Assay technologies facilitating drug discovery for ADP-ribosyl writers, readers and erasers

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Abbreviations:

ADP	adenosine diphosphate
ARC	ankyrin repeat cluster (motif)
ARH	ADP-ribosyl-acceptor hydrolase
ART	ADP-ribosyltransferase
ARTD	diphtheria toxin-like ADP-ribosyltransferase
BRCA	breast cancer (gene)
BRCT	BRCA1 C-terminus
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
DSF	differential scanning fluorimetry
ELISA	enzyme-linked immunosorbent assay
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
HA	hemagglutinin
HRP	horseradish peroxidase

HT	high-throughput
IC_{50}	half-maximal inhibitory concentration
MAR	mono-ADP-ribose
MARylation	mono-ADP-ribosylation
NAD^+	nicotinamide adenine dinucleotide
NADase	NAD-glycohydrolase
PAR	poly-ADP-ribose
PARylation	poly-ADP-ribosylation
PARG	poly-ADP-ribose glycohydrolase
PARP	poly (ADP-ribose) polymerase
PTM	post-translational modification
SAM	sterile α-motif (domain)
SRPK2	serine/threonine-protein kinase
TFMU	4-(trifluoromethyl)umbelliferone
TFMU-IDPr	4-(trifluoromethyl)umbelliferone inosine diphosphate ribose
TNKS1	tankyrase
TR-FRET	time-resolved fluorescence resonance energy transfer

SUMMARY

ADP-ribosylation is a post-translational modification catalyzed by writer enzymes - ADP-ribosyltransferases. The modification is part of many signaling events and can modulate the function and stability of target proteins and often results in the recruitment of reader proteins that bind to the ADP-ribosyl groups. Erasers are integral actors in these signaling events and reverse the modification. ADP-ribosylation can be targeted with therapeutics and many inhibitors against writers exist, with some being in clinical use. Inhibitors against readers and erasers are more sparse and development of these has gained momentum only in recent years. Drug discovery has been hampered by the lack of specific tools, however many significant advances in the methods have recently been reported. We discuss assays used in the field with a focus on methods allowing efficient identification of small molecule inhibitors and profiling against enzyme families. While we focus on human proteins, the methods can be also applied to bacterial toxins and virus encoded erasers that could be targeted to treat infectious diseases in the future.

Introduction

Poly-ADP-ribosylation (PARylation) was discovered in the 1960's and it was later linked to the ADP-ribosyltransferase PARP1, which is capable of post-translationally adding chains of poly-ADP-ribose (PAR) to various polar residues of the protein[1–3] (**Figure 1**). In addition to PARP1, 16 other PARP enzymes are present in humans, most of them catalyzing mono-ADP-ribosylation. Human PARPs are structurally related to diphtheria toxin and thus were classified into the diphtheria toxin-like ADP-ribosyltransferase (ARTD) family[4]. In addition to bacterial toxins and PARPs also other enzyme classes capable of ADP-ribosylation have been discovered like clostridium toxin-like ARTC enzymes[5], some sirtuins[6] as well as some ubiquitin E3 ligases[7]. The modification carried out by writers of ADP-ribosylation is reversible and hydrolyzed by multiple erasers: ADP-ribosylhydrolases of the ARH and macrodomain families[8] (**Figure 1**). Various reader domains and motifs binding to ADP-ribosyl groups facilitating protein localization and protein-protein interactions have been identified[9–12] (**Figure 1**). ADP-ribosylation and its misregulation have been implied in various diseases and the discovery of small molecule inhibitors has been a driving force in development of new methods to study writers, readers and erasers of ADP-ribosylation reviewed here. Efforts to inhibit PARPs in the context of DNA repair have already resulted in new anti-cancer treatments[13] exemplified by the first in 2014 approved PARP inhibitor Olaparib (Lynparza®), which is now in clinical use as a first-line drug either alone or in combination against certain ovarian, fallopian tube and peritoneal cancers[14].

Initially, PARP activity was measured through incorporation of a radioactive ATP into an acid-insoluble material, although the nature of this product of the DNA-dependent enzyme PARP1 was not known at this stage[3]. Later, an assay method to detect PARP protein and PARP activity directly from nitrocellulose blots using radiolabeled substrate [^{32}P]NAD^{+} was

developed by Simonin et al. (1991)[15]. The low-throughput western blot or dot blot methods have been used with other detection methods including biotinylated NAD^+ analogs[16] and streptavidin coupled HRP, PAR H10 or MAR/PAR antibodies[17–19] and various ADP-ribosylation affinity reagents developed recently[20–23].

It is noteworthy that when using purified proteins, it is possible to see the disappearance of a protein band upon addition of NAD^+ with a simultaneous appearance of a high molecular weight smear on a coomassie stained SDS-PAGE. This is a simple and widely available qualitative method of assessing poly-ART activity of enzymes capable of forming PAR chains[24] and can similarly be used to measure PAR hydrolysis activity. Mono-ART activity of enzymes capable of transferring only single ADP-ribose units cannot be detected as a shift on an SDS-PAGE. Some writers and readers can also ADP-ribosylate nucleic acids and remove the modification, respectively. This modification is readily detectable on a gel when small oligonucleotides are used as targets[18,19,25,26].

Interpretation of the modification by mass spectrometry (MS) proves difficult due to the lack of sequence specificity and presence of multiple modification sites per target protein. Toxins however are sequence specific and modify typically only certain residues in the target proteins and therefore MS spectra is easy to interpret[22]. Similarly, MS has been used to detect hydrolysis of MAR and PAR as well as metabolites like *O*-acetyl-ADP-ribose[27,28].

