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A Macrodomein-Linked Immunosorbent Assay (MLISA) for Mono-ADP-Ribosyltransferases

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Abstract

ADP-ribosyltransferases (ARTs) catalyze reversible additions of mono- and poly-ADP-ribose onto diverse types of proteins by using nicotinamide adenine dinucleotide (NAD⁺) as a cosubstrate. In human ART superfamily, 14 out of 20 members are shown to catalyze endogenous protein mono-ADP-ribosylation and play important roles in regulating various physiological and pathophysiological processes. Identification of new modulators of mono-ARTs can thus potentially lead to discovery of novel therapeutics. In this study, we developed a macrodomain-linked immunosorbent assay (MLISA) for characterizing mono-ARTs. Recombinant macrodomain 2 from poly-ADP-ribose polymerase 14 (PARP14) was generated with a C-terminal human influenza hemagglutinin (HA) tag for detecting mono-ADP-ribosylated proteins. Coupled with an anti-HA secondary antibody, the generated HA-tagged macrodomain 2 reveals high specificity for mono-ADP-ribosylation catalyzed by distinct mono-ARTs. Kinetic parameters of PARP15-catalyzed automodification were determined by MLISA and in good agreement with previous studies. Eight commonly used chemical tools for PARPs were examined by MLISA with PARP15 and PARP14 in 96-well plates and exhibited moderate inhibitory activities for PARP15, consistent with published reports. These results demonstrate that MLISA provides a new and convenient method for quantitative characterization of mono-ART enzymes and may allow identification of potent mono-ART inhibitors in a high-throughput compatible manner.

Graphical Abstract

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Keywords

post-translational modification; ADP-ribosyltransferase; macrodomain; immunosorbent assay; enzyme activity

1. Introduction

Protein ADP-ribosylation is a reversible post-translational modification enzymatically catalyzed by ADP-ribosyltransferases (ARTs). Upon binding of nicotinamide adenine dinucleotide (NAD^+) as a cosubstrate, ARTs promote dissociation of nicotinamide leaving group and subsequent addition of single or multiple units (in a linear or branched form) of ADP-ribose onto target proteins through covalent linkage to side chains of many different types of amino acid residues, resulting in mono- or poly-ADP-ribosylation (Figure 1).^{1–3} Human genome encodes 20 ART enzymes that belong to three protein families consisting of intracellular poly-ADP-ribose polymerases (PARPs), sirtuins, and extracellular ARTs.² In the superfamily of human ARTs, 14 members are shown to display mono-ART activity with majority from the PARP family, exemplified by the commonly studied PARP10, PARP14, PARP15, and PARP16.^{4,5} Mono-ART-mediated post-translational modifications are found to be involved in major cellular processes such as DNA replication, cell proliferation, signal transduction, and apoptosis.^{1–4} Cellular mono-ADP-ribosylations are also identified as mechanisms in response to genotoxic stresses and unfolded protein responses to modulate protein functions and cell physiology.⁶ In addition, some mono-ARTs exhibit anti-viral activities upon infections, implicating a role in innate immunity.⁷

Accumulating evidence revealed that overexpressed mono-ARTs are widely seen in many types of human diseases, including cancer, immune disorders, and metabolic diseases.^{8–17} Their expression levels and enzymatic activities often correlate with disease pathogenesis and progression.^{16,18–23} Given their potentially essential functions and roles in pathophysiology, human mono-ARTs have been emerging as highly promising targets for development of new diagnostics and therapeutics.^{4,10,16,22,24} However, potent inhibitors specifically targeting disease-related mono-ARTs are yet to be developed for therapeutic applications as well as for better understanding of their mechanisms involved in pathogenesis. Thus, innovative assays suitable for high-throughput screening are desired for

quantitative determination of mono-ART activities and identification of novel inhibitors with high potency and specificity.

Currently, several methods have been established for characterization of mono-ARTs. Radioactive NAD⁺ compounds are common and sensitive tools for examining ART activities.⁵ Non-radioactive NAD⁺ analogues with remote purine moieties modified by alkyne, biotin, and etheno groups have been generated for studying protein-ADP-ribosylation catalyzed by both poly- and mono-ARTs.^{24–31} An enzyme-coupled spectrophotometric assay was also developed for quantitatively determining ADP-ribosylation activity by measuring decrease of UV absorbance at 340 nm for NADH consumed by glutamate dehydrogenase (GDH) in response to produced ammonia from nicotinamide-catalyzed hydrolysis of nicotinamide.³² Similarly, ART activity could be measured on the basis of the decrease in fluorescence for a fluorophore chemically converted from unused NAD⁺.^{33,34} In comparison to commercially readily available antibodies against poly-ADP-ribose, few monoclonal antibodies specific for mono-ADP-ribose have been developed.^{35,36}

