Measuring Cellular PARylation to Gain Insight into PARP/PARG-Targeted Drug Discovery

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ABSTRACT

Background

Inhibitors of poly (ADP-ribose) polymerases (PARPs) have revolutionized cancer therapy by highlighting the potential of synthetic lethal drugs to target the DNA damage response (DDR) network. More recently, the development of PARG inhibitors was spurred by the discovery that inhibiting PARG (poly (ADP-ribose) glycohydrolase) results in the accumulation of poly (ADP-ribose) on DDR proteins and induces cell death.

Adding PARG or PARP inhibitors to a cell of interest and quantifying the resulting levels of PARylation can provide valuable insight on compound membrane permeability and target engagement in a cellular context. Nevertheless, assessing PARylation (poly-ADP-ribosylation in which multiple ribose units are added in linear or branched forms) in cells is difficult compared to biochemical assays, impeding the progress of candidate drug development.

A sandwich ELISA was developed to analyze the total protein PARylation present in cellular extracts. Experimental protocols were optimized to discern differences in cellular PARylation levels resulting from activating the DDR or from exposure to PARP and/or PARG inhibitors. It is worth noting that our assay specifically detects PARylation, measuring the effects associated with PARP family members 1 to 5, as other family members primarily catalyze MARylation (mono-ADP-ribosylation in which a single ADP-ribose unit is added to a protein).

Results

Titration of known PARP or PARG inhibitors was performed to validate the assay.

1) PARG inhibitor PDD00017273, used in combination with DNA damaging agent H_2O_2, increased the level of cellular PARylation, exhibiting a similar IC_{50} value across the four cell lines tested. This is consistent with the notion that PARG is the major de-PARylating enzyme in most cells. The potency of other known PARG inhibitors PDD00017272 and PDD00017238 ranked the compounds as expected from published results: PDD00017272< PDD00017273< PDD00017238.

2) Titration of the three PARP1/2 inhibitors: Talazoparib, Olaparib and AZD5305, in combination with a DNA damage agent and PDD00017273 inhibitor, shows a dose-dependent reduction in cellular PARylation in HEK293 cells, as expected since PARP1 is activated in response to DNA damage and this activation is blocked by the inhibitors (IC_{50} values, Olaparib = 0.013 µM, AZD5305 = 0.0024 µM, Talazoparib = 0.0016 µM). These IC_{50} are consistent with published values and underscore the usefulness of the assay in measuring dose response effects, allowing accurate determination of compound IC_{50} in living cells.

Conclusion

In summary, our optimized experimental cell treatment protocols and ELISA allow quantification of total cellular PARylation and accurately rank candidate drugs targeting PAR erasers or PAR writers in a high-throughput format, using living cells.

ASSAY PRINCIPLE

The LysA™ Universal PARylation Assay Kit is a sandwich ELISA-based assay designed to analyze the level of total PARylation present in cellular extracts. The kit includes a PAR standard for absolute quantification. The assay detects differences in protein PARylation levels resulting from inducing the DNA damage response or from exposure to PARP/PARG inhibitors.

Principle

A 96-well plate is coated with an (ADP)-ribose Binding Reagent specific for PARylated chains. Lysates from cells are added to the coated wells, and PARylated proteins present in the cell lysates are captured by the reagent. This is followed by an incubation with a detection antibody, then a secondary HRP-conjugated antibody. Addition of a chemiluminescent HRP substrate generates a luminescence signal that directly correlates with the level of cellular PARylation. The assay does not detect mono-ADP-ribosylation.

Keeping in mind that PAR levels reflect the homeostasis between PAR writers and PAR erasers, it is expected that adding specific inhibitors and/or using DNA-damaging agents will affect this balance. For example, adding PARG inhibitors pushes the system toward increased PARylation by blocking PAR removal, whereas adding PARP inhibitors decreases protein PARylation

LysA™ Universal PARylation Assay Kit Methods

A white 96-well Maxisorp™ plate was coated with 50 µl/well of (ADP)-ribose Binding Reagent the day before the experiment. The plate was washed three times with PBST (Phosphate Buffer Saline containing 0.05% Tween-20 (v/v)) and blocked with 200 µl/well of blocking buffer 3 for 2 hours at room temperature (RT). The entire volume of each cell lysate containing protease and PARG inhibitors (50 µl/well) was loaded onto the plate and incubated for 1 hour at RT. The negative control consisted of an equivalent volume of lysis buffer. The plate was washed three times with PBST and the Detection Antibody was added in blocking buffer for 45 minutes at RT, followed by three washes in PBST. A secondary HRP-Labeled Antibody was added in blocking buffer for 30-45 minutes at RT and the plate was washed three times in PBST before addition of the ECL substrate. Chemiluminescence was measured using a Bio-Tek microplate reader. *Illustrations created with BioRender.com*

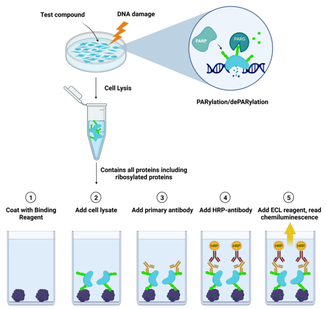

Figure 1. Illustration of the assay principle.