Most of the methods described above are low-throughput and measurement of each sample requires more preparation and analysis time in contrast to if the samples could be measured in a highly parallel fashion. There is interest in efficient assay technologies for ADP-ribosyl writers, readers and erasers in both biochemical research and in drug discovery. In the

following we focus, using selected examples from literature, on assays that can be carried out on multi-well microplates and therefore can be readily applied in a higher throughput setting

Assays described for writers of ADP-ribosylation facilitate drug discovery

Various assay technologies have been described for writers (**Table 1**). In many cases, the assays rely on detection of labelled ADP-ribosyl groups attached to a protein using radiolabeled or a chemical NAD^+ analog as a substrate. Unmodified NAD^+ is more biologically relevant and may also be used, though multi-step conversion of the NAD^+ or hydrolysis products for a detectable readout is required. For example, the accumulation of a side product, nicotinamide, may be detected using coupled enzymatic reactions, decrease in NAD^+ concentration can be quantified by chemical conversion to a detectable product after the enzymatic reaction or ADP-ribosyl groups attached to proteins can be detected using various protein reagents like antibodies or ADP-ribose binding proteins.

Assays for writers using labeled substrate NAD^+ analogs

An early study of inhibition and activation of ADP-ribosylation reactions was conducted by Banasik et al. (1992)[29]. Their method used ^{14}C-labeled NAD^+ as substrate for incorporating radioactivity into ADP-ribosylated products that accumulated as trichloroacetic acid-insoluble material, which was collected on nitrocellulose or glass fiber filters for liquid scintillation counting. This assay was then used for screening a series of compounds for their effect on enzymatic activity, among which were e.g., fatty acids, benzamide derivatives and vitamins. A radiolabeled assay was later developed into a high-throughput (HT) format on microplates by Dillon et al. (2003)[31]. In the assay, activated PARP1, NAD^+ and ^3H-NAD^+ are added to wells of the scintillation counting plate. The resulting incorporation of ^3H into the PAR chains is then detected with scintillation counting after terminating the reaction with acetic acid. The use of

FlashPlate format with wells precoated with scintillant provides signal amplification and easy way of automatable high-throughput measurements tested for 96- and 384-well plates.

A scintillation proximity assay principle has been applied by Cheung & Zhang[30] to measure PARP1 activity. In this assay, a mixture of NAD^+, biotin-NAD^+ and ^{3}H-NAD^+ is used to incorporate two labels to the poly-ADP-ribose polymer. PAR chains are then bound to streptavidin-coated scintillation beads via biotin, through which the radioactive label is brought into proximity of the beads, leading to excitation of the scintillant. This technique removes the need for separation of the substrate and product before the measurement. Suitability for inhibitor screening was also tested and IC_{50} values comparable to the literature for selected compounds were obtained.

A high-throughput assay avoiding radiolabeled substrates in microplate format suitable for inhibitor screening was introduced by Brown and Marala[33]. This colorimetric assay uses biotinylated NAD^+ for detection and measures the auto-ADP-ribosylation of PARP upon activation by nicked DNA. PARP enzyme, or in some applications a target protein, is first immobilized to wells of the plate and this is followed by additions of biotinylated NAD^+ and activating DNA when needed (and the possible inhibitor). Avidin-linked alkaline phosphatase, and its substrate *p*-nitrophenyl phosphate is used for detection. The appropriate washing steps are included in between the additions of assay components. The amount of used and bound biotinylated NAD^+ and subsequently the amount of detected phosphate from the alkaline phosphatase reaction correlates with the PARP activity. Variations of this assay strategy are commonly used for mono- and poly-ARTs and often use luminescent detection with streptavidine coupled horseradish-peroxidase (**Figure 2a**)[24,48–50]. A similar inhibitor screening method for mono-ARTs was developed by Wigle et al.[37]. The principle of this assay is to

immobilize His-tagged mono-ART enzymes onto Ni-NTA plates to create molecular crowding. Consequently, even trace amounts of self-MARylation activity present can be detected using highly sensitive time-resolved lanthanide fluorescence (DELFIA technology): A europium-labeled streptavidin probe binds to biotin-conjugated ADP-ribose, which has been attached by the mono-ART using biotinylated NAD^+ as a substrate (**Figure 2b**). Self-modification assays for all active mono-ART PARP enzymes were developed with concomitant rapid screening of inhibitors in parallel for each of them.

As alternatives to assays using radiolabeled or biotinylated NAD^+, multiple colorimetric or fluorescent substrates have been described. A *p*-nitrophenoxy (*p*NP)-substituted derivative of NAD^+ was developed providing a simple, sensitive, and inexpensive kinetic assay for PARP family of enzymes[32]. The amount of the colored leaving group pNP is determined by measurement of the absorbance at 405 nm. Kinetic parameters K_M, V_{max} and k_{cat} for PARP1, TNKS1 and PARP4 could be calculated using this method and similarly IC_{50} values could be measured for an example inhibitor using these enzymes.

The fluorescent derivative etheno-NAD^+ has been used to determine NAD^+ hydrolase activity of especially NADases and ADP-ribosylating toxins[43,51]. In this assay, the NAD^+ analog nicotinamide 1,N^6-ethenoadenine dinucleotide is used as a substrate to produce a fluorescent product upon hydrolysis. The assay can be used for recombinant enzymes, cells or tissue lysates[42]. Multiple other fluorescent NAD^+ probes have been also recently reported[52,53] and applied to for example measuring NAD^+ dependent ubiquitination of *Legionella pneumophila* SidE proteins[54].

A method to label potential PARP substrate proteins in cells using a clickable NAD^+ was introduced by Jiang et al.[34]. When used in the reaction, clickable NAD^+ analogs provide terminal alkyne groups in the attached ADP-ribosylation, which can be subsequently conjugated with a suitable detectable affinity tag and visualized on SDS-PAGE gels. By using biotin as a label, isolation and identification of such substrate proteins is possible. Assays using target proteins coupled to a microplate and clickable NAD^+ analogs have subsequently been used for the discovery of selective PARP inhibitors[35,36].