Macrodomains are a family of evolutionarily conserved proteins characterized by tight binding affinity and high specificity to mono- and poly-ADP-ribose.^{37–39} By recognizing ADP-ribose units covalently attached to target proteins, macrodomains act as readers of mono- and poly-ADP-ribosylation in regulating ART-mediated signaling pathways.^{40–42} Numerous human macrodomain-containing proteins have been identified, including macroH2A and its variants, MacroD1-3, C6orf130, ALC1, PARP9, PARP14, and PARP15.^{37,38,43} Notably, a subset of macrodomain proteins display hydrolase activities and function as erasers of protein ADP-ribosylation, which include archaeobacterial Af1521 and human MacroD1, MacroD2, and C6orf130.^{44,45} In light of their high affinity and specificity to ADP-ribose, macrodomain proteins have been applied to proteomic and imaging studies to profile and visualize ADP-ribosylated proteome.^{46,47}

We hereby report the development of a macrodomain-linked immunosorbent assay (MLISA) as a generally applicable method for quantitative characterization of mono-ART enzymes. By exploiting macrodomain 2 of PARP14 that binds tightly to both free and protein-linked ADP-ribose *in vitro* and in cells while lacking hydrolase activity,^{44,48} a recombinant agent for recognizing mono-ADP-ribosylated proteins was generated. In combination with an anti-hemagglutinin (HA) antibody, the macrodomain 2-based ADP-ribose binding module was shown to detect protein mono-ADP-ribosylation with good selectivity. As a general approach, the developed MLISA allows rapid quantification of protein ADP-ribosylations catalyzed by distinct mono-ARTs exemplified by PARP15 and PARP14, as well as characterization of PARP15 enzyme kinetics. Furthermore, a panel of commonly used chemical tools for PARPs was examined for inhibitory activities against PARP15 and PARP14 by performing MLISA-based screening in 96-well plates. Our study shows that MLISA provides a convenient and quantitative approach for characterizing mono-ARTs and potentially enables discovery of new mono-ARTs inhibitors in a high-throughput compatible format.

2. Experimental Methods

2.1 Materials and Reagents

cDNA of human PARP15 (accession number: BC101701) and PARP14 (accession number: BC039604), were obtained from GE Dharmacon (Lafayette, CO). Synthetic DNA encoding macrodomain 2 (residue 983-1196) of PARP14 with codon optimized for bacterial expression was purchased from Integrated DNA Technologies (IDT) (Coralville, IA).

Olaparib was purchased from Selleckchem (Houston, TX) and Minocin, XAV939, 1,5-isoquinolinediol, DR2313, 6(5H)-phenanthridinone, nicotinamide, adenine, β -NAD⁺, and β -NADH were purchased from Sigma Aldrich (St. Louis, MO). 96-well high-binding fluorescence plates were purchased from Greiner Bio-One (Monroe, NC). PureGrade microplates and semi-micro polystyrene cuvettes were purchased from BrandTech Scientific, Inc (Essex, CT). Dithiothreitol (DTT) was purchased from VWR International (Radnor, PA). Trichloroacetic acid (TCA) and Tris base were purchased from Fisher Scientific (Waltham, MA). Anti-HA monoclonal antibody-horseradish peroxidase (HRP) conjugate (clone 2-2.2.14), PierceTM Coomassie Plus (Bradford) assay kit, and QuantaBluTM fluorogenic peroxidase substrate were purchased from Thermo Fisher Scientific (Waltham, MA).

2.2 Molecular Cloning and Protein Expression and Purification

The catalytic domains of PARP15 (residue 481-678) and PARP14 (residue 1611-1801), with N-terminal His₆-tags and Factor Xa cleavage sites were amplified through polymerase chain reaction (PCR) using primers P1–2 and P8–9 (Table S1), followed by additions of XhoI and XbaI restriction enzyme sites at 5' - and 3' -end, respectively, using primers listed in Table S1 (P5 and P6 for PARP14; P10 and P11 for PARP15). Macrodomain 2 (residue 999-1196) of PARP14 with an N-terminal His₆-tag and a Factor Xa cleavage site was amplified by PCR using primers P3 and P4, followed by incorporation of XhoI and XbaI restriction enzyme sites and a C-terminal HA-tag using primers P5 and P7 (Table S1). The amplified DNA fragments were digested by XhoI and XbaI restriction enzymes and then ligated into pET-28a(+) using T4 DNA ligase. All generated expression vectors were confirmed by DNA sequencing provided by Genewiz LLC (South Plainfield, NJ).

