Design of Small Molecule Screening Assays for Measuring Poly-(ADP-ribose) Glycohydrolase (PARG) Enzymatic Activity

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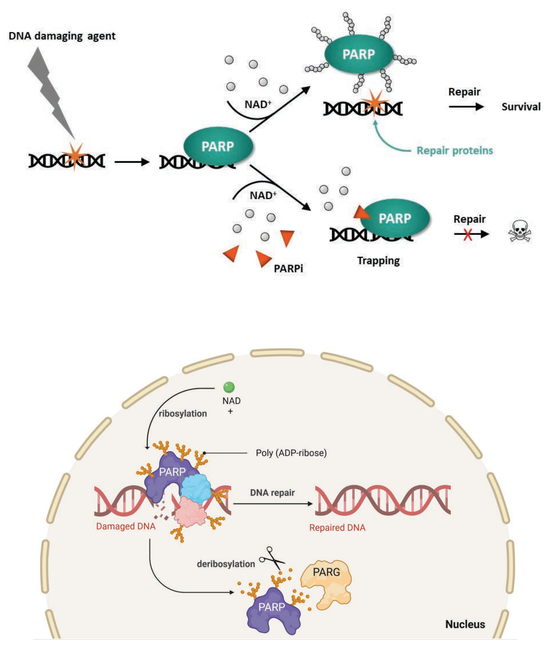
BACKGROUND

As first responders of the DNA Damage Response (DDR), PARP1 and PARP2 (Poly-ADP ribose polymerase) can sense single strand breaks in the DNA and bind to the damaged region before adding poly-ADP ribose (PAR) chains to their own protein backbone, to histones, and to other repair proteins in order to recruit and activate them (1). The PARylated PARP next detaches from the DNA, allowing the other proteins to initiate the repair process. Removal of PAR chains by protein PARG (Poly ADP-ribose glycohydrolase) recycles PARP to its inactive form, ready to sense DNA damage again.

PARG, therefore, plays a crucial role in DNA repair processes, including base excision repair and single-strand break repair, by removing PAR chains from PARP-modified proteins, which enables DNA repair and the recycling of these crucial proteins. Dysregulation of PARG activity has been implicated in various diseases, including cancer (2). Targeting PARG for therapy is gaining traction as a potential strategy for sensitizing cancer cells to DNA damage-inducing agents (3). Inhibition of PARG activity increases the accumulation of PAR on proteins and interferes with DNA repair mechanisms, ultimately leading to cell death. Thus, developing PARG inhibitors as therapeutic agents has emerged as a promising approach to cancer treatment, particularly in combination with DNA damage-inducing agents such as radiation and chemotherapy.

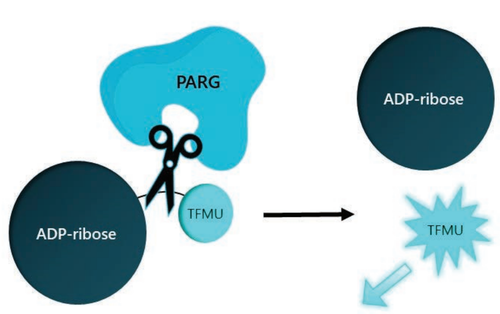


FLUOROGENIC ASSAY

Assay Principle

The PARG fluorogenic assay is a straightforward, high-throughput, homogeneous (no-wash) assay designed to measure the hydrolase activity of PARG for screening and profiling applications.

PARG is incubated with a fluorogenic ADP-ribose substrate (4) in which the TFMU fluorophore is quenched by the presence of the ribose. PARG-mediated hydrolysis of the substrate from the ribose releases the fluorophore from quencing. Fluorescence intensity, therefore, is directly proportional to PARG hydrolase activity.



