

# An enzymatic assay for poly(ADP-ribose) polymerase-1 (PARP-1) via the chemical quantitation of NAD<sup>+</sup>: application to the high-throughput screening of small molecules as potential inhibitors<sup>☆</sup>

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## Abstract

The enzyme poly(adenosine 5'-diphosphate (ADP)-ribose) polymerase (PARP-1) catalyzes the formation of (ADP)-ribose polymers on a variety of protein acceptors in a NAD<sup>+</sup>-dependent manner. While PARP-1 is activated by DNA damage and plays a critical role in cellular survival mechanisms, its overactivation leads to a depletion of NAD<sup>+</sup>/ATP energy stores and ultimately to necrotic cell death. Due to this dual role of PARP in the cell, small-molecule inhibitors of the PARP family of enzymes have been widely investigated for use as potentiators of anticancer therapies and as inhibitors of neurodegeneration and ischemic injuries. Unfortunately, standard assays for PARP inhibition are not optimal for the high-throughput screening of compound collections or combinatorial libraries. Described herein is a highly sensitive, inexpensive, and operationally simple assay for the rapid assessment of PARP activity that relies on the conversion of NAD<sup>+</sup> into a highly fluorescent compound. We demonstrate that this assay can readily detect PARP inhibitors in a high-throughput screen using 384-well plates. In addition, the assay can be used to determine IC<sub>50</sub> values for PARP inhibitors that have a range of inhibitory properties. As existing PARP assays utilize specialized reagents such as radiolabeled/biotinylated NAD<sup>+</sup> or antibodies to poly(ADP-ribose), the chemical quantitation method described herein offers a highly sensitive and convenient alternative for rapidly screening compound collections for PARP inhibition.

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The mammalian cell possesses elaborate systems that regulate both life and death. Upon insult through chemical, biological, or other means, the cell activates multiple mechanisms in an attempt to survive. For example, in response to DNA damage the enzyme poly(ADP-ribose) polymerase (PARP-1)<sup>1</sup> binds to DNA and utilizes the ADP-ribose moiety of NAD<sup>+</sup> to poly(ADP-ribosylate) a variety of proteins (including itself, in an automodification process) on glutamate residues (Fig. 1) [1,2]. The ADP-ribosyl units are added as both linear and branched chains, and more than 100 monomers can

be appended in forming the poly(ADP-ribose) polymer. The formation of these polymers dramatically alters the properties of the acceptor proteins, and this modification initiates the DNA damage control and repair processes. The automodification of PARP-1 with the ribosyl polymer eventually leads to dissociation of the enzyme from the DNA. This poly(ADP-ribosylation) is transient, as the modified proteins are rapidly restored to their original states by a poly(ADP-ribose) glycohydrolase [3]. The DNA repair role for PARP-1 in response to alkylating agents and ionizing radiation is supported by studies in PARP-1-deficient cell lines [4] and organisms [5]. Therefore, PARP-1 enzymatic activity appears to have a cytoprotective role within the cell, and inhibition of PARP-1 with small molecules is known to increase the sensitivity of cells to cytotoxic agents [6–8].

However, it is also apparent that PARP-1 enzymatic activity is a significant contributor to necrotic cell death.

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<sup>1</sup> Abbreviations used: PARP, poly(ADP-ribose) polymerase; NAD<sup>+</sup>, β-nicotinamide adenine dinucleotide; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone; NMN, N-methylnicotinamide; DMSO, dimethyl sulfoxide.