BL21 (DE3) cells were transformed with the generated constructs for bacterial protein expression in LB Broth supplemented with kanamycin (50 μ g mL⁻¹). The overnight bacterial culture (5 mL) was diluted into 1 liter LB Broth with kanamycin (50 μ g mL⁻¹) for growth at 37 °C in an incubator shaker at speed of 250 rpm (Series 25, New Brunswick Scientific, NJ). When OD_{600nm} reached 0.6–0.8, protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for overnight at 22 °C. Cells were then harvested by centrifugation at 4,550 g (Beckman J6B Centrifuge, JS-4.2 rotor), resuspended in equilibrium buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20 mM imidazole), and lysed using a French Press (GlenMills, NJ) at 25,000 psi for three times. Cell debris was removed by centrifugation at 27,000 g for 1 hour (Beckman Coulter centrifuge, JA-17 rotor) and supernatant was filtered through a 0.45 μ m membrane. The filtrate was loaded on a gravity flow column packed with 1 mL Ni-NTA agarose resin (Thermo Fisher Scientific, Waltham, MA), followed by washing with 15 column volumes of wash buffer (20 mM Tris-

HCl, pH 8.0, 200 mM NaCl, 30 mM imidazole). Proteins were then eluted in 15 column volumes of elution buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 400 mM imidazole), dialyzed in storage buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM DTT, 10% glycerol) at 4 °C for overnight and another 6 hours in fresh storage buffer, and concentrated using an Amicon centrifugal concentrator (EMD Millipore, Temecula, CA) with a 10 kDa cutoff. Purified proteins were examined by SDS-PAGE and NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and aliquoted and flash-frozen in liquid nitrogen for storage at -80 °C. Calculated molecular extinction coefficient values are 1.20 for PARP 14, 1.13 for PARP15, and 0.93 for macrodomain 2.

2.3 MLISA Assay

2.3.1 Overall assay design and validation—The general scheme of MLISA is shown in Figure 2. First, 200 µL of PARP-catalyzed automodifications using NAD⁺ as cosubstrate in reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT) were performed in 96-wells plates together with the coating process for 2 hours at room temperature. Following five washes with 200 µL of PBST (0.1% v/v Tween-20 in PBS, pH 7.4) in each well, plates were blocked with 3% BSA dissolved in PBS, pH 7.4, for 2 hours under room temperature. Next, each well was washed five times using 200 µL of PBST and then incubated with 100 µL of purified macrodomain 2 (0.1 µM M2 for PARP15 and 0.3 µM M2 for PARP14) in PBS for 1 hour at room temperature. Subsequently, plates were washed five times with 200 µL of PBST and incubated with 100 µL of anti-HA-HRP conjugate (1: 5000 in PBS) for 1 hour at room temperature. After another five washes with 200 µL of PBST, 75 µL of QuantaBluTM fluorogenic peroxidase substrate was added to each well and incubated for 10 min prior to reading. Fluorescence intensity in each well was then measured using Synergy H1 Multi-Mode Reader (BioTek, Winooski VT). All experiments were performed at least in triplicate.

Control wells were established to evaluate background signal intensity due to non-specific binding in between assay components. Wells for 2-hour PARP enzymatic reactions with 500 µM of NAD⁺ were set as the maximal signal wells while wells with only enzymes and no NAD⁺ addition were set to be the minimal signal wells. Fluorescence signal intensities of wells containing 3% BSA only, 3% BSA with various concentrations of M2, NAD⁺ plus various concentrations of M2, and enzymatic reactions without M2 addition were separately measured after incubation with anti-HA-HRP conjugate and compared to those of the maximal signal wells. MLISA assays for both PARP15 and PARP14 were performed on five plates with two sets of triplicates of the maximal and minimal signal wells on each plate for assay validation and repeatability purposes, of which three plates were carried out on the same day and two plates on different days. Assay quality was assessed through analyzing commonly accepted statistical parameters including signal-to-noise ratio (S/N), signal-to-background ratio (S/B) and screening window coefficient (Z').^{33,49,50} Coefficients of variations (CVs) are calculated by analyzing all data on the assay plates and the normality of both maximal and minimal signal intensities of PARP15 and PARP14 was evaluated through Kolmogorov-Smirnov and D'Agostino and Pearson omnibus tests using Graphpad Prism (GraphPad Software, La Jolla, CA).

2.3.2 Time- and concentration-dependent PARP catalytic activities—To perform activity assays with varied reaction time and concentrations, 96-well plates were coated with reaction mixtures in triplicates that consist of 500 μM of NAD^+ and purified PARP enzymes at varied concentrations (PARP15: 0.25 μM , 0.5 μM , 0.75 μM , 1 μM , 1.5 μM , 2 μM , and 2.5 μM ; PARP14: 1.5 μM , 3 μM , 4.5 μM , 6 μM , 7.5 μM , and 9 μM) in reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT) and incubated for different time points (PARP15: 0, 5, 10, 15, 20, 30, 40, 60, 80, 90, 100, and 120 minutes; PARP14: 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, and 360 minutes) at room temperature. The reactions were quenched with 20% ice-cold TCA at those time points. For each enzyme concentration, the apparent rate constant (k) was calculated in Graphpad Prism using one phase exponential association equation $Y=Y_{\text{max}}*(1-\exp(-k*X))$. The rates were then plotted against enzyme concentrations and fitted through the linear regression model for signal linearity analysis.

