Small molecule screening assay for mono-ADP-ribosyltransferases

Haikarainen Teemu, Murthy Sudarshan, Maksimainen Mirko, Lehtiö Lari*

Faculty of Biochemistry and Molecular Medicine and Biocenter Oulu, University of Oulu, Oulu, Finland

*Corresponding author: lari.lehtio@oulu.fi

Abstract

Mono-ADP-ribosyltransferases of the PARP/ARTD enzyme family are enzymes catalyzing the transfer of a single ADP-ribose unit to target proteins. The enzymes have various roles in vital cellular processes such as DNA repair and transcription, and many of the enzymes are linked to cancer-relevant functions. Thus inhibition of the enzymes is a potential way to discover and develop new drugs against cancer. Here we describe an activity-based screening assay for mono-ADP-ribosyltransferases. The assay utilizes the natural substrate of the enzymes, NAD^+, and it is based on chemically converting the leftover substrate to a fluorophore and measuring its relative concentration after the enzymatic reaction. The assay is homogenous, robust and cost-effective, and most importantly, applicable to mono-ADP-ribosyltransferases as well as poly-ADP-ribosyltransferases for screening of small molecule inhibitors against the enzymes.

Key Words

PARP, ARTD, mono-ADP-ribosyltransferase, screening assay, inhibitor

1. Introduction

The transfer of an ADP-ribose from NAD^+ to a target protein is a post-translational modification catalyzed mainly by proteins belonging to the PARP/ARTD family. These enzymes can be further divided to mono-ADP-ribosyltransferases and poly-ADP-ribosyltransferases. Mono-ADP-ribosyltransferases can catalyze the transfer of a single ADP-ribose unit to target proteins, whereas poly-ADP-ribosyltransferases can catalyze the polymerization reaction resulting in long chains of ADP-ribose units attached to target proteins. Most of the enzymes are also able to modify themselves through automodification. There are 18 members in the human ARTD protein family [1,2]. ARTD1-ARTD6 (PARP1-PARP5) are poly-ADP-ribosyltransferases whereas ARTD7-ARTD18 (PARP7-PARP17, TPT1, potentially also PARP3) are mono-ADP-ribosyltransferases, with the exception of ARTD13, which is an inactive member of the ARTD family [3]. Poly-ADP-ribosyltransferases of the ARTD/PARP family are the most extensively studied in the context of drug discovery especially due to their roles in cancer-related functions, such as DNA repair and Wnt-signaling [4–6]. Recently, the mono-ADP-ribosyltransferases have also gained more attention due to discovery of their roles in various cellular functions such as cell death and transcriptional regulation [7].

Assays based on biophysical methods and on enzyme activity have been described in the literature. Biophysical assays such as differential scanning fluorimetry [8] and microarray [9] are based on measuring binding of the compound to the target protein or *vice versa*. Activity-based ELISA methods utilize a biotinylated NAD^+ as a substrate and the signal is detected via luminescence produced after reaction with streptavidin-conjugated HRP [10]. Similar ELISA-based assays utilizing biotinylated NAD^+ and streptavidin-conjugated HRP are available commercially.

We describe here a robust, time- and cost-effective assay, which can be used for screening of small molecule inhibitors for all active members of the ARTD family [11,12]. The assay was initially adapted and optimized from an assay developed by Putt & Hergenrother [13] for

ARTD1/PARP1, and it is based on quantifying the leftover of NAD^+ after enzymatic hydrolysis by ARTDs. In the reaction, an ARTD enzyme uses NAD^+ to attach a single ADP-ribose to a substrate protein such as SRPK2. It should be noted that the assay does not differentiate between tans- and automodification (of ARTD itself) or NAD^+ hydrolysis. Leftover NAD^+ is converted to a fluorophore *via* a chemical reaction with acetophenone and formic acid and relative activity can be measured when compared to control without the enzyme. This screening method can be directly applied for all mono-ADP-ribosyltransferases taken that conditions can be found where these enzymes show robust activity. Assay can also be applied for other enzyme families carrying out similar cleavage of NAD^+. The assay conditions optimized for the catalytic domains of human ARTD7/PARP15, ARTD8/PARP14, ARTD10/PARP10, ARTD12/PARP12 and ARTD15/PARP16 using a 96-well plate are described in Table 1 and Figure 1. In the following we will describe the assay in detail and address specific points to take into account when applying the assay for these enzymes.