Assays using natural β-NAD+

Many enzymes that consume NAD^+ produce nicotinamide as a side product. This can be used to generate equivalent amount of ammonia using a nicotinamidase enzyme allowing quantification. An additional enzymatic or chemical reaction step to generate a detectable signal is however required. When the reaction is coupled with glutamate dehydrogenase it provides a continuous absorbance signal at 340 nm through the conversion of NADPH to $NADP^+$ [55]. If the chemical ortho-phthalaldehyde is added in an end-point assay, it provides a sensitive fluorescent signal (**Figure 2c**)[46].

Putt & Hergenrother developed an assay to monitor PARP1 activity which removed the practical inconvenience of using a radiolabeled compound in the assay[38]. This assay quantitates the remaining NAD^+ after PARP-catalyzed reaction (**Figure 2d**). Acetophenone, KOH and formic acid are used for stopping the enzymatic reaction as well as converting NAD^+ into a fluorophore. With an excitation at 372 nm and registering fluorescent emission at 444 nm, one can detect NAD^+ concentrations down to pM range however with linearity between 1 nM and 100 μM. We modified the assay for tankyrases[40] and for mono-ART PARP

enzymes[39] and demonstrated that it is suitable for screening of inhibitors. This assay has become a standard assay in our laboratory for profiling of inhibitors with PARP enzymes and it can be run on 384-well plates in semi-automated manner. Notably, the assay measures the total NAD^+ consumption: both NADase activity that some enzymes possess as well as ADP-ribosylation.

A nanoparticle PARP1 assay has been evaluated by Wang and coworkers[41]. This assay is based on synthetic peptide-templated copper nanoparticles forming an interaction with phosphate groups, which are plentiful in PAR chains. Array plates are modified by dsDNA followed by addition of NAD^+ and PARP1. ADP-ribosyl groups produced by the action of PARP1 attach to the Copper-loaded nanoparticles. Finally, oxidation by nitric acid liberates nanoparticles into solution, which can be quantitated using voltammetry. Due to the abundance of phosphate groups in PAR chains compared to phosphates in the short activating dsDNA the assay is not adversely affected.

Eskonen et al.[45] developed a time-resolved TR-FRET method for detecting cysteine-specific PTM's such as ADP-ribosylation. In this method, the thiol-reactive AlexaFluor 680 is used as acceptor and a Europium label is used as the donor to produce a FRET signal, which will be prevented upon cysteine ADP-ribosylation. The assay works with a cysteine-containing peptide as the substrate which simplifies its use compared to protein substrates. Dependence on the identification of a suitable peptide for a writer may limit its general applicability.

Recently an assay describing a fluorescently GFP-tagged macrodomain was reported, which also allowed assays using microplates[23]. The detection is based on high affinity protein reagent and this provided a convenient method for measuring inhibitor potencies but requires

the use of a plate washer for automation. Automodified PARP catalytic domains have also been detected in an ELISA-like assay with HA-tagged macrodomains[47]. Alternatively, mono- or poly-ADP-ribose groups can be detected with highly sensitive nanoluciferase linked macrodomains in HT dot blot format[22].

Assays for new drug targets - readers and erasers of ADP-ribosylation

Readers of ADP-ribose belong to multiple different domain families, while the enzymes that completely remove protein ADP-ribosylation all belong to ARH or macrodomain families. Many of these enzymes have been implied as drug targets and significant advances in assay technologies for these proteins have been reported recently. The assays can be divided into activity assays for the erasers and binding assays for the readers (**Table 2**). Moreover, the use of non-hydrolyzable ADP-ribosylated reagents allows setup of universal binding assays for both readers and erasers (**Table 2**). However, these reagents may display altered binding affinities for some proteins compared to native ADP-ribosyl groups[56].

Activity based assays for ADP-ribosylation erasers

Poly-ARTs PARP1-2 and TNKS1-2 can generate PAR chains, while hydrolases limit this signaling event by degrading the polymer. The main eraser of polymers is PARG which degrades the polymer, but cannot remove the proximal ADP-ribose unit from the protein[68]. ARH3 enzyme can degrade PAR slowly, but it is the main eraser of serine linked MAR left attached after PARG activity in the context of DNA repair[8]. As PARG degrades PAR, it generates free ADP-ribose units and these can be quantified chemically (**Figure 3a**)[57]. In this protocol, benzamidine reacts with the reducing sugar moiety of ADP-ribose to produce a fluorescent product, which is stable for 30 min and shows a linear signal for ADP-ribose between 500 nM and 100 μM concentration with a detection limit as low as 50 nM. ADP-ribose

can also be converted enzymatically to AMP that can be subsequently detected [58,59,69,70]. ADP-ribose is released from the MARylated protein by the macrodomain and the assay utilizes a NudF or a Nudt5 ADP-ribose pyrophosphatase that cleave ADP-ribose to AMP and ribose 5-phosphate. The amount of AMP can be measured with a commercial AMP-Glo reagent.

Drown et al.[60] devised assays that use 4-(trifluoromethyl)umbelliferone (TFMB)-based ADP-ribose conjugates to measure PARG and ARH3 activities both in biochemical assays and from whole-cell lysates. In the enzymatic reaction, the TFMU moiety is liberated and can be subsequently detected as it is fluorescent when not conjugated to ADP-ribose. TFMU-ADPr, which has ADP-ribose in the structure, is a general probe for both PARG and ARH3, while TFMU-IDPr, which contains instead an inosine unit, is a specific probe for ARH3.

A HT activity assay for PARG and ARH3 reported by Stowell et al.[61] uses biotinylated NAD^+ to automodify His-tagged PARP1. PARG activity on degrading the PAR chains subsequently causes a loss of the time-resolved FRET signal generated by anti-his antibody coupled to a long-lived fluorescent dye XL665 and biotin-europium cryptate. The assay was used to discover PARG inhibitors in a large screening campaign, which were shown to be selective as the hits did not inhibit ARH3 or PARP1.