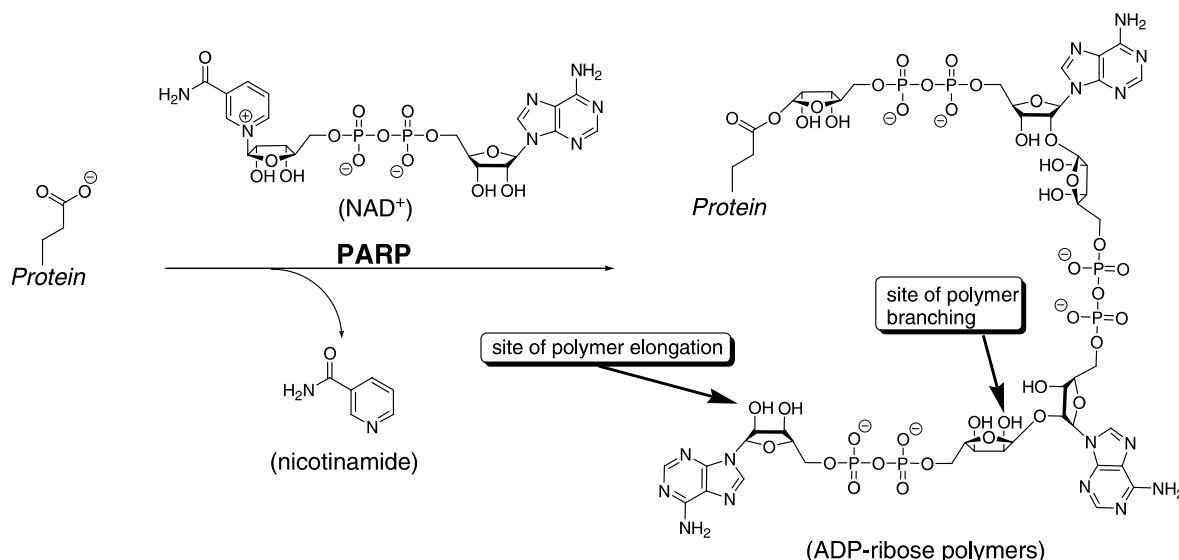


Fig. 1. Reaction catalyzed by PARP. The ADP-ribose moiety of  $\text{NAD}^+$  is appended to carboxylate residues of target proteins. Additional ADP-ribose units can be added to the 2'-OH (elongation) or the 2''-OH (branching).

In this scenario, it is believed that PARP-1 activity leads to the depletion of  $\text{NAD}^+$  and ATP energy stores inside the cell, ultimately leading to necrosis [9–11]. In this case, PARP-1 activity has a cytotoxic role within the cell, and inhibitors of PARP-1 have been shown to prevent necrotic cell death in a wide variety of in vivo models of ischemic and reactive oxygen species-induced injury [10–13].

These contrasting and seemingly paradoxical roles for PARP-1 (Fig. 2) have made it the subject of considerable biochemical and medicinal interest. To date, at least six members of the PARP family of enzymes have been identified. PARP-1, a 1014-amino acid, 113-kDa nuclear enzyme, was the first PARP discovered and remains the most well-characterized member of the family. During apoptosis PARP-1 is cleaved after a DEVD sequence by caspase-3 into p89 and p24 fragments; this cleavage separates the DNA binding domain from the catalytic domain and thus renders the enzyme catalytically inactive [14–17]. Caspase-mediated PARP-1 cleavage during apoptosis is believed to prevent futile cycles of DNA damage and repair, while helping to save cellular ATP/ $\text{NAD}^+$  for the apoptotic death program. The other five members of the PARP family, PARP-2, PARP-3, VPARP, tankyrase I, and tankyrase II, have varying degrees of sequence homology with PARP-1, but at this point their in vivo functions remain poorly understood [18].

X-ray crystal structures of the catalytic domain of PARP-1 have been solved in the presence of small-molecule inhibitors, which appear to occupy the nicotinamide binding site of the enzyme [19,20]. Because PARP inhibitors have been shown to both enhance the effects of cytotoxic agents and avert necrotic cell death,

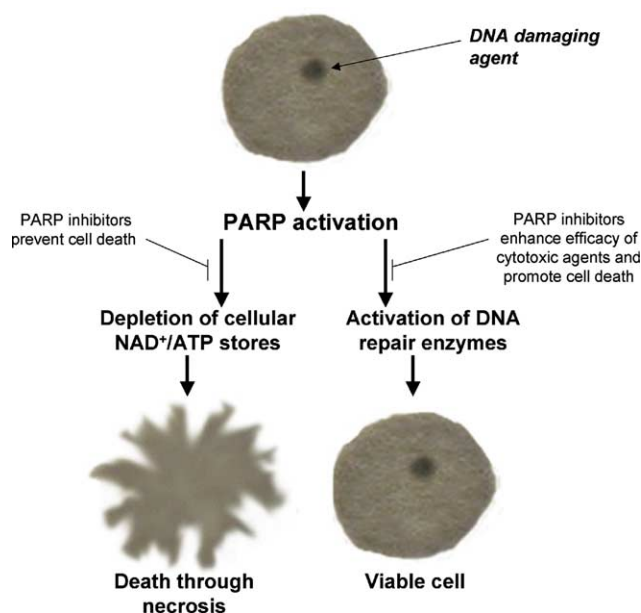


Fig. 2. Dual nature of PARP. PARP activation can lead to DNA repair and recovery of normal cellular function. Alternatively, it can cause energy deprivation and cell death. PARP inhibitors have been shown to inhibit both recovery and cell death, depending on the circumstances.