Methods

Experiments were performed in duplicate in black, low-binding 384-well assay plates. The purified protein was thawed on ice and diluted to 2.5 ng/reaction in a final volume of 25 µl. When using serial dilutions of PARG inhibitors that are dissolved in DMSO, the final concentration of DMSO in the assay was kept constant at 1% and was also included in all the controls. PARG was pre-incubated with the inhibitors for 15 minutes at room temperature followed by the addition of the fluorescent-conjugated substrate, at a final concentration of 4 µM, for 60 minutes at room temperature and in the dark. The fluorescence signal was detected at λ=502 nm (excitation λ=385 nm) using a Bio-Tek fluorescence plate reader. The blank value (determined in the absence of PARG enzyme) was substrated from all other values.

Results

The PARG fluorogenic assay yielded a robust fluorescence signal and could be completed in two hours or less. Validation experiments demonstrated that this assay can reliably separate positive and negative controls (signal-to-background ratio = 52; Z-factor = 0.81). Three PARG inhibitors were evaluated, as well as PARP inhibitor olaparib. As expected, olaparib did not affect PARG enzymatic activity. PARG inhibitors PDD00017273, PDD00017272 and PDD00017238 (MedChem Express) had IC_{50} values in the range of 0.5, 0.3 and 1 nM, respectively, when using 2.5 ng of enzyme per reaction.

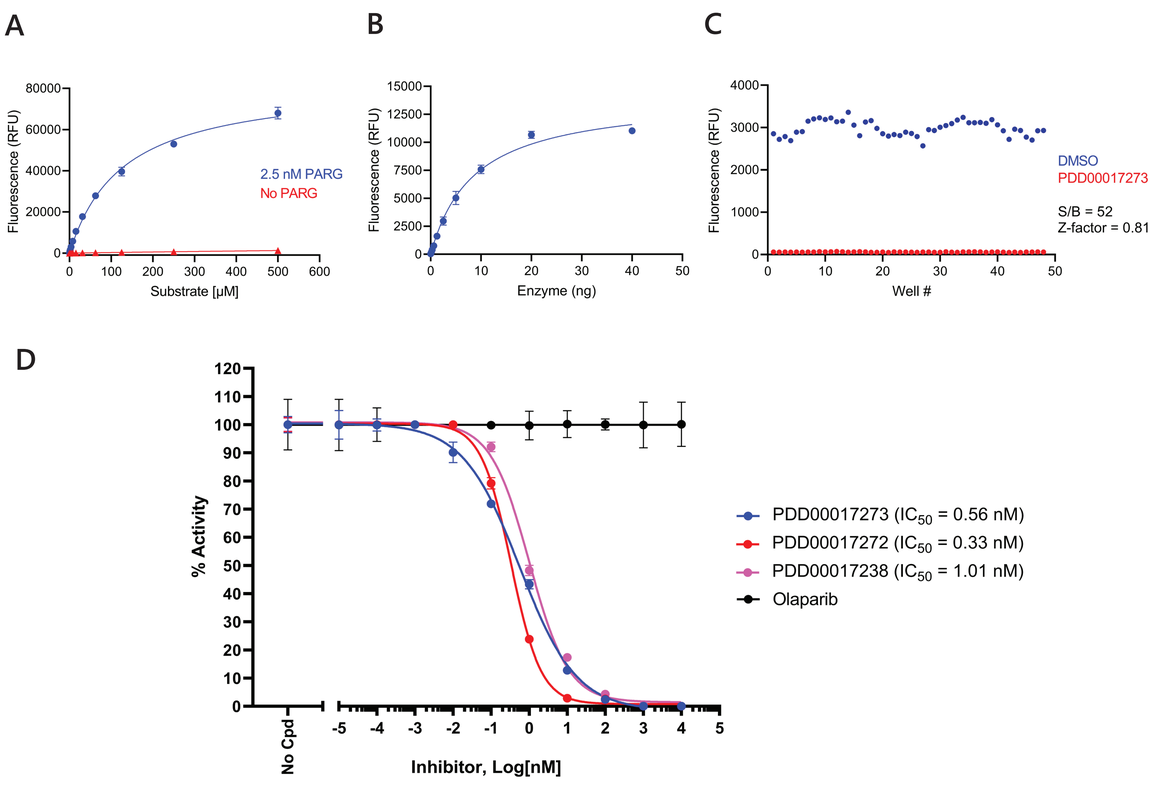
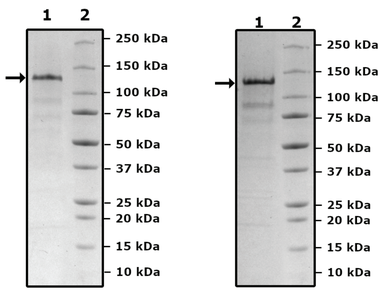