As PARG is the main eraser of PAR in the nucleus PAR hydrolysis can be quantified also in cells by immunofluorescence using an anti-PAR antibody and this strategy has also been used in inhibitor screening[71]. PAR antibodies have also been used in enzymatic assays. Affar et al.[72] devised a PARG assay based on dot-blots and Okita et al.[62] further developed it into a high-throughput screen for PARG inhibitors. This assay utilized commercial bovine PARG and poly-ADP-ribose spotted on nitrocellulose or positive charged nylon membranes. Dot-

blots were incubated with anti-PAR antibody and HRP-conjugated anti-mouse IgG for detection with a chemiluminescent substrate.

We have described an assay based on the activity of the erasers, where the α-configuration of a nicotinamide moiety present in α-NAD^+ mimics the protein-linked ADP-ribosyl group[63]. The amount of α-NAD^+ is converted to a fluorophore and quantified using a similar procedure as described for writers using β-NAD^+ described in Table 1.

Hydrolysis of ADP-ribosyl groups from a target protein is measured in an AlphaScreen method described by us[64]. This activity-based assay is suitable for high-throughput screening of inhibitor libraries and as inhibition prevents the loss of the signal. The assay does not have as many false positives as typically found in AlphaScreen assays where compounds have a tendency to quench the signal. The basis for the assay are His-tagged SRPK2 proteins, which are MARylated by PARP10 using a biotinylated NAD^+ as substrate. The resulting double-tagged SRPK2 binds to AlphaScreen streptavidin donor beads and to nickel chelate acceptor beads, which come into favorable distance for singlet oxygen transfer and give luminescence signal after excitation with a laser. Erasers which accept the biotin-ADP-ribose tagged SRPK2 as a substrate lower this enhanced luminescence signal.

Assay technologies designed for readers

Individual PARP macrodomain interactions with mono-ADP-ribosylated ADP-ribosyltransferase domains were studied by Ekblad and coworkers[65] using an AlphaScreen technology. They first assayed the altogether seven His-tagged macrodomains of PARP9, PARP14 and PARP15 with five biotinylated PARPs. This resulted in characterization of

several such interactions ranging in dissociation constants from 0.3 to 2.5 μM by the subsequent surface plasmon resonance analysis. The AlphaScreen assay uses auto-MARylation of the *in vivo* biotinylated catalytic PARP as a basis for the setup. Streptavidin donor beads bind to the modified PARP, while nickel chelate acceptor beads bind to the tested his-tagged macrodomains. As PARP macrodomains do not hydrolyze the modification, the interaction causes an amplified luminescence signal. The assay was subsequently also validated for inhibitor screening.

Another example of the use of AlphaScreen technology is the work by Schuller et al.[56] to screen for inhibitors of macrodomain 2 (MD2) of PARP14. A biotinylated ADP-ribose peptide with a ring-opened ADP-ribose moiety was custom synthesized and used together with the His-tagged macrodomain to bring the donor and acceptor beads into proximity (**Figure 3b**). Due to the non-hydrolysable nature of the ring-opened ADP-ribosyl group used, the assay works for a wide range of catalytically active and inactive macrodomains. Potential inhibitors preventing MD2 from binding the peptide were searched for from a set of 48,000 compounds resulting in discovery of a highly selective inhibitor, demonstrating the usefulness of this assay for a high-throughput setting.

Lambrecht et al.[66] developed a fluorescence polarization assay for measuring the binding of PAR to PARG (**Figure 3c**). Central to the assay is synthesis of a fluorescent ADP-ribose dimer, which was shown to bind to enzymatically inactive human PARG mutants E756N and E755N with K_D values of 83 ± 7 and 208 ± 14 nM, respectively. Although the work was motivated by finding a compound that binds to PARG for crystallographic purposes – and therefore also inactive mutants were used – it was also noted that it functions as a general assay for

determining PAR-binding of proteins and that it could be applied to high-throughput screening efforts.

Recently, we developed a method to measure binding of ADP-ribosyl readers and erasers to mono- or poly-ADP-ribosyl groups[22]. This method is based on enzymatic introduction of a cysteine-linked mono-ADP-ribosyl group to a peptide tag, which can be recombinantly fused to proteins in contrast to chemical synthesis of cysteine MARylated peptides used in low throughput assays[70]. The 9-residue peptide tag is derived from the C-terminal sequence of human G-protein subunit α and is site-specifically MARylated by pertussis toxin subunit S1. Cysteine-ADP-ribosylation is stable against hydrolysis and therefore allows for detection of binding also for binders with otherwise hydrolytic activity[22,70]. This technique was shown to work with different binding technologies such as FRET (**Figure 3d**), BRET, AlphaScreen and bio-layer interferometry. As a proof of concept, we utilized this method to screen for inhibitors of the SARS-CoV-2 macrodomain and identified the drug Suramin as inhibitor.

Ando et al.[67] developed a method to enzymatically label PAR/MAR groups opening opportunities for multiple assay technologies for readers and erasers. They used an enzyme 2'-5'-oligoadenylate synthase 1 (OAS1) to attach dATP or its various labeled analogs to 2' position of either free or protein-bound ADP-ribose units. This way MARylated or PARylated proteins can be labeled and their functions in cells determined and followed with a suitable detection technique, such as autoradiography in case of a ^{32}P-labeled dATP. This method makes it also convenient to analyse PAR chain lengths with e.g., mass spectrometry, or study interaction of certain length of PAR chains with PAR-binding proteins. The technique can be

used to generate also fluorescently labelled PAR chains to allow for example fluorescent polarization assays to be developed.

Universal biophysical assays for drug discovery

Biophysical binding assays like differential scanning fluorimetry (DSF) are typically used as orthogonal assays in hit validation stage, but they can also be used effectively in profiling of inhibitors across the family or even as primary screening tools. The lack of selectivity of known and potential PARP inhibitors was studied by Wahlberg et al.[73] and it was realized that most of the compounds, including clinical candidates, were not selective towards the target as they caused a thermal shift in melting temperature of also other PARP enzymes. DSF is often used in screening of small molecule fragments that provide starting points for medicinal chemistry. This has been used to identify fragments binding to different domains of human tankyrases[74,75].Typically biophysical assays are low throughput and most screening facilities use exclusively microplate assays.

