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ARTICLE *in* ANALYTICAL BIOCHEMISTRY · NOVEMBER 2004

Impact Factor: 2.22 · DOI: 10.1016/j.ab.2004.04.032 · Source: PubMed

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A nonradiometric, high-throughput assay for poly(ADP-ribose) glycohydrolase (PARG): application to inhibitor identification and evaluation

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Received 8 April 2004
Available online 3 July 2004

Abstract

The enzyme poly(ADP-ribose) glycohydrolase (PARG) catalyzes the hydrolysis of glycosidic bonds of ADP-ribose polymers, producing monomeric ADP-ribose units. Thus, in conjunction with poly(ADP-ribose) polymerase (PARP), PARG activity regulates the extent of in vivo poly(ADP-ribosyl)ation. Small molecule inhibitors of PARP and PARG have shown considerable promise in cellular models of ischemia–reperfusion injury and oxidative neuronal cell death. However, currently available PARG inhibitors are not ideal due to cell permeability, size, and/or toxicity concerns; therefore, new small molecule inhibitors of this important enzyme are sorely needed. Existing methodologies for in vitro assessment of PARG enzymatic activity do not lend themselves to high-throughput screening applications, as they typically use a radiolabeled substrate and determine product quantities through TLC analysis. This article describes a method whereby the ADP-ribose product of the PARG-catalyzed reaction is converted into a fluorescent dye. This highly sensitive and reproducible method is demonstrated by identifying two known PARG inhibitors in a 384-well plate assay and by subsequently determining IC_{50} values for these compounds. Thus, this high-throughput, nonradioactive PARG assay should find widespread use in experiments directed toward identification of novel PARG inhibitors.

Cells commonly use posttranslational modifications (PTMs)[1] to control enzymatic activity and regulate protein function. These chemical modifications of amino acid side chains come in all shapes and sizes, ranging from the simple addition of a phosphate group to highly complex multistep glycosylations. Such PTMs allow for a rapid cellular response to environmental stimuli and play pivotal roles in signal transduction.

One such PTM is the poly(ADP-ribosyl)ation of a variety of proteins in response to cellular DNA damage. The formation of this polymer is catalyzed by the poly(ADP-ribose) polymerase (PARP) family of enzymes in a NAD^+-dependent manner, and much is known about the biochemistry and cellular biology of the PARP-1 isozyme [1,2]. As with many PTMs, poly(ADP-ribosyl)ation of proteins is transient and readily reversible (Fig. 1). Thus, the cell uses the hydrolytic enzyme poly(ADP-ribose) glycohydrolase (PARG) in both an exoglycosidic and an endoglycosidic manner to catabolize poly(ADP-ribose) polymers (PAR) to monomeric ADP-ribose units (Fig. 2) [3]. PARG is much more active toward long-chain polymers and has a molecular weight of approximately 110 kDa [4,5], although it is easily proteolyzed to 59- and 74-kDa fragments during its purification from mammalian cells [6]. The specific activity of PARG is considerably higher than that of PARP, and as such, the in vivo half-life of PAR is only approximately one minute in DNA-damaged cells [7]. There is evidence to suggest that PARG is largely localized to the cytoplasm with small amounts being translocated into the nucleus, the major site of PARP-1 activity [4]. Interestingly, although there appear

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[1] *Abbreviations used*: PTM, posttranslational modification; PARP, poly(ADP-ribose) polymerase; PARG, poly(ADP-ribose) glycohydrolase; PAR, poly(ADP-ribose) polymer.

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doi:10.1016/j.ab.2004.04.032

