Assay Strategies to Evaluate Potency and Selectivity of Next-Generation PARP Inhibitors

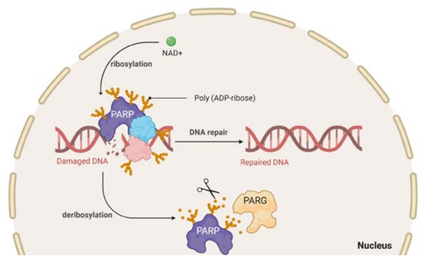
K. Zientara-Rytter, E. Jones, M. Awad, O. Okakpu, B. Kennedy, R. Ken, Y. Luo, S. Plouffe, V. Baron, M. Kinbara, J. Mikolosko, P. Shashkin

BPS Bioscience, San Diego, CA 92121

INTRODUCTION

The PARP (poly(ADP-ribose) polymerase) family catalyzes the ADP-ribosylation of proteins, playing a key role in the response to DNA damage, and is therefore highly relevant to cancer therapy. Several inhibitors are clinically approved, with newer generations focusing on increased specificity.

Here, we describe complementary assays for evaluating the potency and selectivity of candidates against the PARP family, including enzymatic assays for catalytic inhibitors, DNA-trapping, and olaparib competition assays. We also discuss a cellular PARylation assay that measures drug activity in cells to obtain information on parameters not captured in biochemical assays.

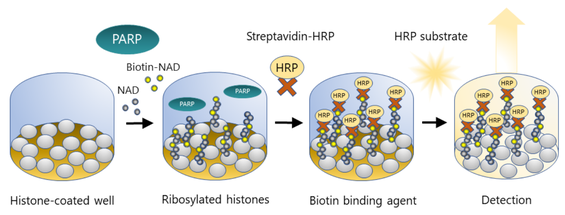


ENZYMATIC PARYLATION ASSAYS

These ELISA-type assays were designed to measure the mono or poly-ADP-ribosylation activity (depending on the enzyme) of the entire PARP family, resulting in one of the most extensive PARP portfolios commercially available. Validated 96 and 384-well formats can assess the efficacy of compounds across PARP family members, allowing selectivity assessment. These enzymatic assays were optimized for colorimetric or chemiluminescence detection options for flexibility.

Principle

Histone proteins are coated on a 96-well plate. A biotinylated NAD+ mix (termed PARP Substrate Mixture) is incubated with the enzyme in an optimized assay buffer. The plate is then treated with streptavidin-HRP followed by addition of the ELISA ECL substrate to produce chemiluminescence that can be measured using a chemiluminescence reader. The chemiluminescence signal is proportional to PARP activity.