Figure 1. Optimization and performance of the fluorogenic assay measuring PARG activity. ***(A)*** *Experiments were performed in the presence or increasing amounts of fluorogenic substrate, with or without PARG, to control for intrinsic fluorescence signal in the absence of enzyme.* ***(B)*** *Optimal PARG concentration to use in the assay was determined with an enzyme titration.* ***(C)*** *PARG activity was measured in a series of positive (wells containing 1% DMSO) and negative (wells containing 10 µM PDD00017273) controls. Z-factor was determined as described in (5). S/B: signal-to-background ratio.* ***(D)*** *PARG activity was measured in the presence of increasing concentrations of four inhibitors and the IC_{50} was determined for each inhibitor.*

PROTEIN PRODUCTION

The chemiluminescent assay and the fluorogenic assay both rely on the quality of the enzymatically active protein. The assays use 112 kDa full length, His-tag PARG recombinant, produced in Sf9 cells and affinity purified. Protein concentration is determined using Bradford/BCA analysis. Each new lot of protein is confirmed to be active using the Fluorogenic Assay (not shown).

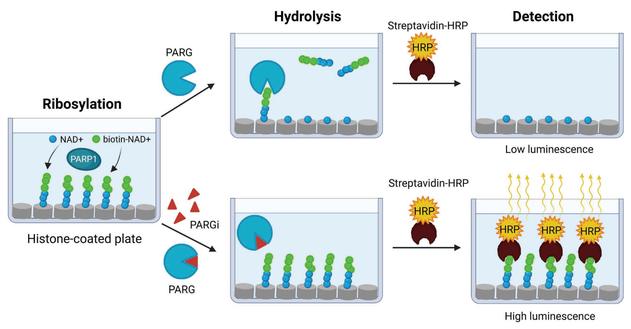
Figure 2. SDS-PAGE electrophoresis followed by Coomassie blue staining was performed on two lots of the recombinant PARG enzyme (BPS Bioscience #101726). Lane 1 shows 2 µg of PARG protein and lane 2 shows the molecular weight standards. The arrows point to PARG.



CHEMILUMINESCENT ASSAY

Assay Principle

The PARG Chemiluminescent Assay is an ELISA (enzyme-linked immunosorbant assay) that measures the enzymatic activity of PARG and is designed for screening and profiling applications. First, histones are coated on a 96-well plate. The second step is the *de novo* poly (ADP)-ribosylation (PARylation) of histones by PARP1 using a biotin-labeled NAD^+ substrate, so that the newly formed PAR chains are biotinylated. PARP1 is washed away and PARG is added, resulting in hydrolysis of the biotinylated PAR polymers. Addition of streptavidin-conjugated HRP (horseradish peroxidase) is followed by measurement of HRP activity using a chemiluminescent substrate. Thus, the assay quantifies the remaining biotinylated PAR polymers relative to the control condition (measured in the absence of PARG), resulting in a loss of the luminescence signal proportional to the activity of PARG. Addition of a PARG inhibitor prevents removal of PAR chains and results in a dose-dependent increase in signal.



Methods

Experiments were performed in triplicate using 96-well modular plates. A 96-well plate was coated with histones at 4°C overnight. The plate was washed 3 times and blocked using Blocking buffer 3 (BPS Bioscience #79743) for 90 minutes at room temperature, followed by 4 more washes.

PARP1 was diluted to 7 ng/reaction in a final volume of 50 µl. Histone ribosylation was performed by adding PARP1 in the presence of NAD^+ and an activated DNA probe for 30 minutes at room temperature, followed by addition of biotinylated NAD^+ for 30 minutes. The plate was washed 4 times before initiation of the hydrolysis reaction.