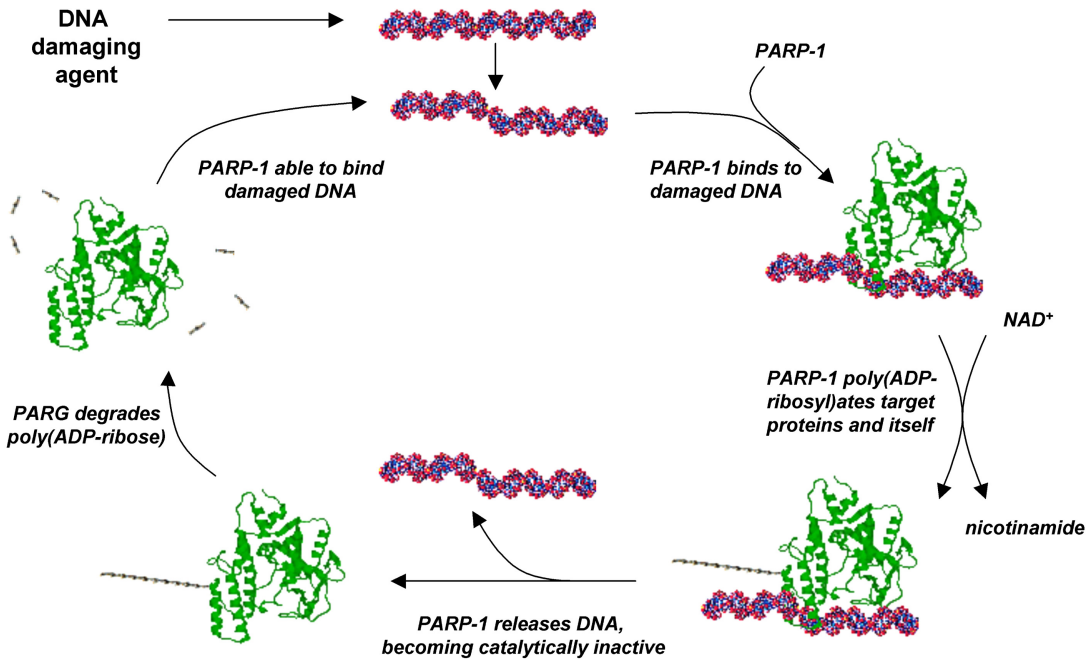

Fig. 1. The cycle of protein poly(ADP-ribosyl)ation. On damage to DNA, PARP-1 binds the DNA, is activated, and catalyzes the formation of poly(ADP-ribose) polymers (PARs) on a variety of protein acceptors, including itself. Electrostatic repulsion between the newly formed polymer and DNA causes the release of PARP-1, thereby inactivating it. The poly(ADP-ribose) glycohydrolase (PARG) enzyme degrades the PAR, thereby allowing for PARP-1 to once again bind to damaged DNA and initiate poly(ADP-ribosyl)ation.

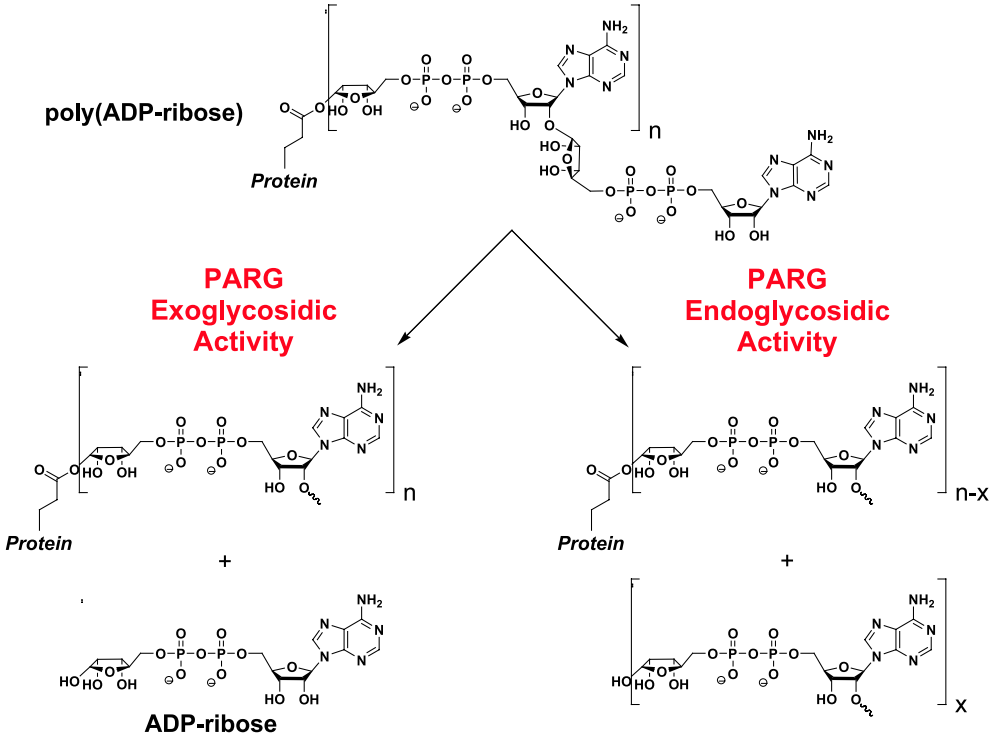

Fig. 2. PARG catabolizes poly(ADP-ribose) polymers in both an exoglycosidic and an endoglycosidic manner. The exoglycosidic activity results in the immediate production of ADP-ribose monomers, whereas the endoglycosidic mode of action gives smaller ADP-ribose polymers; these polymers can be converted into ADP-ribose through further exoglycosidic processing.

to be at least 18 PARP isozymes [8], only one human PARG has been identified thus far and its sequence is highly conserved among mammals [3].