Protein crystallography is a preferred method to experimentally verify inhibitor binding modes, but when crystals amenable for soaking with small molecules are available it can also be used for screening of binding fragments. This is exemplified by the joint effort to discover SARS-CoV-2 macrodomain binding low affinity fragments that can be used as a basis for synthesis of inhibitors in a structure-guided manner[76]. Structures of many writers, readers and erasers have been determined and this has inspired efforts to use structures as a basis for computational virtual screening of large compound libraries. Examples of such hit discovery efforts where compounds have been robustly verified using biochemical and biophysical methods include docking-based virtual screening for inhibitors of PARP14 and PARP15 [77], discovery of first

inhibitors for ADP-ribosylhydrolase ARH3[78] and discovery of inhibitors for SARS-CoV-2 macrodomain[79].

Indirect cell-based screening assays

Specific response in cells has been used also for hit identification for proteins involved in ADP-ribosylation based signaling. The main benefit over the biochemical protein-based assays is that the discovered compounds already have a desired effect in cell context, although it is not ensured that the effect is due to the expected mechanism. The compound potencies do often not correlate with target binding affinities due to e.g. bioavailability differences or off-target-binding and therefore deriving a structure-activity relationship for inhibitor series may not be straightforward. Writers are often toxic to the host cell when overexpressed as they consume NAD^+ and may modify proteins in the cell. This feature has been used in rescue assays to discover inhibitors for a specific target.

Overexpression of PARP10 causes apoptosis-like cell death in human cells and we have used this feature to validate the PARP10 inhibitors that engage with the target in the cell context as inhibitors rescue the cells[80]. PARP10 expression in bacteria is also toxic and bacterial growth inhibition screening may provide a method for screening of PARP10 inhibitors as well as other writers[81]. Yeast cells have been used in a similar context and Yashiroda and co-workers[82] took advantage of tankyrase toxicity to discover flavone as an inhibitor of the writer. Corroborating this finding, we also identified flavones as tankyrase inhibitors in protein-based assay[83]. Yeast *Schizosaccharamyces pombe* was also used in a HT screening of a large compound libraries to identify spiroindoline compounds as tankyrase inhibitors that caused a recovery of a tankyrase expression induced growth defect[84].

Cell lines that are sensitive to inhibitors have been used to validate the mechanism of identified compounds, but this feature could be also used for inhibitor screening. Tankyrase sensitive DLD-1 cells could be used for screening inhibitors[85], but the use of tankyrase inhibitor insensitive cell lines in parallel to filter out generally toxic compound causes complication to the experimental setup. Synthetic lethality of BRCA deficient cells to PARP inhibitors could be used in a similar setting[86].

Typically, cell-based screening assays are utilize reporter assays for certain cell signaling pathways. As such, these assays are not widely available for writers, readers and erasers perhaps due to their recent discoveries. Emerging knowledge of their roles in the cell and the observed toxicity has been taken as advantage in the ADP-ribosylation field. Tankyrases are an exception, as the first inhibitors were discovered through identification of Wnt-signaling inhibitors, which only subsequently were found to target tankyrases[85,87–89].

Protein-protein interaction inhibitors as an alternative way to affect ADP-ribosylation

Inhibitor discoveries have been focused on catalytic or ADP-ribosyl binding domains, but the enzymes involved contain multiple additional domains with different function and they are often involved in macromolecular interactions and bind to e.g. damaged DNA, acceptor proteins to be modified or mediate activation signals in the enzyme. The catalytic domains are conserved within the protein family and targeting of additional domains could provide a route for development of specific inhibitors. Na et al.[90] showed that (±)-gossypol bound to the BRCT domain of PARP1 causing dimerization and subsequent inhibition of PARP1 enzymatic activity. They used a fluorescently-labelled heptapeptide optimized for binding to PARP1 BRCT domain as the probe to establish a HT screening method. DNA damage activated PARPs

have multiple domains and DNA binding causes structural changes. These changes could be blocked by small molecule inhibitors and a FRET- or FP-based assay system could provide a method for screening of inhibitor libraries preventing structural changes and domain-domain interactions, respectively[91,92].

Tankyrases contain ankyrin repeat clusters (ARCs) for binding interaction partners and sterile α-motif (SAM) domains for oligomerization. These scaffolding domains are necessary for the signaling function and therefore could be targeted by small molecules. ARCs have been used for fragment screening using DSF, resulting in low affinity compounds that can be used in further development[74]. We developed a high-throughput screening assay based on FRET, which is applicable for all ARCs as well as SAM domain oligomerization [93]. The assay can be automated and run even on 1536-well plates in a homogenous format, hopefully facilitating inhibitor discovery for the challenging protein-protein interaction targets. Computational approaches may also facilitate the discovery of protein-protein inhibitors as demonstrated by the discovery of an early stage cell active ARC inhibitor through large virtual screening effort[94].

CONCLUSIONS

PARPs were discovered 60 years ago and there are already several drugs targeting writers of ADP-ribosylation. The field has a lot to study regarding the roles and potential targeting of readers, writers and erasers as many of them are involved in diseases, but also in immunity against pathogens. Discovery of specific small molecule inhibitors can facilitate the studies and provide tools for target validation. The lack of tools has especially in recent years been overcome by advanced assay technologies that allow even HT screening of modulator compounds. Assay formats using microplates are available now to follow the reactions

continuously, with robust end-point assays for efficient screening or binding assays providing the convenience in establishing HT screening campaigns. Multiple detection techniques are available and can be applied for new targets or changed to suit the specific needs.

Profiling of inhibitor specificity is a key point in validating the chemical probes and this has been addressed now in the literature describing new tool compounds in contrast to earlier studies. The comparison of readout from different assays and reports is not always straightforward as determined IC_{50} values depend on the assay conditions like concentrations of the substrate or binding partners. Sensitivity remains a concern in multiple technologies described due to e.g., slow activity of the enzymes or unidentified protein targets and their availability. Complementation of the screening assays with biophysical methods allowing determination of affinities, thermodynamics and kinetics of binding allow researchers to overcome these and provide critical information to improve the hit compounds towards optimized leads. The advanced methods reviewed here indicate that there will be a significant number of compounds and drug targets validated in the future for all writers, readers and erasers of ADP-ribosylation.