2.3.3 Inhibition of PARP enzymatic activities—To carry out inhibition assays, 96-well plates were coated with reactions in triplicates that consist of 500 μM of NAD^+ , purified PARP enzymes (PARP15: 500 nM; PARP14: 3 μM), and various inhibitors at multiple concentrations (0, 0.5, 1, 2.5, 5, 7.5, 10, 12.5, and 15 μM for PARP15, 0.1% DMSO or water and 0, 1, 2.5, 5, 10, 25, 50, 75, and 100 μM for PARP14, 0.6% DMSO or water) in reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT). Additional inhibitor concentrations (ranging from 1 nM to 1 mM for Olaparib, 1,5-isoquinolinediol, 6(5H)-Phenanthridinone, and DR2313) were included for PARP15-catalyzed reactions for determination of IC_{50} values. DMSO-soluble inhibitors (Olaparib, XAV939, 1,5-isoquinolinediol, minocin, 6(5H)-phenanthridinone, and adenine) were initially dissolved in 100% DMSO and diluted in water to reduce DMSO content to either 0.1% (for PARP15) or 0.6% (for PARP14) in each well. In the presence of inhibitors at various concentrations, fluorescence intensities (FI) for PARP-catalyzed reactions were measured by MLISA. Inhibition activities were normalized on the basis of the measured fluorescence intensities (FI_0) for the wells without inhibitors and with 0.1% DMSO or water for PARP15 and 0.6% DMSO or water for PARP14. IC_{50} and pIC_{50} values of individual inhibitors were calculated by fitting the dose-response curves with four parameters in Graphpad Prism for both PARP15 and PARP14.⁵¹

2.3.4 Enzyme kinetics of PARP15-catalyzed automodification—To characterize enzyme kinetics, 96-well plates were coated with reactions in triplicates that consist of 500 nM of PARP15 and NAD^+ at varied concentrations (5, 10, 20, 30, 40, 200, and 400 μM) in reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT). The reactions were quenched with 20% ice-cold TCA at different time points (0, 2.5, 5, 7.5, 10, 12.5, 15, and 20 minutes). The plates were further incubated for up to 2 hours under room temperature followed by five washes with 200 μL of PBST. Kinetic parameters were calculated by fitting data to Michaelis-Menten model implemented in GraphPad Prism.

To determine reaction rates, standard curves were generated for each reaction plate. In brief, PARP15-catalyzed automodification reactions were incubated for 2 hours under room temperature in 1.5 mL microcentrifuge tubes that contained 50 $\mu\text{g mL}^{-1}$ of purified PARP15 and 500 μM of NAD^+ in reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT). Reaction mixtures were then serially diluted and plated simultaneously on both the 96-well ELISA

plates and clear 96-well pureGrade plates. Upon additions of Coomassie Plus (Bradford) assay reagents to clear 96-well pureGrade plates, the absorbance at 595 nm of each well was measured by Synergy H1 Hybrid Multi-Mode Microplate Reader. The concentrations of automodified PARP15 were calculated through fitting to linear regression curves generated with BSA standards. Standard curves were constructed through linear correlation of the determined concentrations of automodified PARP15 with the fluorescence intensities of the corresponding wells measured on 96-well ELISA plates.

2.4 High-performance liquid chromatography (HPLC)-based activity assay

PARP-catalyzed automodification reactions were performed at room temperature in 100 μ L assay solutions containing 50 mM Tris-HCl, pH 7.4, 2 mM DTT, and varied concentrations of NAD⁺ and purified PARP enzymes. The reaction mixtures after varied lengths of incubation were separated by reverse phase HPLC using a semipreparative C18 Kinetex[®] column (5 μ m, 100 Å, 150 x 10.0 mm, Phenomenex Inc, Torrance, CA) with a gradient of methanol (0–50% in 12 min) in water containing 0.1% formic acid. Reaction rates were determined on the basis of the assigned peaks of nicotinamide and NAD⁺.

3. Results

3.1 Overall assay design

Previous studies indicated that macrodomain proteins possess high affinity and specificity towards protein ADP-ribosylation and could be utilized to identify and visualize ADP-ribosylated proteins in the cells.^{46–48} We thus envisioned that recombinant macrodomains can possibly act as powerful detection agents of mono-ADP-ribosylation for direct and quantitative characterization of mono-ARTs through a novel immuno-like assay potentially adaptable for high-throughput screening. To test this notion, macrodomain 2 of PARP14 was chosen for developing the MLISA, since it shows high affinity to mono-ADP-ribose, but displays no hydrolase activity seen in many other macrodomain proteins.^{44,48} The bacterial expression construct for macrodomain 2 was designed with an N-terminal His₆ tag followed by a Factor Xa cleavage site for purification and a C-terminal HA tag for recognition by a secondary antibody. Recombinant macrodomain 2 was stably expressed in *E. coli* and purified by Ni-NTA affinity chromatography with a final yield of 1.4 mg per liter. SDS-PAGE revealed that the generated macrodomain 2 migrated as a single band around 24 kDa (Figure S1).