Small-molecule inhibitors of PARP and PARG have shown considerable medicinal promise. There are a myriad of inhibitors of PARP-1, and their in vivo role to

both potentiate the effect of anticancer agents and inhibit degenerative-type cell death has been well documented [9–11]. In contrast, very few inhibitors of PARG have been described [12–16]. The most commonly used PARG inhibitors are from the tannin class of natural products [17,18]; however, these molecules are modestly potent, high-molecular weight species that are far from ideal from a drug development perspective. ADP-ribose analogues [13,14,19] and DNA intercalators [16,20] also give reasonable PARG inhibition, but the former compounds lack cell permeability and the latter have considerable toxicity. Although an ideal small-molecule inhibitor of PARG does not yet exist, it is apparent that PARG inhibitors have tremendous potential as pharmacological agents as they have been shown to reduce ischemia injury [21], suppress tumor virus gene expression [17], and prevent oxidative neuronal cell death [22,23]. This role of PARG in apoptosis was underscored by the recent finding that PARG is a substrate for caspase-3, although the functional consequences of this cleavage are not yet clear [6].

We are actively engaged in an effort to identify novel PARG inhibitors to use as tools both for the further elucidation of PARG function in vivo and for the purpose of exploring their therapeutic potential. Unfortunately, PARG inhibitor development is limited by existing enzymatic activity assays that are neither convenient nor high-throughput. The standard assay for measuring PARG activity and for evaluation of putative PARG inhibitors uses ^{32}P-labeled PAR as the substrate and relies on TLC to separate the monomeric product [24]. Although this assay has been useful for obtaining IC_{50} values for individual compounds [13–15,19] and for investigating PARG enzymology [12,18], it is obviously not suitable for high-throughput screening of combinatorial libraries or compound collections. Other assays using radiolabeled PAR suffer from similar limitations [25]. This article describes a nonradiometric PARG assay method based on the conversion of the ADP-ribose product into a fluorescent molecule. We show that this quantitation of ADP-ribose is sensitive and accurate and that it can be used both to determine IC_{50} values for individual PARG inhibitors and as a general high-throughput screening method in 96- or 384-well plates. As such, this represents the first truly high-throughput PARG activity assay.

Materials and methods

Reagents

PARG isolated from bovine thymus was purchased from Biomol (Plymouth Meeting, PA). ADP-HPD was purchased from Calbiochem (San Diego, CA). Ethacridine (6,9-diamino-2-ethoxyacridine) and ADP-ribose were purchased from Sigma (St. Louis, MO). XL1-Blue *Escherichia coli* was purchased from Stratagene (La Jolla, CA). A plasmid containing the full-length human PARP-1 gene (pSD6.3) [26] was the kind gift of Serge Desnoyers (University of Laval, Quebec, Canada). The 96- and 384-well fluorescence plates and all other reagents were purchased from Fisher (Chicago, IL). The PARP assay buffer consisted of 100 mM Tris, 10 mM $MgCl_2$, 8 mM DTT, 10% (v/v) glycerol, and 10% (v/v) ethanol, pH 8.0. The PARG assay buffer consisted of 50 mM KCl and 50 mM KH_2PO_4, pH 7.2. PAR buffer A consisted of 250 mM ammonium acetate, 6 M guanidinium HCl, and 10 mM EDTA, pH 9.0. PAR buffer B consisted of 1 M ammonium acetate and 1 mM EDTA, pH 9.0.

PARP-1 expression and purification

An overnight culture of *E. coli* XL1-Blue (1 ml) harboring the plasmid pSD6.3 was used to inoculate 1 L of LB media. The bacteria were grown at 37 °C to an $OD_{600} = 0.4$, at which point protein expression was induced by addition of IPTG to 250 μM, and the cells were grown an additional 4 h. Cells were harvested by centrifugation, resuspended in 15 ml of PBS, and passed twice through a French press. The cell lysate was then spun at 15,000*g* for 30 min. The supernatant was decanted, and ammonium sulfate was added to the supernatant to 55% saturation and incubated at 4 °C for 1 h with stirring. The solution was then spun at 18,000*g* for 30 min at 4 °C. The supernatant was decanted, and ammonium sulfate was then added to the supernatant to 70% saturation and incubated at 4 °C for 1 h with stirring. The solution was again spun at 18,000*g*, and this time the supernatant was discarded. The pellet was resuspended in 2 ml of PARP assay buffer and dialyzed (3 × 1:1000) in PARP assay buffer. The lysate was concentrated using a 100-kDa centricon centrifugal filter (Millipore, Bedford, MA). The total protein concentration was determined using the Bradford assay, calibrated with BSA.