2 Materials

Prepare all solutions in ultrapure water. Store all reagents at room temperature unless otherwise stated. Follow local regulations when disposing hazardous waste.

2.1 Enzymatic reaction

1. Active, purified recombinant mono-ADP-ribosyltransferase and substrate proteins (see Tables 1 and 2). The recombinant proteins can be purchased or the catalytic fragments can be expressed in *E. coli* host using expression vectors listed in Table 2. Recombinant proteins can be purified using Ni-affinity chromatography with optional removal of tag and a polishing size exclusion chromatography. Purified proteins should be flash frozen in liquid nitrogen in small aliquots (e.g. in PCR tubes). Only use freshly thawed protein samples for the assays.

2. Assay buffer depends on the enzyme and the buffers we have tested are listed in Table 1.

3. 1 mM NAD^+ in water (see Note 1). Store in -20C and dilute before use in assay buffer to appropriate concentration. Assay pH will be set by the buffer used in dilution of all the reagents (Table 1).

4. Assay plates: Black polypropylene 96-well plates with U-shaped or flat bottom.

5. Compound dilution plates: Transparent polypropylene V-shaped 96-well plates.

6. Plate shaker. Optional, but recommended due to long incubation times to achieve efficient mixing and temperature control.

7. Adhesive PCR foil seals for plates. Other seals can be used, but PCR seals have performed best for us.

2.2 Chemical reaction

1. 20% acetophenone in ethanol (see Note 2).

2. 2 M KOH made in ultrapure H_2O.

3. 100% Formic acid.

4. Plastic plate covers.

5. Plate reader based on filters or monochromators with fluorescence intensity capability (ex. 372 nm, em. 444 nm).

3 Methods

3.1 Testing enzymes

Before screening of small molecule inhibitors is it advisable to test the enzymatic activity using the assay and to carry this out by varying both the time and the enzyme concentration [11]. This should be repeated for each new batch of protein in order to confirm the suitable assay parameters. The activity of each protein batch should be evaluated using the optimized assay conditions (Table 1). A dilution series of the enzyme using two-fold serial dilution (*e.g.* 50, 100, 200, 400, 800, 1600, 3200 nM) should be assayed using different time points usually ranging few hours up to 24 h (see Table 1). The conversion-% (see Data analysis) for a robust screening assay should be 60-70 %. It is advisable to select lowest possible enzyme concentration, which still gives 60-70 % conversion within 24 h. For convenience, one should also ensure that there is enough of the protein preparation to carry out a screening campaign with the same protein batch.

3.2 Before screening

When screening small focused libraries we recommend using quadruplicate wells for both the enzymatic reactions and compound control wells (Figure 1). If planning screening of large compound libraries we recommend the researcher to get familiar with the assay optimization and validation for high throughput screening and to confirm that e.g. the screening window coefficient (Z´) indicates that the assay would be suitable for screening of compounds in singlets (two wells per compound; control and reaction). A good source of information would be the assay guidance manual from the National Institutes of Health [14].

Assay conditions can be optimized for buffer components, incubations times and NAD^+ concentration [11]. The same assay protocol described below can be used for these optimizations. We have observed that many enzymes of the ARTD family are very sensitive to DMSO and this requires special attention. It may be necessary to add equal amount of DMSO to all the wells taking

into account the amount transferred with the compound, which are commonly stored as DMSO stocks. Same is true for the dose response measurements where the compound concentration varies.

3.3 Compound screening

Compound screening is carried out by mixing the appropriate concentration of the compound, proteins used and NAD^+ followed by incubation for the enzymatic reaction. Blank and control wells are needed to quantify the inhibition.

1. Dilute the compounds to be screened (stock usually 10 mM in 100% DMSO) to e.g.100 µM in assay buffer (Table 1) or in assay buffer supplemented with 1% DMSO in compound dilution plates (see Note 3). Use always fresh compound dilutions in screening. As the volume of the enzymatic reaction is 50 µl, only 5 µl of the diluted compound solution is needed for each well. Dilution can be made directly from the compound stock to a microtube or microplate and the solution should be thoroughly mixed.