Next, to test the generality of the resulting macrodomain 2 in binding and reporting mono-ADP-ribosylation, two human mono-ARTs PARP15 and PARP14 were recombinantly produced. Overexpression of these mono-ARTs are frequently detected in many types of cancer, but little is known about the characteristics of these enzymes.⁵² All PARPs were stably expressed in *E. coli* and purified using the same method as macrodomain 2 with final yields of 1–1.5 mg per liter. SDS-PAGE showed single bands of 25 kDa for PARP15 and 23 kDa for PARP14 (Figure S1). The catalytic activities of the generated PARPs were verified through automodification reactions using HPLC-based activity assay.

The MLISA was designed by utilizing the HA-tagged macrodomain 2 as a primary detection agent for recognition of mono-ADP-ribosylated proteins that are immobilized on ELISA plates (Figure 2). An anti-HA antibody-HRP conjugate was included as a dual agent for detecting bound macrodomain 2 and reporting levels of activity through enzyme-mediated signal amplification. First, automodification reactions by mono-ARTs were performed in the wells for direct coating of ADP-ribosylated proteins on 96-well plates. The reactions could be quenched by adding 20% TCA to each well. By dispensing libraries of compounds onto the plates, their effects on mono-ART activities could be quantitatively measured for identification of new activators/inhibitors. Second, 3% BSA in PBS pH 7.4 was utilized as blocking agent following the coating step, similar to conventional immunoassays. Then, HA-tagged macrodomain 2 and anti-HA antibody-HRP conjugate were added to each well in a sequential order for complex assembly. It should also be noted that each of these steps was incubated for 1–2 hours at room temperature and thorough washes (5×) with PBST were carried out prior to additions of any reagents for next step. Lastly, upon addition of fluorogenic substrates of HRP to each well, enzymatic activities were determined on the basis of recorded fluorescence intensities that closely correlate with the levels of immuno-complexes formed in the wells.

3.2 Validation of MLISA

By performing MLISA with auto-ADP-ribosylation catalyzed by different mono-ARTs, the specificity of macrodomain 2 towards mono ADP-ribosylated proteins was examined. Reactions containing PARP enzymes with and without NAD^+ were incubated for two hours in the wells, followed by blocking with 3% BSA, detecting with macrodomain 2, and the reporting step as established for the MLISA. In comparison to the control wells where no reactions occur or no macrodomain 2 existed, the respective ones with NAD^+ -dependent auto-ADP-ribosylation showed dramatically increased fluorescence intensities for both PARPs (Figure 3). This indicated that the generated macrodomain 2 binds specifically to mono-ADP-ribosylated proteins. Using macrodomain 2 as a detection agent of mono-ADP-ribose, MLISA allows quantitative measurements of mono-ADP-ribosylation on distinct proteins. It was found that relative to reaction wells, the control wells with PARP14 revealed higher background fluorescence intensities than those with PARP15, likely resulting from the nonspecific binding caused by high concentration of PARP14 (3 μM for PARP14 and 500 nM for PARP15). Importantly, by increasing the concentrations of PARP14 to 6 and 9 μM for enhanced mono-ADP-ribosylation, lower contributions of fluorescence signals from the background were observed (Figure S2), supporting high specificity of the generated macrodomain 2 for mono-ADP-ribosylated proteins. Relative to PARP15, higher macrodomain 2 concentration (0.3 μM versus 0.1 μM) was used for PARP14 to improve signal-to-background ratio (>2.5). Additions of anti-HA antibody-HRP conjugate with and without macrodomain 2 to the wells with only 3% BSA resulted in minimal fluorescence intensity, showing that neither of these reagents binds nonspecifically to BSA. In the absence of macrodomain 2, incubation of anti-HA antibody-HRP conjugate with PARP-catalyzed reactions led to low fluorescence signals, indicating the lack of specific binding to ADP-ribosylated proteins for the anti-HA secondary antibody. Compared with reaction wells, the control wells without PARP enzymes gave minimal fluorescence signals, showing no affinity between macrodomain 2 and NAD^+ . Taken together, these results support the use of

recombinant macrodomain 2 in MLISA as a specific detection agent for mono-ADP-ribosylated proteins. The well-to-well, plate-to-plate, and day-to-day variations and frequency distribution of maximal and minimal signals were evaluated through analysis of five independent assay plates performed on different days (Tables 1 and S2; Figure S3). The Z' factors for PARP15- and PARP14-catalyzed automodifications are 0.8 and 0.6, respectively, indicating that the developed MLISA assay is suitable for screening purposes.