Determination of PARP activity

The activity of PARP-1 was determined as described previously [27]. Briefly, PARP-1 was incubated in the presence of 1 μM NAD^+ and activated DNA (this DNA was isolated from HL-60 cells and subsequently cleaved nonspecifically with restriction enzymes) for 20 min in a 96-well plate at a final volume of 50 μl. After the incubation, 20 μl of a 2 M KOH solution and 20 μl of a 20% (v/v) acetophenone (in EtOH) were added, and the plate was incubated for 10 min at 4 °C. Then 90 μl of 88% formic acid was added, and the plate was incubated for 5 min in an oven set at 110 °C. The fluorescence was measured at an excitation of 360 nm and an emission of 445 nm. The specific activity was found to be approximately 500 units/mg (1 unit is defined

as the amount of enzyme required to cleave 1 nmol of NAD^+ in 1 min at 25 °C).

Production of PAR

PARP-1 (20 units) was added to 900 µl of PARP assay buffer containing 1 mM NAD^+ and 22.5 µg activated DNA (this DNA was isolated from HL-60 cells and subsequently cleaved nonspecifically with restriction enzymes). This reaction was run at 37 °C for 30 min and stopped by the addition of 300 µl of cold 100% trichloroacetic acid. The proteins thus precipitated were collected by centrifugation at 18,000*g* for 30 min at 4 °C. After removal of the supernatant, 1 ml of 1 M NaOH and 100 mM EDTA were added to the pellet and incubated for 2 h at 37 °C to cleave the PAR from the PARP protein acceptor. Then 9 ml of PAR buffer A was added to the PAR, and this solution was immediately column purified (see below) or frozen for future use.

Purification of PAR

A PROSEP-PB (Millipore) phenyl boronate column was preequilibrated by applying 10 ml of PAR buffer A, followed by 15 ml of water and then another 10 ml of PAR buffer A. PAR was bound to the column by applying the PAR solution (in PAR buffer A) generated from the PARP reaction above to the column. The flow-through was then reapplied to the column to ensure that all of the PAR was bound. The column was then washed with 25 ml of PAR buffer A, followed by 10 ml of PAR buffer B. The bound PAR was eluted with 4 ml of water in 1-ml fractions. Fractions containing PAR were then pooled and concentrated. The concentration, measured as monomer, was determined by measuring the absorbance at 258 nM. PAR has an extinction coefficient of 13,500 cm^{-1} at 258 nm [28].

ADP-ribose calibration curve

A 0.5- to 10-µM ADP-ribose solution (50 µl) in PARG assay buffer was added in quadruplicate to the wells of a Nunc 384-well fluorescent plate. Then 5 µl of an aqueous 8.5 M KOH solution and 5 µl of an aqueous 850 mM benzamidine solution were added to each well, resulting in a final concentration of 708 mM KOH and 70.8 mM benzamidine. The plate was then incubated for 10 min in an oven set at 110 °C. The plate was allowed to cool and then read on a Criterion Analyst AD (Molecular Devices, Sunnyvale, CA) with an excitation of 360 nm and an emission of 445 nm (see exact settings below).

Fluorescence plate reader settings

Fluorescence was measured on a Criterion Analyst AD using a 360 ± 15-nm excitation filter, a 445 ± 15-nm emission filter, and a 400-nm cutoff dichroic mirror. The fluorophore was excited using a 1000-W flash lamp for 1.0×10^5 µs with 25 reads performed per well.

Generation of fluorescence spectra

PARG assay buffer (55 µl) and an aqueous 8.5 M KOH solution (5 µl) were added as the blank to a fluorescent 96-well plate. To determine the background fluorescence of benzamidine in the reaction, 50 µl of PARG assay buffer, 5 µl of an aqueous 8.5 M KOH solution, and 5 µl of an 850 mM aqueous solution of benzamidine were added to the 96-well plate. Similarly, to determine the fluorescence of ADP-ribose, 55 µl of a 100 µM ADP-ribose solution in PARG assay buffer and 5 µl of an aqueous 8.5 M KOH solution were added. Finally, to generate a spectrum of the fluorescent molecule produced, 50 µl of a 100 µM ADP-ribose solution in PARG assay buffer, 5 µl of an aqueous 8.5 M KOH solution, and 5 µl of an 850 mM aqueous solution of benzamidine were added to the 96-well plate. The 96-well plate was then heated for 10 min in an oven set at 110 °C. The excitation and emission spectra were then measured on a Gemini fluorescent plate reader (Molecular Devices).