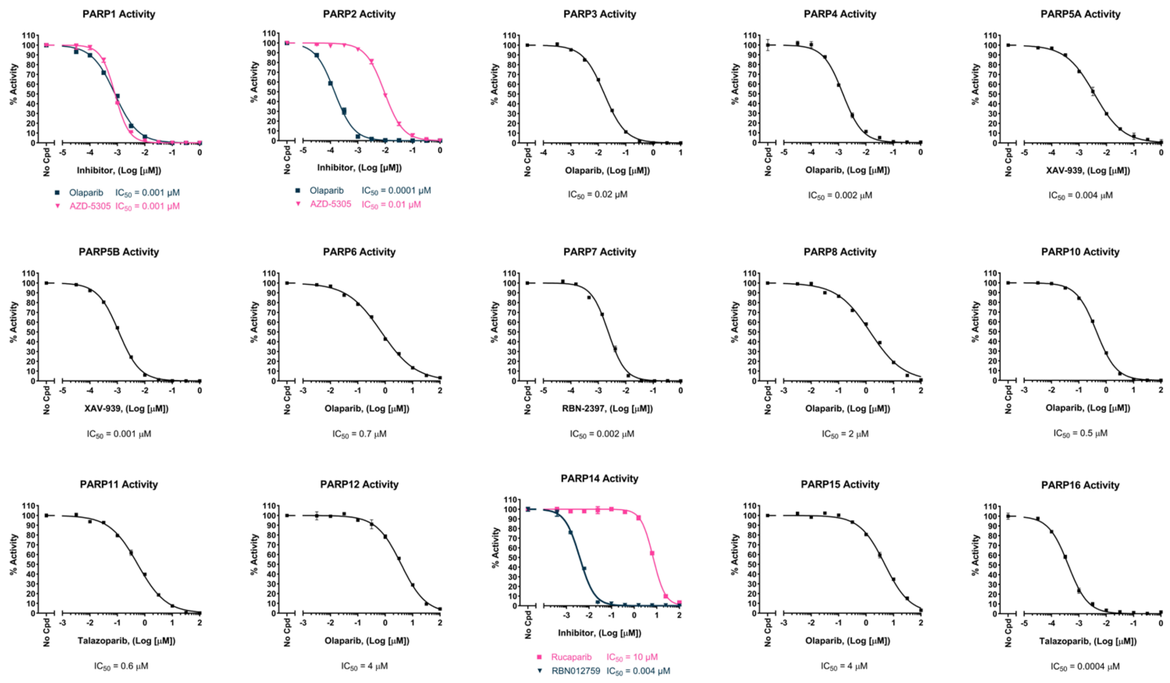


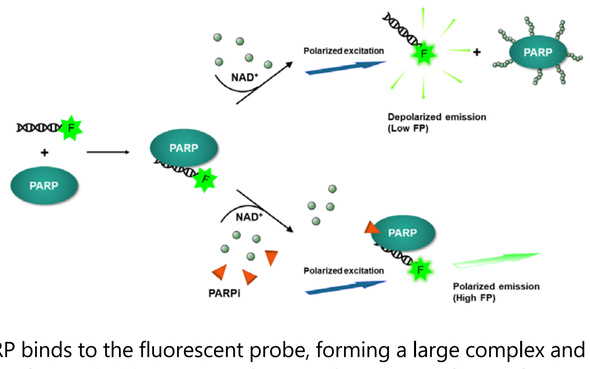
Figure 1: PARP inhibitors decrease ADP-ribosylation activity of PARP enzymes. *White 96-well plates were coated with a histone substrate overnight followed by a blocking step. Purified recombinant PARP enzymes were added to the plate in the presence NAD⁺ and increasing concentrations of the indicated inhibitors. ADP-ribosylation was measured by adding streptavidin-HRP (horseradish peroxidase) followed by a chemiluminescent HRP substrate. The IC_{50} was determined by curve fitting using Prism software v11.0.*

PARPTRAP ASSAYS

PARP1 and PARP2 bind to sites of DNA damage, which activates their auto-ribosylation and promotes dissociation of the protein-DNA complex. Inhibitors that block auto-ribosylation prevent this dissociation, effectively trapping PARP proteins on DNA. Because trapped PARP-DNA complexes are highly cytotoxic to cancer cells, PARP trapping is desirable for PARP-directed anticancer therapies.

Principle

We designed a unique homogeneous (no-wash) PARPTrap™ assay using principles of fluorescence polarization (FP). The fluorescent probe is a small molecule that can rotate freely in solution, resulting in low FP. In the absence of ribosylation, PARP binds to the fluorescent probe, forming a large complex and resulting in the emission of highly polarized light. After auto-ribosylation, PARP dissociates from the fluorescent oligonucleotide duplex, which can then rotate freely again (low FP). In the presence of a PARP inhibitor, the protein is trapped by the fluorescent oligonucleotide duplex, contributing to its high FP. **Thus, an increase in FP signal is directly proportional to PARP trapping.**



