared with WT (Fig. 2C), similar to that of catalytic mutant E988A. Other interdomain mutants exhibited delayed release kinetics (Fig. 2C), consistent with their slower rates of PAR formation (Fig. 1C). Collectively, cell-based and biochemical analyses indicate that disruption of domain-domain contacts critical for activation does not influence PARP-1 binding to DNA damage, and that the deficiency in DNA damage-dependent activation causes PARP-1 to remain engaged with DNA in a manner that is not effectively reversed by automodification.

Disruption of PARP-1 allosteric regulation sensitizes pancreatic cancer cells to platinum-based agents

To determine whether allosteric disruption of PARP-1 catalytic activity could deliver comparable therapeutic potential as current PARP inhibitors, isogenic MIA PaCa-2 cells overexpressing PARP-1 mutants (Fig. 3B) were treated with a variety of chemotherapeutic agents following two criteria: (i) they currently or previously had been part of a chemotherapeutic regimen for pancreatic cancer and (ii) they are classified as DNA-damaging agents. PARP-1/WT- and PARP-1/W318R-transfected cell lines were analyzed, as well as the cell line bearing the E988A mutation that targets a catalytic active site residue, and should therefore mimic drugs that target the catalytic domain. Overexpression of PARP-1/WT had no effect in combination with chemotherapy (Fig. 3A). However, overexpression of PARP-1/W318R or PARP-1/E988A rendered cells 4 to 8 times more sensitive to platinum-based anticancer agents (Fig. 3A). Increased sensitivity was not seen in combination with other chemotherapeutic agents (Supplementary Fig. S3), indicating a preferential synergistic potential between PARP-1 inhibition and platinum-based therapies, in line with current clinical trial outcomes (17). In addition, allosteric disruption of catalytic activity seems to be equally effective at sensitizing cells as direct disruption at the catalytic domain. The molecular mechanisms responsible for sensitization with platinum agents are not completely understood. However, sensitization from this combination has been observed in MIA PaCa-2 cells (18), as well as other cell-based models (19–21), and is likely cell-type specific (22). One possible mechanism of action could relate to the ability of mutant PARP-1 to recognize platinum-DNA damage (23) and form trapped complexes that shield the toxic platinum-modified lesions from repair and promote accumulation of unrepaired damage.

Allosteric disruption of PARP-1 catalytic activity does not prevent AR transcriptional regulatory functions

In addition to the DNA damage response, PARP-1 is involved in a number of transcriptional regulation events (24). For example, PARP-1 catalytic activity is necessary for AR ligand-dependent transcription (15). To determine if allosteric regulation is specific to the DNA damage response, the AR transcriptional function was assessed in PARP-1−/− MEFs transiently transfected with PARP-1/WT, PARP-1/W318R, and PARP-1/E988A. The catalytic mutant PARP-1/E988A is deficient in AR transcription compared with WT (Fig. 3C, ref. 15), whereas the interdomain mutant PARP-1/W318R exhibited substantial ligand-induced AR reporter activity. Furthermore, the ligand-induced AR reporter activity of PARP-1/W318R was decreased in the presence of PARP inhibitor ABT-888, similar to PARP-1/WT (Fig. 3C). Thus, disruption of PARP-1 allosteric regulation shuts down DNA damage–dependent activity.
without altering the catalytic domain per se, thus allowing PARP-1 to maintain other important cellular functions outside of the DNA damage response.