Determination of IC_{50} values for PARG inhibitors

To determine IC_{50} values of the PARG inhibitors, 39 µl of a 25.6 µM solution of PAR in PARG assay buffer and 1 µl of the inhibitors at varying concentrations (in PARP assay buffer) were added into the wells of a 384-well plate. The reaction was initiated by adding 10 µl of PARG at a concentration of 1 ng/ml (in PARG assay buffer), bringing the final concentration to 200 pg/ml PARG and 20 µM PAR with varying concentrations of inhibitors in a total volume of 50 µl. The plate was incubated for 20 min at room temperature. The amount of free ADP-ribose hemiacetal was determined exactly as described for the calibration curve above. The average value of control wells containing only PAR was set as 0% PARG activity, whereas the average value of control wells containing PAR and PARG (but no inhibitor) was set as 100% PARG activity. Any intrinsic fluorescence exhibited by the PARG inhibitors was subtracted out, and the values obtained from the various concentrations of inhibitors were converted into a percentage of PARG activity and plotted. All data points were determined in triplicate, and no more that 7.5% of total PAR was cleaved during the course of the PARG assay.

Library screen

Stock solutions of 88 compounds were prepared, each at a concentration of 12.5 mM in DMSO. Contained within this compound collection were the known PARG inhibitors ethacridine and ADP-HPD. Then 50 µl of

each of these 88 stock solutions was placed into the wells of a 96-well plate (the parent plate). To test the library for PARG inhibition, 40 μl of a 25.6-μM solution of PAR in PARG assay buffer was added to the wells of a Nunc flat-bottom 384-well fluorescent plate (the experimental plate). Subsequently, 0.2 μl of the test compounds was transferred from the parent plate to the experimental plate using a pin transfer apparatus (V & P Scientific, San Diego, CA). To initiate the reactions, 10 μl of PARG at a concentration of 1 ng/ml (in PARG assay buffer) was added. This brought the final concentration of PARG to 0.2 ng/ml, the final concentration of PAR to 20 μM, and the final concentration of compounds to 50 μM. The plate was incubated for 20 min at room temperature, and the amount of monomeric ADP-ribose hemiacetal was then determined exactly as described above for the calibration curve. Within the experimental plate, this assay was performed in duplicate. To control for any potential fluorescence inherent in the compounds under evaluation, wells containing only the compound (at 50 μM) in PARG assay buffer (total volume of 50 μl) were analyzed alongside the experimental samples, in duplicate, within the same 384-well plate. The value of any intrinsic fluorescence detected in the compounds was subtracted out during the final analysis. No more that 7.5% of total PAR was cleaved during the course of the PARG assay. The average value of control wells containing only PAR was set as 100% PARG inhibition, whereas the average value of control wells containing PAR and PARG was set as 0% PARG inhibition. Any intrinsic fluorescence exhibited by the test compounds was subtracted out, and the values obtained were converted into a percentage of PARG activity and plotted.

Data analysis

Graphs were analyzed using Table Curve 2D software. PAR standard curves were fitted with a least squares linear model, and inhibitor curves were fitted with a logistic dose–response curve.

Results and discussion

Through the action of PARG, the PAR polymer is catabolized into ADP-ribose monomer units. Thus, the immediate products of the PARG-catalyzed reaction are the ADP-ribose hemiacetal and the remaining polymer chain. Given that the standard PARG enzymatic assay employs a radioactive substrate and a TLC-based quantitation, we speculated that analysis of the ADP-ribose-reducing sugar might offer a practical, convenient, sensitive, nonradioactive, and high-throughput alternative for assessment of PARG activity. Analysis of hemiacetals and aldehydes through spectroscopic techniques is a venerable method for quantitation of reducing sugars. For example, the Tollens "silver mirror" test and methods employing 3,5-dinitrosalicylic acid are familiar to the student of general chemistry.

Preliminary studies were conducted on the ability to accurately and sensitively quantitate ADP-ribose through a variety of transformations. For instance, reducing sugars can be converted into colored products using Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) [29,30], tetrazolium blue [31], PAHBAH (4-hydroxybenzhydrazide) [32–34], and ferricyanide [35]. Unfortunately, in our trials, the lack of sensitivity of all of these colorimetric methods precluded their use in the PARG enzymatic assay. Reducing sugars can also be converted into fluorescent derivatives; thus, ethylenediamine sulfate [36], resorcinol [37], and 2-cyanoacetamide [38,39] all were evaluated in this context. However, these three reagents either required prolonged heating times or gave inconsistent results in the quantitation of ADP-ribose.