2. Transfer 5 µl of the compounds from the dilution plates to assay plates in reaction wells. Add 5 µl of buffer with 1% DMSO to control wells to match the DMSO concentration in the compound wells. Screening is usually done in singlets (control + reaction) when assay reproducibility is sufficient [14] (see Figure 1 for layout).

3. Add 25 µl NAD^+ to the reaction and control wells (see Table 1 for concentration and Figure 1 for locations).

4. Add 10 µl of substrate (SRPK2) (or buffer) to reaction wells and control wells (see Table 1 for concentration and Figure 2 for layout).(see Note 4)

5. Add 10 µl ADP-ribosyltransferase to the reaction wells (see Figure 1 for layout).

6. Add 10 µl assay buffer to control wells (see Figure 1 for layout). (see Note 5)

7. Add 50 µl assay buffer to blank wells (see Figure 1 for layout).

6. Seal the plates with adhesive PCR foil seals. Incubate in plate shaker at 300 rpm at room temperature until 50-70 % conversion is reached (see Table 1 for examples of incubation times). Please note that the incubation time needs to be optimized for each protein batch. Relatively long incubation times, efficient mixing and temperature control are often required in order to obtain robust and stable signal.

7. Some compounds can produce fluorescence signal with used wavelengths and therefore interfere with the assay. This is more typical than quenching of the signal (see Note 6). Fluorescence signal caused by the compound can be observed by comparing the compound control and control wells (Figure 1).

3.4 Chemical reaction

After the enzymatic reaction a chemical reaction is carried out in order to convert NAD^+ to a fluorophore.

1. Move the plate to the chemical fume hood (see Note 7). The chemicals used in the conversion of NAD^+ to a fluorophore are hazardous. Acetophenone also has a distinctive and strong odor.

2. Add 20 µl 20% acetophenone in ethanol to all the wells. Add 20 µl 2 M KOH to all the wells in the plate. Insert plastic plate cover on the plate. Incubate for 10 minutes. This will stop the enzymatic reaction and convert left over NAD^+ to a fluorophore after the incubation of excess acid in step 3.

3. Add 90 µl of 100% formic acid to all the wells in the plate. Insert plastic plate cover on the plate. Incubate for 20 minutes (see Note 8).

4. Measure the fluorescence intensity with a plate reader. Use 372 nm excitation and 444 nm emission wavelength. It is advisable to optimize parameters, such as slits and Z-focus for the plate reader and detector gain for the NAD^+ condition used.

3.5 Data analysis

The flow for the simple evaluation of the screening result plate is described below.

1. Average the blank wells and subtract the blank from all the wells.
2. Activity of an enzyme can be calculated by dividing the difference between mean of the control wells from mean of the reaction wells and dividing this by the mean of the control wells. Typically this is expressed as % of conversion of NAD^+ (for robust screening assay ~60%), and can be set to 100% activity.
3. Repeat the above calculation, but by taking the difference between the compound control and the compound reaction and divide this by the mean of the control wells. This procedure assumes that compound contribution is the same for both the compound control and reaction wells. (see Note 9).
4. Now the conversion-% observed can be compared to the reaction wells and plotted as activity % using e.g. Graphpad Prism.
5. Set the hit limit according to the purpose [14].
6. Proceed to dose-response measurement of the hit compounds and validation using an orthogonal assay.

3.6. Dose-response measurements

For dose response measurement the exactly same procedure can be used, while instead of different compounds varying concentration of a single compound (half-log dilution in quadruplicates) can be used. Pay attention to DMSO concentration (see Note 3) and use assay condition yielding approximately 30% conversion of NAD^+. This is a compromise between the effect of lowering the substrate concentration during the enzymatic assay and the robustness of the signal [11]. Fluorescence signal can be used directly, and is recommended, for fitting the dose response (e.g. in Graphpad Prism). Control and reaction results can be added 2-log units away from the lowest and highest concentration, respectively. In case of compound interference conversion % (or activity %) must be used for fitting.