Development of a high-throughput method to detect allosteric regulation

As PARP-1 inhibitors continue to hold tremendous promise as targeted anticancer agents, there is a growing interest in specific PARP-1 inhibitors. The unique structural aspects of the PARP-1 complex of essential domains is therefore of particular interest as a therapeutic target. Using the core components of this complex as our target model (Fig. 4A), we developed a high-throughput assay that is capable of detecting complex formations in the presence of DNA. The assay is based on fluorescent polarization techniques and detects the binding of WGR-CAT to a Zn1–Zn3 saturated DNA complex as an increase in fluorescence polarization (Fig. 4B). To validate that the readout accurately represented allosteric activation, we tested several mutants at the domain interfaces. Four mutations were made along the Zn1–Zn3 interface: E76A, R78A, W79A, and N242W; two mutations were made along the Zn3-WGR-HD interface: W318R and W318A. Mutations in the Zn1–Zn3 construct did not affect their DNA-binding affinity (Supplementary Fig. S4). When WGR-CAT was added to the Zn1–Zn3 variants, the maximum change in polarization (ΔPolarization) was notably lower for R78A, W79A, W318A, and W318R, although the estimated binding affinity was comparable with WT (Fig. 4C). The deficiency in ΔPolarization correlated well with the observed deficiencies in DNA-dependent catalytic activity (Fig. 4D). The N242W mutant was designed to mimic the steric clash of a potential small allosteric inhibitor bound at the Zn1–Zn3 interface. In this instance, the binding affinity of WGR-CAT dropped 2-fold, and the rate of PAR synthesis was reduced consistently. Mutations made in WGR-CAT further validated the assay. When WGR residues K633 and R735 were mutated to alanine, decreases in maximum polarization were seen in the high-throughput assay that corresponded to the level of DNA-dependent PAR synthesis (Fig. 4C and D). The mutant W589A is known to affect DNA binding of WGR, and showed no increase in polarization, which correlated well with its deficient DNA-dependent activation. Mutant E988A was used to show that direct disruption of the catalytic domain does not influence the assay readout and, therefore, catalytic site inhibitors would not be detected by this assay.

Perspective

Although the use of PARP-1 inhibitors has been widely deployed, the therapeutic benefit from pan-PARP inhibition (i.e., targeting multiple PARP family proteins), as opposed to specific PARP-1 inhibition, is not clear. The functional roles among PARPs are diverse, and cross-inhibition creates the
potential for deleterious off-target effects (13, 25). On the other hand, increased therapeutic effectiveness can often accompany the complexities of polypharmacology, as exemplified by promiscuous kinase inhibitors (26). Selective pharmacologic targeting of PARP-1 (and other PARPs) will be useful in distinguishing therapeutic effectiveness from toxicity in complex diseases.

In this study, disruption of allosteric regulation was used to model selective inhibition of PARP-1. Single-point mutations at domain interfaces were used to disrupt allosteric regulation. These mutants do not affect high-affinity binding to DNA damage, although they do have a pronounced effect on DNA-dependent catalytic activation. In a cellular environment, these observations are paralleled as mutant PARP-1 recruits to sites of DNA damage, but fails to generate detectable PAR. Interestingly, the role of PARP-1 in AR-dependent transcription was not severely affected, revealing applications of allosteric inhibitors in reducing potential off-target effects. We also find that high-affinity DNA binding combined with catalytic deficiency stalls release from DNA in the presence of NAD\(^+\). The prolonged occupancy of mutant PARP-1 likely contributes to sensitization of DNA-damaging agents by retaining unrepaired DNA damage.

We have identified a synergistic combination between allosterically disrupted PARP-1 and platinum-based agents in pancreatic cancer cells with a WT BRCA2-related DNA damage repair pathway. We believe that allosteric inhibition will likely hold therapeutic potential in other types of cancers when used as combination therapy (in a BRCA2-WT setting) or as monotherapy, especially in the context of BRCA1/2/Fanconi anemia–deficient tumors (27). We have developed a high-throughput assay to detect the allosteric activation status of PARP-1 to facilitate the identification of pharmacologic inhibitors. This study initiates an innovative pipeline to identify an optimal compound that will disrupt PARP-1 function in cancer cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed by the authors.

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**Figure 4.** A high-throughput (HT) fluorescent polarization (FP) assay to detect allosteric PARP-1 inhibitors. **A,** schematic of PARP-1 proteins used in this assay (Zn1–Zn3 and WGR-CAT), mapped with mutation sites. **B,** dose–response of WGR-CAT binding to fluorescein-labeled DNA alone or to fluorescein-labeled DNA saturated with Zn1–Zn3. **C,** WGR-CAT binding in the fluorescent polarization assay in the presence of Zn1–Zn3 (top) or WGR-CAT (bottom) mutations. **D,** catalytic activity of Zn1–Zn3 and WGR-CAT combinations using the colorimetric assay with mutations made in Zn1–Zn3 (top) or WGR-CAT (bottom).
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Lal, J.R. Brody

Study supervision: J.R. Brody, J.M. Pascal

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