Aromatic amidines such as benzamidine are known to react with hemiacetals to give fluorescent products that enable the quantitation of low micromolar concentrations of simple reducing sugars [40–42]. Ultimately, benzamidine was used to rapidly, consistently, and sensitively convert ADP-ribose into a fluorophore; therefore, we have devised a general PARG assay method based on this reaction. The fluorophore produced through the reaction of ADP-ribose with benzamidine was found to be stable for approximately 30 min and has the excitation and emission spectra shown in Fig. 3; the limited stability of the fluorophore precluded its precise chemical characterization. The fluorescence is linear with ADP-ribose in the concentration range between 500 nM and 100 μM (see calibration curve in Fig. 4) and can be used to detect ADP-ribose at concentrations as low as

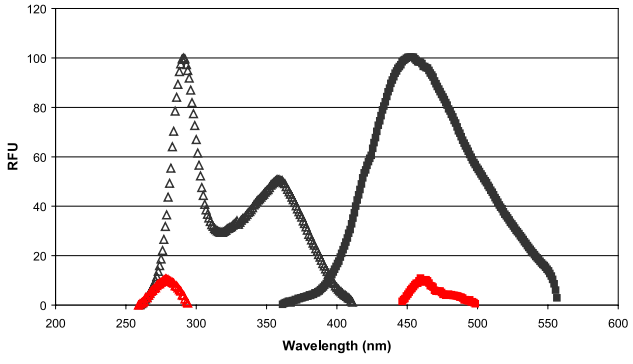

Fig. 3. Excitation (open triangles) and emission (closed squares) spectra of the fluorophore generated from the reaction of benzamidine and ADP-ribose. The excitation and emission spectra were measured on a Gemini fluorescent plate reader (Molecular Devices). The fluorescence excitation (open red triangles) and emission (closed red squares) spectra for ADP-ribose itself are also shown for comparison. Benzamidine did not show any significant fluorescence over 250 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

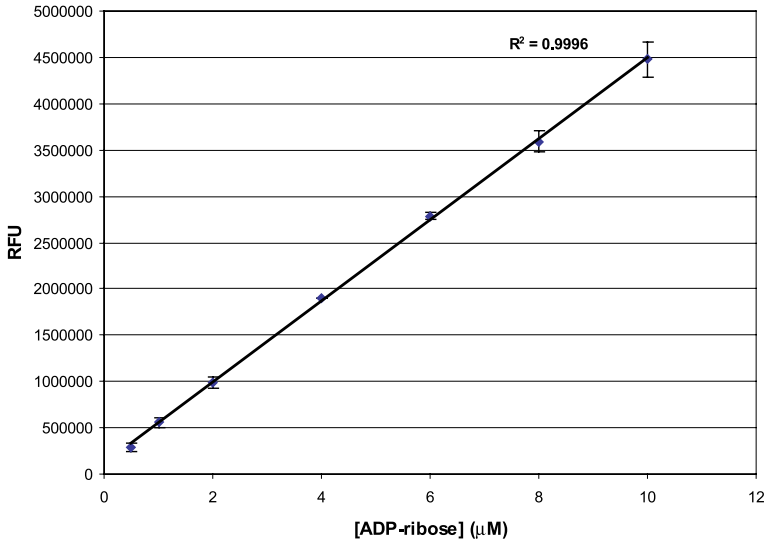

Fig. 4. Calibration curve for the PARG assay. A range of ADP-ribose concentrations was added to the wells of a 384-well plate in quadruplicate. After addition of KOH and benzamidine followed by heating, the fluorescence in the wells was quantitated through an excitation at 360 nm and an emission at 445 nm.