Purified PARG was diluted to 1 ng/reaction in a final volume of 50 µl. When using serial dilutions of inhibitor PDD00017273, which is dissolved in DMSO, the final concentration of DMSO in the assay was kept constant at 1% and was also included in the controls. PARG was pre-incubated with the inhibitor for 15 minutes at room temperature before being added to the plate containing the ribosylated histones for 30 minutes.

The plate was washed 4 times and streptavidin-HRP was added in blocking buffer and incubated for 30 minutes at room temperature, followed by 4 more washes, and by the addition of a chemiluminescent HRP substrate. Results were measured immediately using a plate reader capable of reading chemiluminescence. The blank value (determined in the absence of PARG enzyme) was subtracted from all other values.

Results

PARG inhibitor PDD00017273 blocked the hydrolysis of PAR chains in a dose-dependent fashion, as indicated by a gradual increase in signal compared to the no-inhibitor negative control. When results were analyzed and expressed as percent of control PARG activity (measured in the absence of inhibitor and set to 100%), the IC_{50} of the inhibitor was determined to be 0.029 µM (B).

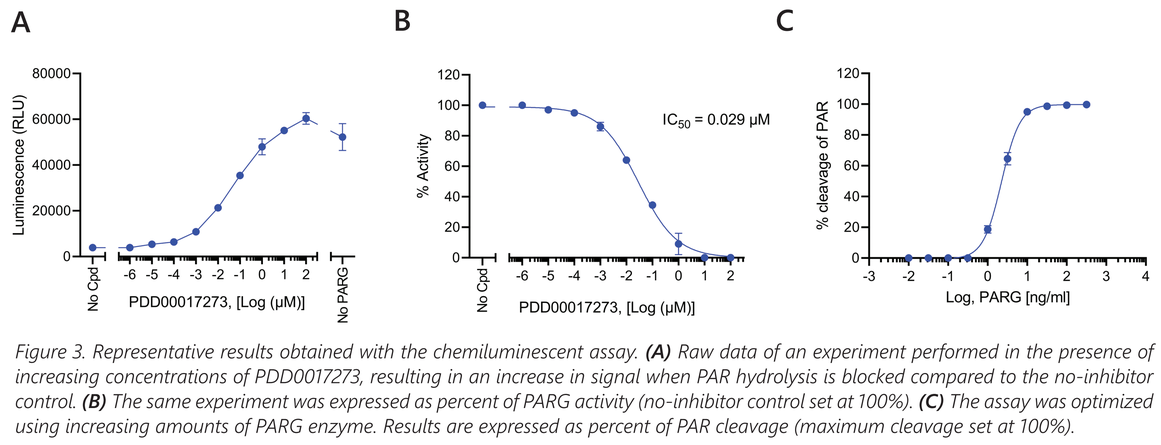


Figure 3. Representative results obtained with the chemiluminescent assay. ***(A)*** *Raw data of an experiment performed in the presence of increasing concentrations of PDD0017273, resulting in an increase in signal when PAR hydrolysis is blocked compared to the no-inhibitor control.* ***(B)*** *The same experiment was expressed as percent of PARG activity (no-inhibitor control set at 100%).* ***(C)*** *The assay was optimized using increasing amounts of PARG enzyme. Results are expressed as percent of PAR cleavage (maximum cleavage set at 100%).*

CONCLUSION

Here we describe two multi-well format assays developed for monitoring PARG enzymatic activity and suitable for screening and titrating small molecule inhibitors.

The PARG Fluorogenic Assay uses a straightforward, fast, no-wash method that allows for the measurement of enzyme kinetics and is ideal for high-throughput screening applications. However, some test compounds may have intrinsic fluorescence that can interfere with results, requiring a non-fluorescent method. The PARG Chemiluminescent Assay, although inappropriate for kinetic studies, offers an alternative that allows to complement the fluorogenic assay and overcome its limitations.

References

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