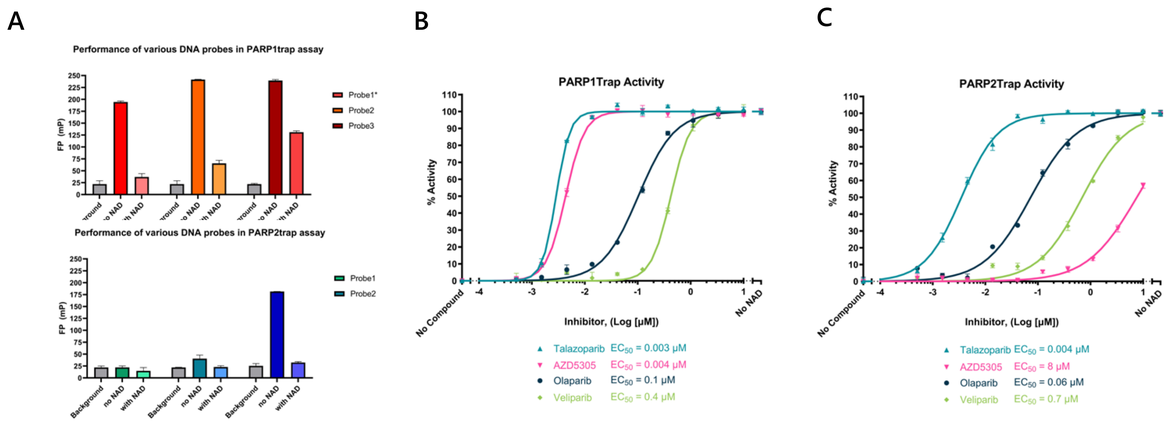


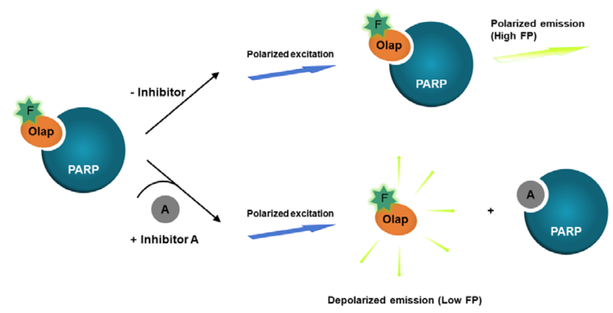
Figure 2. (A) Optimized specific PARP1 or PARP2 DNA substrates provided the best results. Various probes specific for PARP1 or PARP2 were assayed in parallel with pre-determined amounts of PARP proteins. Each probe was assayed with or without NAD+ to determine the signal range. Results are shown as raw signal (mP) with either PARP1 (#80584) or PARP2 (#78296). ***(B, C) PARP inhibitors increase trapping with various efficacy.*** PARP/DNA trapping was measured in the presence of increasing concentrations of inhibitors using the PARPtrap™ Assays (#80584 and #78296). "No compound" corresponds to the "Low FP control" and "no NAD+" corresponds to the "High FP control".

OLAPARIB COMPETITION ASSAYS

Olaparib was the first PARP inhibitor approved for clinical use, marking a milestone in targeted cancer therapy for BRCA-mutated tumors. It has become a benchmark PARP1/2 inhibitor and is widely used as a reference compound in drug discovery programs. The homogeneous (no-wash) assay described here was designed to screen and evaluate compounds that bind to PARP1 or PARP2 at the same site as Olaparib, using an FP–based competitive displacement format.

Principle

PARP1/2 binds to an Olaparib-derived fluorescent probe, forming a high-molecular-weight complex. When excited with polarized light, this complex emits highly polarized fluorescence due to its restricted rotational mobility. A test compound (A) that competes for the Olaparib binding site can displace the fluorescent probe, releasing it into solution. The free probe rotates rapidly, resulting in a decrease in fluorescence polarization. Thus, the reduction in FP signal is directly proportional to the competitive binding of the test compound to PARP1/2.



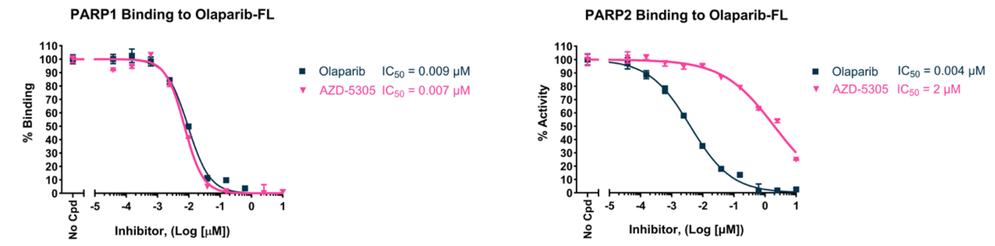


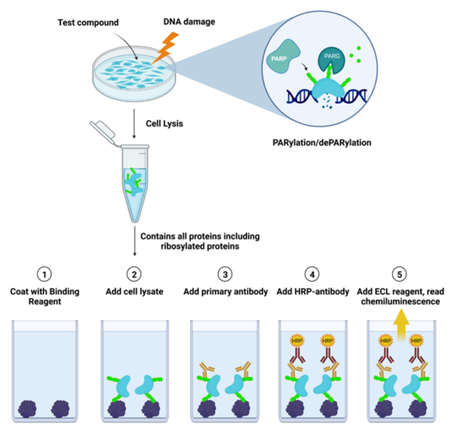
Figure 3. Olaparib and AZD-5305 compete with Olaparib-FL for PARP1/2 binding. *Purified recombinant PARP1 or PARP2 were incubated with increasing concentrations of the indicated inhibitor before addition of the fluorescent olaparib probe for 30-90 minutes at room temperature (PARP1 assay #82293 and PARP2 assay #82294). Results are expressed as percent of binding, in which binding in the absence of inhibitor is set to 100%. The IC_{50} was determined by curve fitting using Prism software v11.0.*

CELLULAR PARYLATION

Assessing cellular PARylation levels is critical for accelerating drug development. We designed a sandwich ELISA to analyze the total protein PARylation present in cellular extracts. Experimental protocols were optimized to discern differences in cellular PARylation levels resulting from activation of the DNA damage repair pathways or from exposure to PARP inhibitors. Our assay specifically detects PARylation, measuring the effects associated with PARP family members 1 to 5, as other family members primarily catalyze mono-ADP-ribosylation.