Notes

1. Store the NAD^+ stock solution in 20 µl aliquots at -20 °C. Do not re-use the thawed aliquots.
2. Prepare and store 20% acetophenone in ethanol in fume hood. Protect the solution from light.
3. The assay has low DMSO tolerance and control wells have to include same DMSO concentration as reaction wells [11]. Use of 1% DMSO has shown to give enough precision to the DMSO concentration in dose response measurements and this shortcut simplifies the pipetting procedure.
4. In order to simplify the pipetting procedure it is possible to pre-mix SRPK2 into the NAD^+ containing buffer.
5. If using higher protein concentration than 1 µM, 10 µl of protein (same concentration as used in the reaction wells) should be added to the compound controls instead of buffer just before stopping the reaction by acetophenone and KOH.
6. One way to overcome the interference is to increase the NAD^+ concentration and therefore the signal, but this may not be possible due to e.g. limited number of ADP-ribosylation sites available and inhibition through automodification.

7. Carry out all the pipetting and incubations in section 3.4 inside the fume hood.
8. The fluorophore is stable in the dark at room temperature for 2 hours. We have observed that high protein concentration can affect the fluorescence signal.
9. This procedure does not work in case compounds quench the reaction, which has not been very typical in our experiments. In this case the calculation should be carried by taking the difference between the compound control and the reaction and by dividing this by the mean of the compound control wells.

References

1. Schreiber V, Dantzer F, Ame J-C, de Murcia G (2006) Poly(ADP-ribose): novel functions for an old molecule. Nat. Rev. Mol. Cell Biol. 7: 517–528
2. Hottiger M O, Hassa P O, Lüscher B, Schüler H, Koch-Nolte F (2010) Toward a unified nomenclature for mammalian ADP-ribosyltransferases. Trends Biochem. Sci. 35: 208–219
3. Kleine H et al. (2008) Substrate-assisted catalysis by PARP10 limits its activity to mono-ADP-ribosylation. Mol. Cell 32: 57–69
4. Gupte R, Liu Z, Kraus W L (2017) PARPs and ADP-ribosylation: recent advances linking molecular functions to biological outcomes. Genes Dev. 31: 101–126
5. Vyas S, Chang P (2014) New PARP targets for cancer therapy. Nat. Rev. Cancer 14: 502–509
6. Haikarainen T, Krauss S, Lehtiö L (2014) Tankyrases: structure, function and therapeutic implications in cancer. Curr. Pharm. Des. 20: 6472–6488
7. Daniels C M, Ong S-E, Leung A K L (2015) The Promise of Proteomics for the Study of ADP-Ribosylation. Mol. Cell 58: 911–924
8. Wahlberg E et al. (2012) Family-wide chemical profiling and structural analysis of PARP and tankyrase inhibitors. Nat. Biotechnol. 30: 283–288

9. Peng B, Thorsell A-G, Karlberg T, Schüler H, Yao S Q (2017) Small Molecule Microarray Based Discovery of PARP14 Inhibitors Angew. Chem. Int. Ed Engl. 56: 248–253
10. Ekblad T et al. (2015) Towards small molecule inhibitors of mono-ADP-ribosyltransferases. Eur. J. Med. Chem. 95: 546–551
11. Venkannagari H, Fallarero A, Feijs K L H, Lüscher B, Lehtiö L (2013) Activity-based assay for human mono-ADP-ribosyltransferases ARTD7/PARP15 and ARTD10/PARP10 aimed at screening and profiling inhibitors. Eur. J. Pharm. Sci. 49: 148–156
12. Narwal M, Fallarero A, Vuorela P, Lehtiö L (2012) Homogeneous screening assay for human tankyrase. J. Biomol. Screen. 17: 593–604
13. Putt K S, Hergenrother P J (2004) An enzymatic assay for poly(ADP-ribose) polymerase-1 (PARP-1) via the chemical quantitation of NAD(+): application to the high-throughput screening of small molecules as potential inhibitors. Anal. Biochem. 326: 78–86
14. Assay Guidance Manual. (Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2004).

Figure legends Figure 1. The layout of the assay for a 96-well plate.