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CONFLICT OF INTEREST

LL & STS are inventors on a patent application concerning an assay technology for ADP-ribosyl binders.

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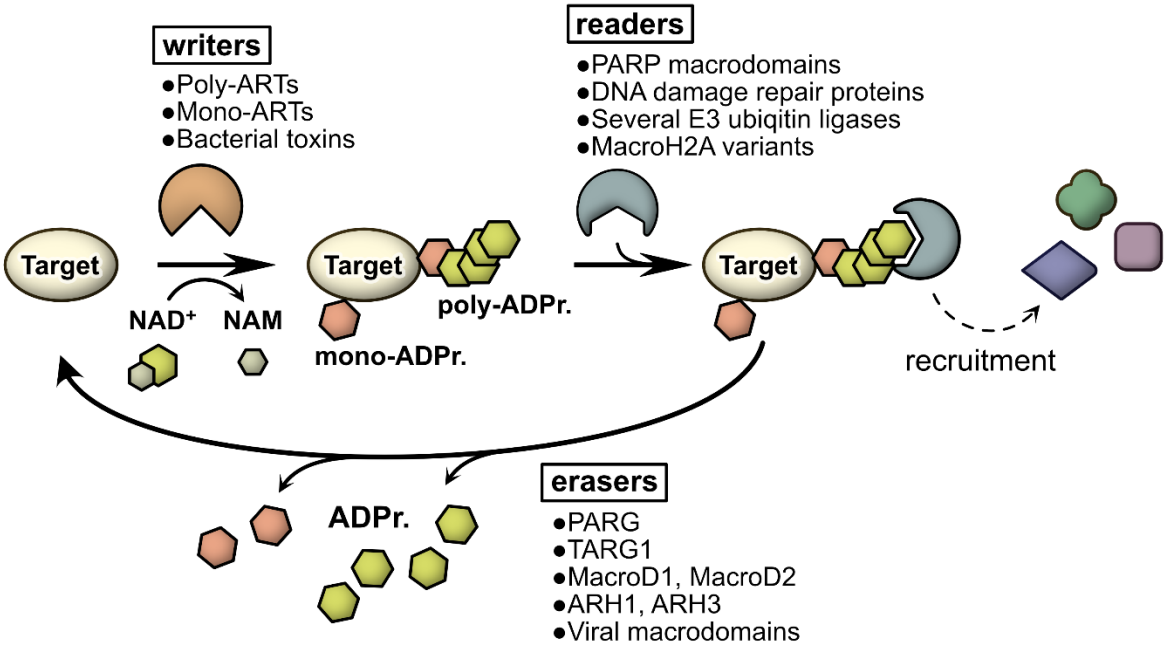

Figure 1. Model for function of ADP-ribosyl writers, readers and erasers. Writers catalyze the transfer of mono- or poly-ADP-ribosyl groups from NAD^+ to target proteins and release nicotinamide (NAM) in the reaction. Readers bind to the ADP-ribosyl groups, facilitating localization and often involving recruitment of other binding partners. Erasers hydrolyze the ADP-ribosyl groups. Erasers of poly-ADP-ribose possess exo- or endoglycosidic activity, while other erasers remove the mono-ADP-ribosyl groups from the target proteins.