Next, catalytic activities of PARP15 and PARP14 were characterized by MLISA at varied lengths of reaction times and different enzyme concentrations. Automodification of PARP15 was incubated for 0–120 minutes in an enzyme concentration range of 0–2.5 μM . The reaction wells showed that the fluorescence intensities for PARP15-catalyzed automodification reactions increased in time-dependent manners (Figure S4). Moreover, the determined apparent rate constants show linear dependence on the concentrations of PARP15 enzyme from 0.25 μM to 2.5 μM (Figure 4A), allowing quantitative measurements of PARP15 activities by MLISA. Similarly, the time-dependent increases in fluorescence signals were seen for PARP14-catalyzed automodifications which were carried out at both higher enzyme concentrations and longer time duration (Figure S5). The measured apparent rate constants also display a linear correlation with the concentrations of PARP14 enzyme in the range of 1.5–9 μM (Figure 4B). These data indicated that PARP14 enzymatic activity can also be quantitatively determined by MLISA. In addition, it was found that further decreased concentrations of PARP15 resulted in significantly reduced apparent rate constants, which were determined to be 0.003 min^{-1} for 0.1 μM and 0.001 min^{-1} for 0.05 μM . Due to low activity, no apparent rate constants could be determined at concentrations below 0.05 μM for PARP15 and 1.5 μM for PARP14. To more accurately determine the apparent rate constants, the fluorescence intensities at time 0 (y-intercept) in Figures S4 and S5 could be included in the exponential association equation. Alternatively, the measured fluorescence intensities in the initial linear region could be fitted to a linear equation with the included y-intercept for determination of the reaction rates. In both cases, the measured rates are linearly dependent on the enzyme concentrations. Collectively, MLISA is shown as a general method for qualitative and quantitative characterization of mono-ARTs.

3.3 Characterization of enzyme kinetics by MLISA

The developed MLISA was then utilized to characterize enzyme kinetics of mono-ARTs. PARP15 was selected as a model enzyme since a published K_m value for its automodification reaction was available⁵³. MLISA-based PARP15-catalyzed automodifications were performed with NAD^+ at varied concentrations. The enzymatic reactions were quenched with ice cold 20% TCA at various time points. As described in the experimental methods, standard curves were created on each plate for determining the concentrations of generated automodified PARP15 on the basis of measured fluorescence intensities. By fitting the kinetic data to Michaelis-Menten equation, the k_{cat} and K_m of PARP15 for automodification were calculated to be $0.011 \pm 0.001 \text{ min}^{-1}$ and $4.5 \pm 2.9 \mu\text{M}$ (Figure 5), respectively, which is consistent with the K_m value of $5.8 \pm 1.9 \mu\text{M}$ as reported previously.⁵³ This supports the use of MLISA as a direct method for examining kinetics of mono-ARTs. It was noted that the reaction rates for PARP15-catalyzed automodifications could be confidently measured by MLISA with a NAD^+ concentration of 5 μM or above.

Reactions with lower NAD^+ concentrations resulted in little fluorescence signals over the background and relatively large variations.

3.4 Evaluation of PARP inhibitors by MLISA

We next applied the MLISA for inhibitor screening of PARP15 and PARP14, given that both mono-ART enzymes are involved in many human diseases including cancer.^{8–17,24} A panel of eight commonly used chemical tools for PARPs was examined in 96-well plates for their inhibitory effects on automodifications of PARP15 and PARP14 at different concentrations (Table S3). Since some of those compounds were tested in 0.1% or 0.6% DMSO, control wells containing 0.1% or 0.6% DMSO only were used as no inhibitor controls to even out the inhibitory effect of DMSO on PARP enzymes. It was found that several inhibitors exhibit dose-dependent inhibition on catalytic activities of PARP15 with IC_{50} values in the range of 2–9 μM (Table 2 and Figure S6). Both Olaparib and 6(5H)-phenanthridinone display moderate inhibition activity for PARP15 with determined IC_{50} values of 4.6 ± 0.9 and 1.5 ± 0.5 μM , respectively, which are consistent with previous studies.^{33,54} Similarly, 1,5-isoquinolinediol and DR2313 were found to inhibit PARP15 activity with IC_{50} of 8.5 ± 2.7 and 8.6 ± 0.6 μM , respectively. The other four compounds including XAV939, minocin, nicotinamide, and adenine gave no significant inhibition effects on PARP15 activity at concentrations up to 100 μM . In contrast to PARP15, none of the eight tested compounds revealed dose-dependent inhibition activity against PARP14 at concentrations up to 100 μM (Figure S7), suggesting large differences in the active sites and/or catalytic mechanisms of PARP15 and PARP14. It was shown that MLISA offers a direct and convenient approach for characterization of mono-ART modulators, which is likely to be suitable for high-throughput screening.