Principle

A 96-well plate is coated with a Binding Reagent specific for PARylated chains. Lysates from treated and control cells are added to the coated wells, allowing capture of the PARylated proteins. This is followed by an incubation with a detection antibody specific for PAR chains, then a secondary HRP-conjugated antibody, and addition of a chemiluminescent HRP substrate. The luminescent signal directly correlates with the level of cellular PARylation. The assay does not detect mono-ADP-ribosylation.



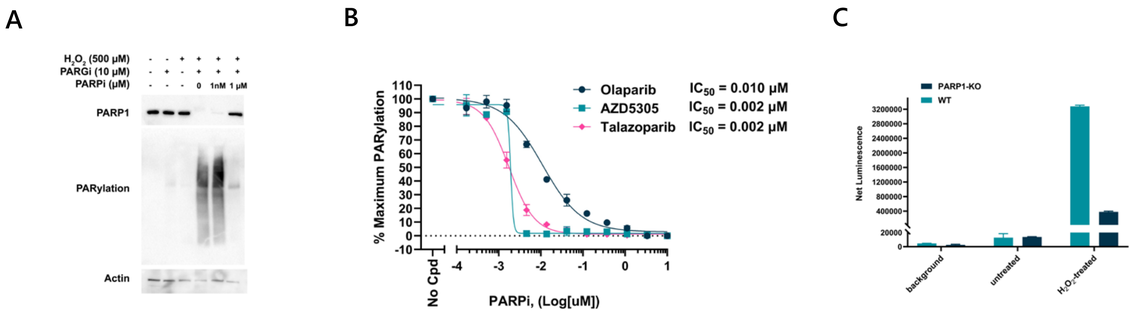


Figure 4. (A) Treatment with 15 min H_2O_2 is sufficient to induce protein PARylation. Cells were preincubated with or without PARGi PDD00017273 (10 µM) and with or without Olaparib for 1 hour and 45 minutes before adding H_2O_2 (500 µM) for 15 minutes to induce DNA damage. Cells were washed, lysed on ice for 10 minutes, and analyzed by western blot using anti-PARP1 antibody (ThermoFisher #436400) followed by our Binding Reagent. ***(B) PARP inhibitors decrease DNA damage-induced PARylation in intact cells.*** HEK293 cells were treated with PARGi in combination with the indicated PARP inhibitors (#78318) for 1 hour and 45 minutes at 37°C. H_2O_2 (500 µM) was added for 15 minutes. Cells were washed and lysed in Modified RIPA Lysis Buffer containing protease and ADP-Ribosylation Inhibitor Mix (#82130). PARylation levels were analyzed immediately using the LysA™ Universal PARylation Assay Kit (#82123). Results are expressed as percent of total PARylation. IC_{50} was determined by curve fitting using Prism software v 11.0. ***(C) PARP1 knockout decreases PARP-mediated PARylation.*** Cells were incubated with 10 µM PARGi for 1 hour and 45 minutes before DNA damage was induced. Cells were washed, lysed, and analyzed immediately for total protein PARylation.

CONCLUSION

PARP inhibitors represent an important class of anti-cancer therapies. For drug developers, it is crucial to have reliable and reproducible assay platforms. Our panel of different PARP assays comprehensively evaluates the various features of PARP enzymes and can be leveraged for PARP inhibitor characterization studies.

- Enzymatic PARylation ELISA and AlphaLISA assays successfully measure mono and poly-ADP-ribosylation activity across the PARP family.
- The PARPtrap FP assays detect PARP-DNA trapping events, a critical mechanism of action for cancer cell cytotoxicity. Of note, the combo PARPTrap Assay for PARP1 and PARP2 (#78317) is especially useful to compare, in a single plate, the potency of a candidate on both PARP1 and 2, facilitating the selection of PARP1 or PARP2-specific compounds.
- The Olaparib displacement assay uses a reduction in fluorescence polarization signal to indicate the competitive binding of an inhibitor candidate to PARP1 or PARP2.
- The LysA™ Universal PARylation sandwich ELISA measures PARylation levels in cell extracts, reflecting drug effects in PARP family members 1 to 5 in biologically relevant contexts.
- PROTAC® based PARP1-Cereblon Binding assay (AlphaLISA format) for optimizing PAR1-directed molecular glues/PROTACs (not shown).

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