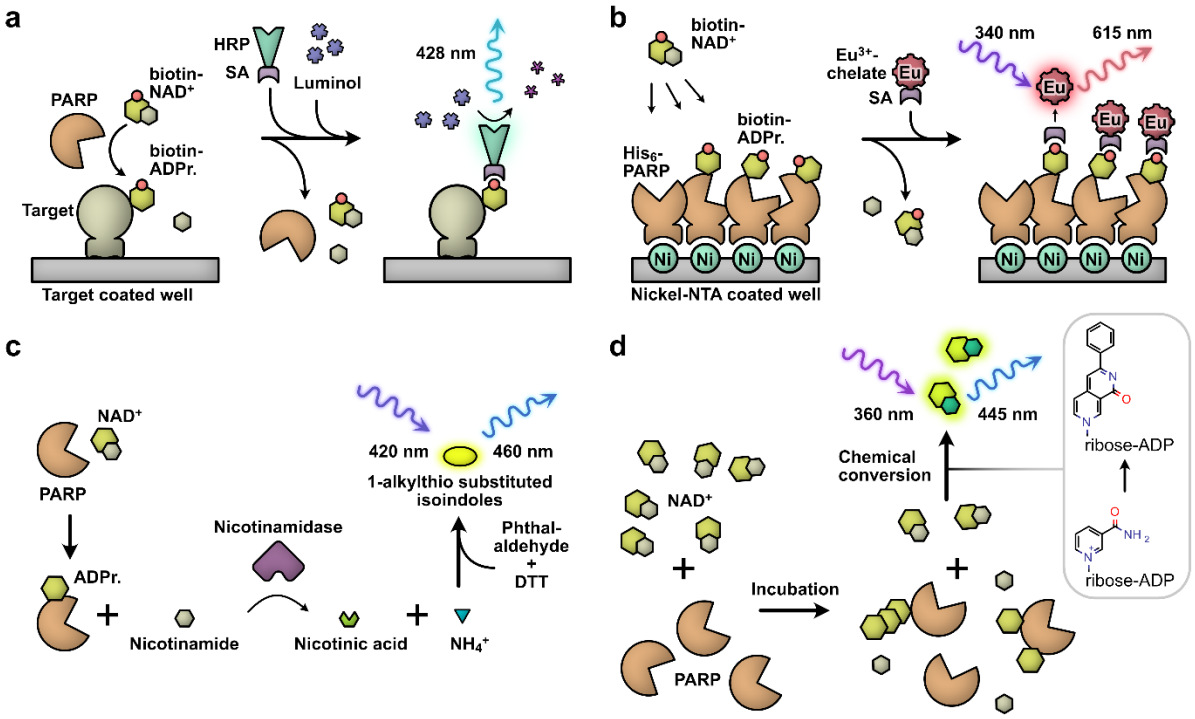

Figure 2. Examples of assays described for writers of ADP-ribosyl groups using various detection technologies. a) ELISA-like target modification assay. Target proteins are immobilized on microplate wells and modified by PARP proteins with biotinylated NAD^+. After washing of the wells, the biotinylated ADP-ribosyl groups are detected with streptavidin (SA) coupled horseradish peroxidase (HRP). b) Forced self-modification assay. His-tagged PARP proteins are immobilized on nickel-NTA coated wells, leading to increased auto-modification activity. Biotinylated NAD^+ is used for the reaction. After washing steps, the resulting biotinylated ADP-ribosyl groups are detected using streptavidin coupled europium chelate using DELFIA technology. c) Nicotinamidase-coupled assay. Nicotinamidase is used to convert nicotinamide released by the reaction of PARP proteins to nicotinic acid and ammonium. The reaction is detected through the chemical conversion of ammonium to fluorescent 1-alkylthio substituted isoindoles. d) NAD^+ quantification assay. PARP proteins use NAD^+ to catalyze the ADP-ribosylation reactions. The remaining NAD^+ is chemically converted to a fluorescent compound that can be detected.

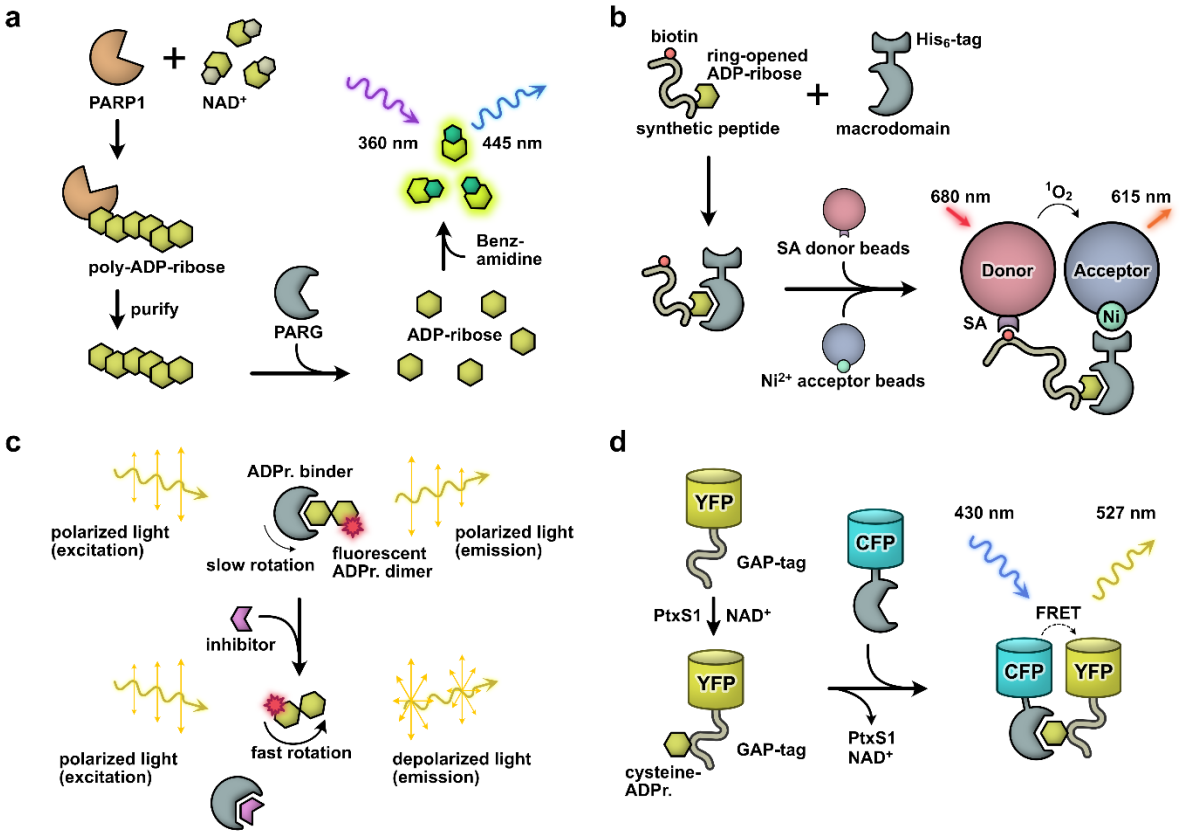

Figure 3. Examples of the assays described for readers and erasers. a) PARG poly-ADP-ribose hydrolysis assay. Poly-ADP-ribose chains are purified from auto-modified PARP1 and hydrolysed by PARG. The resulting ADP-ribose is chemically converted to a fluorescent compound using benzamidine. b) Peptide-based AlphaScreen binding assay. His-tagged macrodomains bind to ring-opened ADP-ribosyl group of a biotin-labeled synthetic peptide. Streptavidin labelled donor beads and nickel-NTA labelled acceptor beads bind to the biotin or His-tag, respectively. Binding is then detected by AlphaScreen technology. c) Fluorescence polarization assay. The assay is based on the binding of a fluorescently labelled ADP-ribose dimer probes by readers or erasers. Inhibitors causing the release of the probes result in the measurable depolarization of the emitted light. d) GAP-tag FRET binding assay. The binding probe is prepared by introducing a single cysteine ADP-ribosyl group by pertussis toxin subunit S1 (PtxS1) to the GAP-tag fused to YFP. After purification of the probe readers or erasers fused to CFP bind to the probe resulting in a FRET signal.

Table 1. Representative assays reported for screening of ADP-ribosyltransferase inhibitors.