4. Discussion

We herein reported the development of an innovative MLISA assay for studying mono-ARTs by exploiting the macrodomain protein as a binding module of mono-ADP-ribose. As a general approach, it may be applicable to investigation of various types of mono-ART enzymes and qualitative and quantitative characterization of mono-ART activities and their modulators. Similar to conventional ELISA, the developed MLISA can possibly be performed in different formats through uses of modified and/or new reagents, including direct, sandwich, and competitive manners. The versatility in assay style would further expand its applications in identifying readers and erasers of mono-ADP-ribosylation and examining enzyme-specific modifications. Through the use of a secondary antibody-HRP conjugate to amplify levels of modifications detected by the macrodomain protein, MLISA is characterized by a wide range of signal intensity, allowing measurements of rapid turnovers of NAD^+ by mono-ARTs and identification of enzyme inhibitors. Additionally, MLISA requires no radioactive NAD^+ or specialized NAD^+ analogues and utilizes reagents which need no special handling and are readily accessible, providing a relatively low-cost and high-accessibility approach for evaluating mono-ART enzymes. By directly measuring and reporting levels of enzyme activities, MLISA reduces complexity of the reaction systems and minimizes experimental variations.

In contrast to those established assays for protein mono-ADP-ribosylation, MLISA also has some potential limitations in characterization of mono-ARTs. The sensitivity of MLISA is dependent on the binding affinity of macrodomain to mono-ADP-ribosylated proteins. Thus, a macrodomain protein with sub-micromolar binding affinity is unlikely to afford an ADP-ribosylation assay with sensitivity comparable to assays using radioactive NAD⁺ or biotinylated NAD⁺. The reaction rates of PARP15-catalyzed automodification can be measured by MLISA with NAD⁺ concentration as low as 5 μ M. MLISA provides a direct endpoint assay for qualitative and quantitative evaluation of protein mono-ADP-ribosylation, whereas the enzyme-coupled assay allows continuously monitoring of mono-ART-catalyzed reactions. In addition, despite tight affinity to both free and protein-linked ADP-ribose,⁴⁸ the specificity of macrodomain for modified peptide/protein needs further studies. To assess other mono-ARTs with MLISA, assay conditions such as concentrations of macrodomain 2 and mono-ARTs need be optimized.

Significant levels of fluorescence intensity were observed in MLISA for the control wells with 3 μ M PARP14 enzyme only, suggesting that in the absence of ADP-ribosylation the recombinant macrodomain 2 binds to catalytic domain of PARP14. It is likely that the macrodomain 2 of PARP14, which is a multidomain protein with a molecular weight of approximately 203 kDa,⁵⁵ can form interactions with the catalytic domain within the protein architecture to coordinate or regulate biological functions. When recombinantly produced, these subdomains of PARP14 may still interact with each other, causing higher background signals as observed in the wells with PARP14 enzyme only.

Olaparib, a potent inhibitor of poly-ARTs, showed less inhibitory activity against mono-ARTs comparing to PARP1 ($IC_{50}=5$ nM⁵⁶), consistent with previous studies.^{33,54} The preferential binding of olaparib to poly-ARTs possibly resulted from differences in catalytic mechanisms, overall structural folds, catalytic elements, and active site interactions. Extensive mechanistic and structural studies have been performed with poly-ARTs with diverse groups of inhibitors, which facilitate elucidation of principles underlying the catalysis and inhibition of poly-ARTs. In comparison, limited information is available for selective inhibition of mono-ARTs.²⁴ In our study, several compounds including olaparib revealed differential inhibition effects on catalytic activities of PARP15 and PARP14. This suggested that new inhibitors specific for individual mono-ARTs could possibly be identified through mechanism and/or structure-based rational design or compound library-based screening. In fact, a potent PARP14 inhibitor was recently identified using a small molecule microarray, which exhibits more than 20-fold selectivity over PARP1.⁵⁷

In addition to macrodomain 2 of PARP14, other macrodomains in the superfamily can possibly be utilized as detection agents for the ADP-ribose moiety, depending on their binding affinities, specificity, and capabilities in hydrolysis. For example, macrodomain 3 of PARP14 was also shown to bind tightly to mono-ADP-ribose *in vitro*.^{37,48} Similarly, PARP15 is a macrodomain-containing protein. In contrast to these mono-ADP-ribose binding modules, macrodomain proteins recognizing poly-ADP-ribose units have been identified, such as PARP9 and Af1521,⁴² suggesting the possibility of characterizing poly-ARTs with macrodomains. Indeed, macrodomains have been utilized to identify and visualize cellular ADP-ribosylated proteins.^{46–48,58} It is of note that some of the

macrodomain proteins were found to have ADP-ribosylhydrolase activities including Af1521, human MacroD1, MacroD2, and C6orf130, preventing them from use in detecting protein ADP-ribosylation.^{44,45} Besides macrodomains, recent studies discovered that WWE domains can uniquely recognize poly ADP-ribose units,^{59–62} representing a new class of protein tools for studying protein ADP-ribosylation. Moreover, the high specificity of macrodomains and WWE domains for ADP-ribose possibly allow studies of extracellular ADP-ribosylation by distinct ART enzymes. Guided by X-ray crystal structures of various macrodomains, protein engineering can be performed to create variants with improved affinity and specificity or orthogonal pairs of macrodomain and non-canonical ADP-ribose. The resulting engineered macrodomains may provide important tools for investigating protein ADP-ribosylation and modulating signaling pathways and biological processes regulated by macrodomains.