ASSAY OPTIMIZATION

As shown in Figure 2A, treating cells with H_2O_2 (500 µM) for 15 minutes was sufficient to induce PARylation, indicated by the diminishing non-PARylated PARP1 band in the Western-Blot concomitantly with the appearance of a "smear" in PARylation bands, indicating the PARylation of cellular proteins. Several Detection Antibody/Binding Reagent combinations were evaluated for performance and sensitivity (Figure 2B). A PARylation standard curve established the sensitivity at 100 pM and a linear regression within the tested range of 100 pM to 14 nM (Figure 2C).

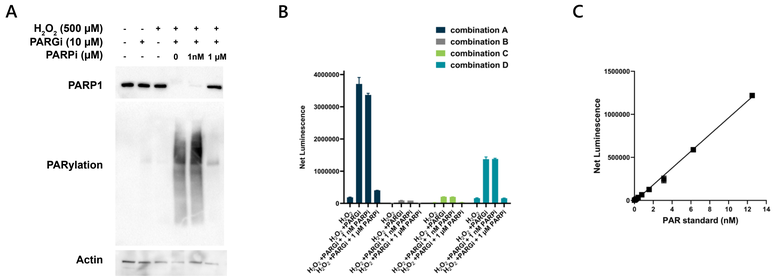

Figure 2. Assay optimization. (A) Evaluation of PARP1 PARylation in response to H_2O_2. *HeLa cells were preincubated with or without 10 µM PARGi PDD00017273 and with or without PARPi Olaparib for 1 hour and 45 minutes before adding H_2O_2 (500 µM) for 15 minutes to induce DNA damage. Cells were washed and lysed on ice for 10 minutes. Lysates were analyzed by western blot using anti-PARP1 antibody (ThermoFisher #436400) and (ADP)-ribose Binding Reagent.* ***(B) Optimization of antibody/reagent combination.*** *Several combinations of Detection ntibody/(ADP)-ribose Binding Reagent were tested by ELISA. Combination A provided the strongest signal and was chosen from then on.* ***(C) Determination of sensitivity and linear range.*** *Increasing amounts of the standard PAR polymer were run in duplicate in the LysA™ PARylation assay (BPS Bioscience #82123).*

STUDY OF PARG INHIBITORS

The effect of PARG inhibitor PDD00017273 was assessed in several cell lines (Figure 3). Similar EC_{50} were measured in all cells, demonstrating the reliability of the assay across cell lines.

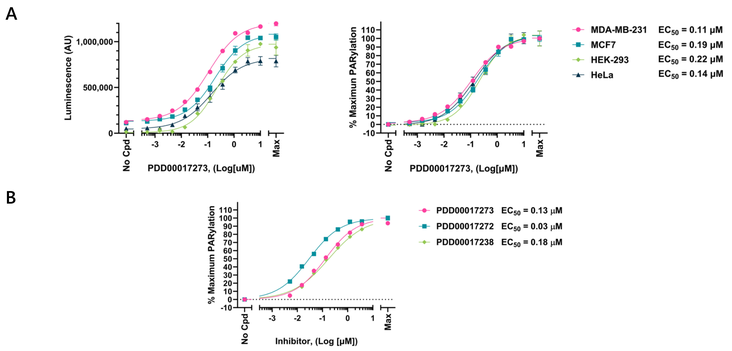

Figure 3. Cell-based evaluation of PARG inhibitors. (A) *Four cell lines were treated with increasing concentrations of PARGi PDD00017273 for 1 hour and 45 minutes at 37°C.* ***(B)*** *HeLa cells were treated with increasing concentrations of three PARGi. All PARGi were purchased from (MedChemExpress). DNA damage was induced by addition of H_2O_2 (500 µM) for 15 minutes. Cells were washed with ice-cold PBS and lysed in 50 µl/well of Modified RIPA Lysis Buffer (BPS Bioscience #82126) supplemented with protease inhibitor cocktail and ADP-Ribosylation Inhibitor Mix (BPS Bioscience #82199 and #82130, respectively). After 10 minutes on ice, lysates were collected and PARylation levels were analyzed immediately using the LysA™ Universal PARylation Assay Kit. Luminescence was measured using a Bio-Tek microplate reader. Results are expressed as raw luminescence signal (left) or as percent of total PARylation (right), in which the maximum PARylation level was set to 100% for each cell line. EC_{50} was determined by curve fitting using Prism software v 11.0.*