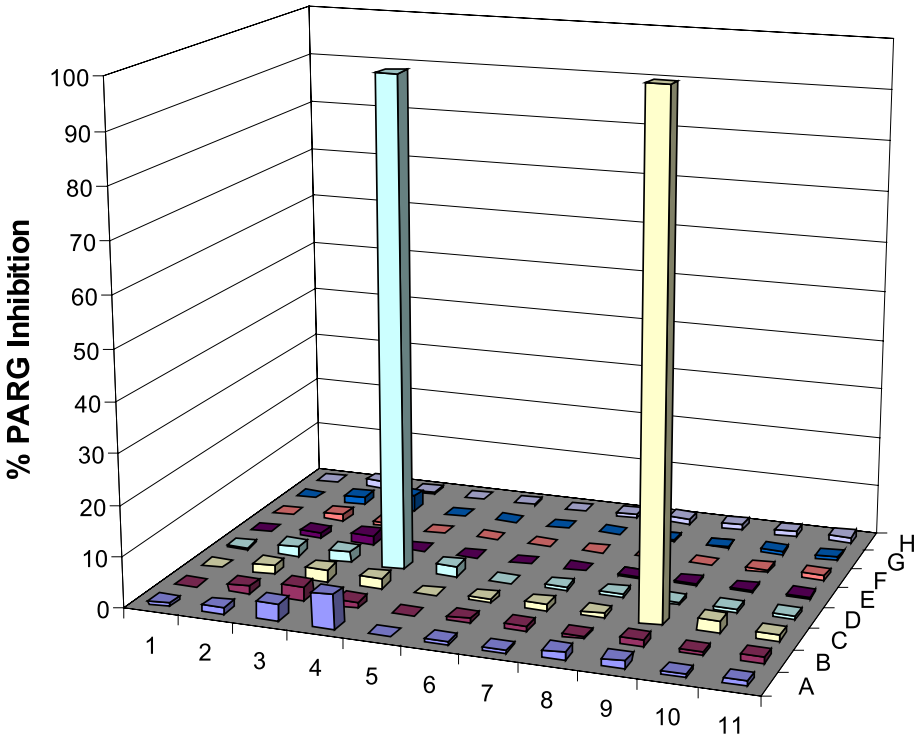

Fig. 5. Screen of a compound collection spiked with known PARG inhibitors. Stock solutions of 88 compounds were prepared, and within this compound collection were the known PARG inhibitors ethacridine and ADP-HPD. To test the library for PARG inhibition, PAR was added to the wells of a 384-well plate. The test compounds were transferred from the parent plate to the experimental plate using a pin transfer apparatus. PARG was added to initiate the reactions, and the final concentrations were 0.2 ng/ml PARG, 20 μM PAR, and 50 μM of each compound. The plate was incubated for 20 min at room temperature, and the amount of monomeric ADP-ribose hemiacetal was then determined as described. The average value of control wells containing only PAR was set as 100% PARG inhibition, whereas the average value of control wells containing PAR and PARG was set as 0% PARG inhibition. ADP-HPD and ethacridine were in positions 9C and 4D, respectively.

50 nM. ADP-ribose alone has very little intrinsic fluorescence (Fig. 3), and benzamidine has no significant fluorescence at the concentration employed in the assay.

The recent cloning and expression of PARG has set the stage for major advances in understanding and controlling levels of poly(ADP-ribosyl)ation. It is apparent from several studies that inhibitors of PARG could be of great value in the treatment of a variety of medical conditions, including ischemia–reperfusion injury and neurodegeneration. However, all currently available