5. Conclusions

We have developed MLISA for characterization of mono-ART enzymes by using the recombinant macrodomain protein as a detection agent of mono-ADP-ribose. It was demonstrated to be applicable to both PARP15 and PARP14. MLISA provides a novel approach for qualification and quantification of protein mono-ADP-ribosylation and identification of mono-ART inhibitors in a potentially high-throughput compatible fashion. Future studies include extending this assay to characterize other mono-ARTs with distinct cellular locations and examining alternative macrodomains for enhanced specificity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A novel immuno-like assay for protein mono-ADP-ribosylation
- Qualitative and quantitative characterization of mono-ADP-ribosyltransferases
- Applicable to distinct mono-ADP-ribosyltransferases

Figure 1.
Protein ADP-ribosylation by mono-ARTs. Approximately 80% mono-ART enzymes (highlighted in red) are implicated in various human diseases.

Figure 2.

General scheme of MLISA. Five washes of PBST were conducted in between each step. 3% BSA, macrodomain 2 (M2), and anti-HA antibody-HRP conjugate were diluted in PBS, pH 7.4.

Figure 3. Macrodomain 2 (M2) is specific for mono-ADP-ribosylation by (A) PARP15 and (B) PARP14. Lane 1: anti-HA antibody-HRP conjugate (Ab) has no binding to BSA. Lane 2: M2 has no binding to BSA as examined by Ab. Lane 3: M2 has low to moderate binding to non-ADP-ribosylated PARPs as examined by Ab. Lane 4: Ab has low binding to ADP-ribosylated PARPs in the absence of M2. Lane 5: M2 has no binding to NAD⁺ in the assay system. Lane 6: M2 specifically binds to ADP-ribosylated PARPs from 2-hour reactions as measured by Ab. Values displayed were calculated as mean values of triplicates $\pm$ SD.

Figure 4.

Concentration-dependent PARP-catalyzed mono-ADP-ribosylations as measured by MLISA. At each enzyme concentration, the apparent constant (k) was calculated in Graphpad Prism using one phase exponential association equation $Y=Y_{\max}*(1-\exp(-k*X))$ on the basis of the measured fluorescence intensities by MLISA at different time points (Figures S4 and S5). The determined apparent rate constants were then plotted versus the concentrations of PARP enzymes. (A) PARP15: 0.25 μM , 0.5 μM , 0.75 μM , 1 μM , 1.5 μM , 2 μM , and 2.5 μM . M2: 0.1 μM ; (B) PARP14: 1.5 μM , 3 μM , 4.5 μM , 6 μM , 7.5 μM , and 9 μM . M2: 0.3 μM . The values were calculated as mean values of triplicates $\pm$ SD.

Figure 5.

Enzyme kinetic parameters of PARP15-catalyzed automodification. Reaction rates measured by MLISA were plotted against varied NAD⁺ concentrations used in the reactions. The values were calculated as mean values of triplicates $\pm$ SD.

Table 1

Statistical parameters of optimized MLISA for PARP15 and PARP14.

	PARP15	PARP14
S/B ¹	19.9 ± 3.4	2.7 ± 0.7
S/N ²	12.4 ± 1.7	6.3 ± 0.2
Z' ³	0.8 ± 0.1	0.6 ± 0.1
Day to day, CV ⁴ (max/min;%)	7.6 ± 1.0/37.1 ± 22.4	6.8 ± 0.7/18.8 ± 11.1
Well to well, CV (max/min;%)	8.5/35.7	8.7/31.0
Plate to plate, CV (max/min;%)	5.5 ± 0.9/11.4 ± 4.5	6.9 ± 1.1/2.8 ± 1.1

¹S/B: Signal to Background ratio.²S/N: Signal to Noise ratio.³Z': Z factor.⁴CV: Coefficient of variation

Table 2

Inhibitory potency of eight compounds for PARP15.

Compound	PARP15 IC ₅₀ (μM)	CI ¹ (μM)	pIC ₅₀
Olaparib	4.6 ± 0.9	3.7 to 5.5	5.3
XAV939	>15 μM	N.D. ²	N.D.
1,5-Isoquinolinediol	8.5 ± 2.7	5.8 to 11.2	5.1
Minocin	>15 μM	N.D.	N.D.
DR2313	8.6 ± 0.6	8.0 to 9.2	5.1
6(5H)-Phenanthridinone	1.5 ± 0.5	1.0 to 2.0	5.8
Nicotinamide	>15 μM	N.D.	N.D.
Adenine	>15 μM	N.D.	N.D.

¹CI: 95% Confidence Intervals.²N.D.: not determined