STUDY OF PARP INHIBITORS

The following experiments compared several PARP1 inhibitors in HEK293 cells and looked at PARP1 contribution to the total cellular PARylation using PARP1-KO cells (BPS Bioscience #82169). These cells were generated using CRISPR/Cas9 technology and single-clone isolation following antibiotic selection. PARP1 is known to account for about 80-90% of cellular PARylation in many cells. Accordingly, most PARylation observed in HeLa cells is due to PARP1 activity, since PARP1-KO cells displayed only 16% of the WT cells PARylation levels.

Finally, we observed that the IC_{50} values for the tested compounds were identical when cellular PARylation was evaluated using the LysA™ PARylation assay or using a homogeneous AlphaLISA® format, which confirmed the reliability of the assay.

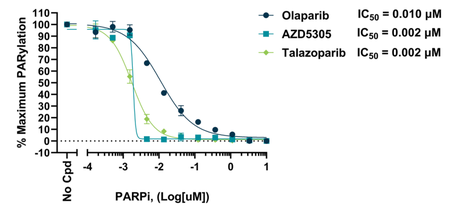

Figure 4. Cell-based evaluation of PARP inhibitors. *HEK293 cells were seeded in 96-well cell culture plates at a density of 50,000 cells/well for approximately 24 hours. Cells were treated with 10 µM PARG inhibitor PDD00017273 in combination with the indicated PARP inhibitors (PARPi sample kit, BPS Bioscience #78318) for 1 hour and 45 minutes at 37°C. H_2O_2 (500 µM) was added for 15 minutes to induce DNA damage. Cells were washed once with ice-cold PBS, placed on ice, and lysed using 50 µl/well of Modified RIPA Lysis Buffer supplemented with protease inhibitor cocktail and ADP-Ribosylation Inhibitor Mix. After 10 minutes on ice, lysates were collected and PARylation levels were analyzed immediately using the LysA™ Universal PARylation Assay Kit. Results are expressed as percent of total PARylation, in which PARylation level in the absence of PARP inhibitor was set to 100% for each cell line. IC_{50} was determined by curve fitting using Prism software v 11.0.*

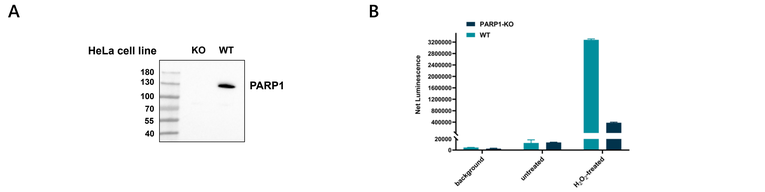

Figure 5. Effect of PARP inhibitors in PARP1-knockout (KO) HeLa cells. (A) PARP1 expression in HeLa wild-type (WT) and PARP1-KO cells. *Cells were lysed and analyzed by western blot using an anti-PARP1 antibody (Invitrogen #436400).* ***(B) Evaluation of PARP1-mediated PARylation in WT and PARP1-KO cells.*** *Cells were incubated with 10 µM PARGi PDD00017273 for 1 hour and 45 minutes before DNA damage was induced by adding H_2O_2 (500 µM) for 15 minutes at 37°C. Cells were washed, lysed, and analyzed immediately for total protein PARylation.*

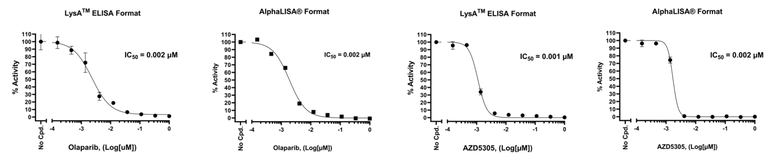

Figure 6. Comparison of two assay formats to determine total PARylation. *HeLa cells were treated with 10 µM PARG inhibitor and increasing concentrations of PARP inhibitor Olaparib for 1 hour and 45 minutes before inducing DNA damage with H_2O_2 (500 µM) for 15 minutes. Cells were washed and lysed. PARylation levels were analyzed immediately using the LysA™ Universal PARylation Assay Kit (BPS Bioscience #82123) or using an AlphaLISA® format. The AlphaLISA® format is a homogeneous (no-wash) assay that relies on the concomitant recruitment of donor and acceptor beads on the same poly-ribose chain to allow for energy transfer, meaning that this assay detects only PARylation and not MARylation. Results are expressed as percent of PARP activity with control (no inhibitor) set to 100%. IC_{50} was determined by curve fitting using Prism software v 11.0.*

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