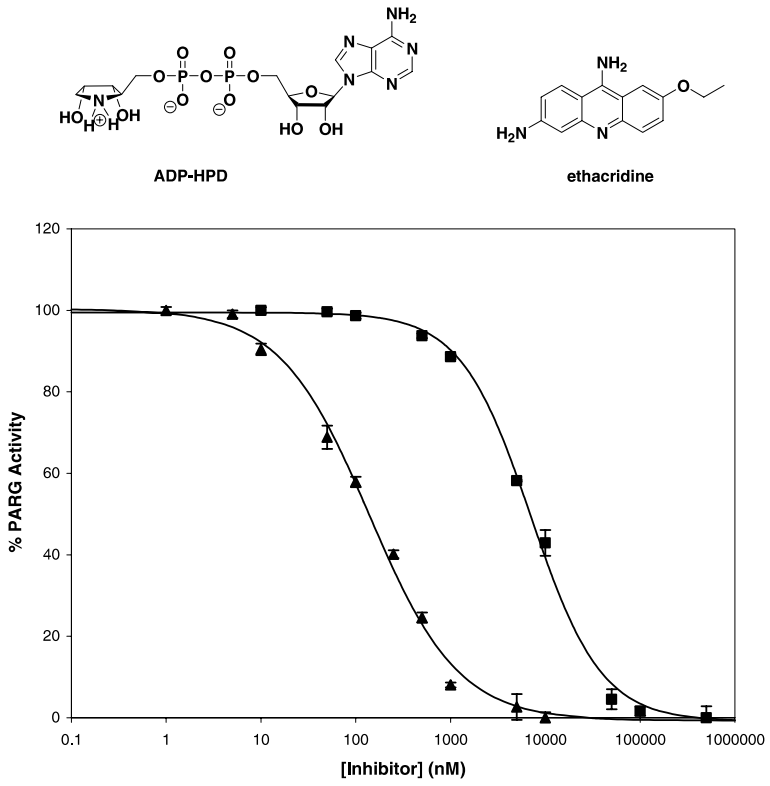

Fig. 6. Determination of IC_{50} values with the fluorescent-based PARG assay. The inhibitors at varying concentrations were added to the wells of a 384-well plate. The reaction was initiated by adding PARG, bringing the final concentration to 200 pg/ml PARG and 20 μM PAR with varying concentrations of inhibitors in a total volume of 50 μl. The plate was incubated for 20 min at room temperature. The amount of free ADP-ribose hemiacetal was determined as described. The values obtained from the various concentrations of inhibitors were converted into a percentage of PARG activity and plotted. All data points were determined in triplicate, and no more than 7.5% of total PAR was cleaved during the course of the PARG assay.

PARG inhibitors possess serious toxicity, size, and/or cell permeability flaws that severely limit their widespread use. Unfortunately, current PARG assay methods do not allow for a rapid evaluation of potential inhibitors but instead rely on tedious TLC analysis.

Thus, we used our new ADP-ribose quantitation method to rapidly assess 88 compounds for their ability to inhibit the enzymatic reaction of PARG. This assay was performed in duplicate in a 384-well plate. Of the 88 compounds, 2 were the known PARG inhibitors ethacridine and ADP-HPD and the other 86 were from a small in-house compound collection. As is apparent from Fig. 5, the known PARG inhibitors were easily identified in this screen. Subsequently, the assay was used to determine precise IC_{50} values for both ethacridine and ADP-HPD. From analysis of the curves in Fig. 6, an IC_{50} value of 136 nM was calculated for ADP-HPD and an IC_{50} value of 7.2 μM was determined for ethacridine. These numbers are in excellent agreement with the literature values of 120 nM for ADP-HPD [14] and approximately 7 μM for ethacridine [16,20]. Obviously, putative inhibitors that contain aldehydes or reducing sugars will have a large background in this assay; therefore, another assay should be used to quantitate the PARG inhibitory properties of such molecules.

If desired, this assay can also be employed in a kinetic mode whereby the enzymatic reaction is quenched at different time points. In such experiments, we have found the fluorescence obtained after benzamidine quantitation to be linear with respect to time. As expected, the amount of enzyme used dictates the length of time needed for the PARG processing of PAR to give a sufficient fluorescent signal. For example, three times less PARG can be used in the assay if the reaction is simply left to proceed three times as long. The protocol described herein has been explicitly optimized to strike a balance between the amount of PARG used and the time required for the assay.

The glycohydrolase activity of PARG is due to both endoglycosidic and exoglycosidic mechanisms [43,44]. It is believed that PARG first reduces PAR to smaller sized polymer units in the endoglycosidic mode, concomitant with exoglycosidic activity that liberates some monomeric ADP-ribose. After this initial phase, the exoglycosidic mechanism dominates and free ADP-ribose is generated. It should be noted that the standard TLC-based PARG assay method quantitates only the monomeric ADP-ribose; thus, the immediate products from endoglycosidic activity are not detected. Because the assay described in this article relies on the conversion of

the reducing sugar to a fluorophore, any liberated hemiacetal (including those polymeric products of endoglycoside cleavage) will be detected.

The ability to rapidly screen for PARG inhibition will now allow combinatorial libraries and small-molecule collections to be easily evaluated for this important biological activity. PARG can be expressed and purified in *E. coli* [5,45], and the PAR substrate can be made on a large scale using a simple in vitro reaction with PARP and NAD^+ (see Materials and methods); therefore, the assay described herein removes the final barrier to high-throughput screening for PARG inhibitors.

In summary, we have developed a novel, sensitive, inexpensive, convenient, and high-throughput method for the detection of PARG activity. We have shown that this assay can be applied to inhibitor screens and subsequent determination of IC_{50} values for hit compounds. Given the demonstrated medicinal consequences of PARG inhibition and the scarcity of known inhibitors, this assay should find widespread use in the search for superior modulators of PARG activity.

Acknowledgments

We thank Serge Desnoyers (University of Laval, Quebec, Canada) for the gift of the plasmid pSD6.3. This work was supported by the University of Illinois and a grant from the National Science Foundation (NSF-CAREER Award to Paul J. Hergenrother).

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