Assay technology	Assay format	Features	Ref.
Scintillation counting	liquid scintillation counting	Used for the discovery of first-generation PARP inhibitors; radioactivity reduces practicality and scintillation counting limits throughput; ^{14}C-labeled NAD$^+$	[29]
Scintillation proximity	nitrocellulose membrane	Homogeneous; biotinylated substrate renders separation of substrate and product unnecessary; scintillation proximity assay; radioactivity reduces practicality; can be adapted to microplates; biotin-NAD$^+$ and ^{3}H-NAD$^+$	[30]
^{3}H-labeled NAD$^+$	384-well plates	Versatility by HT screening of inhibitor libraries; specific FlashPlates needed; use of radioactivity reduces practicality; detection technology is not available in all plate readers	[31]
Colorimetry	96-well plates	Colorimetric assay is simple, sensitive and inexpensive but requires custom made *p*-nitrophenoxy-substituted NAD$^+$; can be used for kinetic measurements; homogeneous	[32]
Colorimetry Fluorescence Luminescence	96-well plates	PARP enzyme or target protein (i.e., histones) attached to the wells, avidin-linked alkaline phosphatase or streptavidin-HRP is used for detection; non-homogeneous	[24,33]
Colorimetry Fluorescence Luminescence	96-well plates	Requires a custom NAD$^+$ analog; Streptavidin-HRP for detection; non-homogenous; main difference to other biotin-based colorimetric assay is the use of click chemistry	[34–36]
TRF	384-well plates	His-tagged enzymes coupled to Ni-NTA plates, measures automodification; highly sensitive; requires commercial DELFIA reagents and specific mirrors for the plate reader; non-homogeneous	[37]
Fluorescence UV absorbance	384-well plates	Chemical conversion of NAD$^+$; can be applied for enzymes consuming NAD$^+$; inexpensive, commonly available reagents; protein concentration needed may limit sensitivity; requires a fume hood for chemical conversion; homogeneous	[38–40]
Voltammetry	48-hole glass chip	An electrochemical sensor for generated poly-ADP-ribose; phosphate-guanidine interaction provides more intensity and selectivity; requires copper loaded nanoparticles and instruments accordingly	[41]
Fluorescence	384-well plates	Homogeneous assay; Etheno-NAD$^+$ analog required; NAD$^+$ analog may not be accepted as a substrate by all ART enzymes	[42–44]
TR-FRET	384-well plates	Homogeneous assay; time delay reduces interfering fluorescent compounds; detection limited for cysteine ADP-ribosylation of a specific peptide; requires specific reagents	[45]
Colorimetric Fluorescence	96-well plates	Coupled enzyme assay measures generation of nicotinamide; nicotinamidase needed to generate ammonia; glutamate dehydrogenase (continuous) or ortho-phthaldehyde (sensitive) needed for detection	[46]
Fluorescence	384-well plates	Detection based on fluorescent macrodomain protein produced in *E. coli*; some hydrolysis activity present despite inactivating mutations; non-homogeneous assay	[23]
Colorimetry Fluorescence Luminescence	96-well plates	Binding-assay; similar to ELISA; HA-tagged macrodomain for detection; anti-HA HRP conjugate needed; non-homogeneous	[47]
Luminescence	96-well format dot blots	Nanoluciferase coupled macrodomains produced in *E. coli*; some hydrolysis activity present; no sample separation; quantification requires optimization of the detection buffer; blots prepared with an acoustic dispenser allow high density formats	[22]

Table 2. Representative microplate assays reported for screening of ADP-ribosyl readers and erasers inhibitors.

Assay technology	Assay format	Features	Ref.
Fluorescence	384-well plates	Activity-based; chemical conversion of ADP-ribose; described for PARG enzyme degrading PAR; homogeneous	[57]
Luminescence	384-well plates	Activity-based; enzymatic conversion of ADP-ribose to AMP by NudF or Nudt5 ADP-ribose pyrophosphatase; AMP-Glo reagent for detection; homogeneous	[58,59]
Fluorescence	384-well plates	Activity-based; custom ADP-ribose conjugates needed; common PARG and ARH3 probe and ARH3 specific probe; homogeneous	[60]
TR-FRET	1536-well plates	Activity-based; HTRF; biotinylated NAD^+ and PARP1 automodification; Streptavidin-europium and anti-His MAb-XL665 required; homogeneous	[61]
Luminescence	Dot blots	Activity-based; PAR-antibody needed; described for PARG enzyme degrading PAR; not demonstrated for HT	[62]
Fluorescence	384-well plates	Activity-based; chemical conversion of α-NAD^+; can be applied for enzymes consuming α-NAD^+; inexpensive generally available reagents; protein concentration needed may limit sensitivity; requires a fume hood for chemical conversion; homogeneous	[63]
AlphaScreen	384-well plates	Activity-based; amplified luminescence; enzymes must accept biotin-ADP-ribosyl as a substrate; commercial beads needed; requires a plate reader with an AlphaScreen module; homogeneous	[64]
AlphaScreen	384-well plates	Binding assay; amplified luminescence; PARP catalytic domain sequences in pNIC-Bio3 vector co-expressed with BirA; commercial beads needed; requires a plate reader with an AlphaScreen module; homogeneous	[65]
AlphaScreen	384-well plates	Binding assay; amplified luminescence; synthesized biotinylated peptide with a ring-opened ADP-ribose required; commercial beads needed; requires a plate reader with an AlphaScreen module; homogeneous; applicable to readers and erasers	[56]
Fluorescence polarization	384-well plates	Binding assay; synthesized fluorescent ADP-ribose dimer required; for readers or inactivated eraser mutants; homogeneous	[66]
FRET, multiple techniques	384-well plates	Binding assay; GAP-tag attached to proteins and a cysteine labelled with ADP-ribose using a toxin; applicable to readers and erasers.	[22]
Multiple techniques	Not defined	Enzymatic labelling of MAR and PAR; OAS1 enzyme and labelled dATP needed	[67]