these compounds have been touted as putative treatments for disease states ranging from cancers to degenerative disorders [8,21–23], and various screens have identified small molecules that inhibit PARP-1 with reasonable potencies [24]. The standard assay for monitoring PARP activity involves the use of radiolabeled  $\text{NAD}^+$  [13,24,25], although there is also an assay that uses an antibody to ADP-ribose [26] and two recently

described assays utilizing biotinylated NAD<sup>+</sup> [27,28]. Unfortunately, the use of radioactive and/or specialized reagents (such as biotinylated NAD<sup>+</sup> and antibodies) in these assays can make their costs prohibitive when screening large compound collections for PARP inhibition. In addition, these assays often involve either the separation of ADP-ribose polymer product from the NAD<sup>+</sup> substrate or the addition of specialized streptavidin-conjugated scintillation proximity assay beads.

Thus, in searching for an inexpensive, convenient method for identifying PARP inhibitors from large compound collections, we did not find an existing assay that fit our needs. We now report an operationally simple protocol for the identification of PARP inhibitors that relies on the highly sensitive chemical quantitation of NAD<sup>+</sup>. We have adapted this assay to 96- and 384-well plates for the rapid screening of small molecules for PARP inhibition.

## Materials and methods

### Reagents

High-specific-activity PARP-1 and activated DNA were purchased from Trevigen (Gaithersburg, MD). Acetophenone, benzamide, 6(5H)-phenanthridinone, and 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Amino-1,8-naphthalimide was purchased from Calbiochem (San Diego, CA). 96-Well fluorescence plates, 96-well UV-visible transparent plates, 88% formic acid, and all other reagents were purchased from Fisher (Chicago, IL). PARP assay buffer consisted of 50 mM Tris, 2 mM MgCl<sub>2</sub> at pH 8.0. The solutions of aqueous 2 M KOH and 20% acetophenone (in EtOH) were stable for at least 1 month at room temperature in the dark. Stock solutions (50 mM) of 6(5H)-phenanthridinone, 4-amino-1,8-naphthalimide, and DPQ were prepared in DMSO. A 5 mM stock solution of benzamide was prepared in the PARP assay buffer.

### NAD<sup>+</sup> calibration curve

Fifty microliters of 1 to 100 nM (for fluorescence) or 1 to 100 μM (for absorbance) NAD<sup>+</sup> solutions in PARP assay buffer was added in quadruplicate to the wells of a Nunc 96-well round-bottomed fluorescence plate, followed by the addition of 20 μL of an aqueous 2 M KOH solution and 20 μL of a 20% acetophenone (in EtOH) solution. Higher concentrations of acetophenone can be used to increase the fluorescent signal; however, most plastic microtiter plates will dissolve if higher concentrations are used. The plate was then incubated at 4 °C for 10 min. Then 90 μL of 88% formic acid was added,

resulting in a final concentration of 222 mM KOH, 2.2% acetophenone, 44% formic acid, and varying concentrations of NAD<sup>+</sup>. The plate was then incubated in an oven set at 110 °C for 5 min. 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). To quantitate NAD<sup>+</sup> via absorbance, the reaction mixture was transferred from the fluorescence plate into a Falcon UV-Vis transparent 96-well plate and read on a SpectraMax Plus (Molecular Devices) at 378 nm. The above reaction cannot be carried out directly in the UV-Vis transparent plate because the plate is not resistant to heating.

### 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 continuous lamp for 1.6 × 10<sup>6</sup> μs with five reads performed per well.

### Library screen

Stock solutions of 88 compounds were prepared, each at a concentration of 1.25 mM in DMSO. Contained within this compound collection were the known PARP inhibitors 4-amino-1,8-naphthalimide, benzamide, DPQ, and 6(5H)-phenanthridinone. Fifty microliters 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 PARP inhibition, 20 μL of NAD<sup>+</sup> (at a concentration of 1.25 μM in PARP assay buffer) was added to the wells of a Costar flat-bottomed 384-well fluorescent plate. Subsequently, 0.2 μL of the test compounds was transferred from the parent plate into the experimental plate using a pin transfer apparatus (V & P Scientific, San Diego, CA). To initiate the reactions, 5 μL of a solution containing both PARP-1 (at 12.5 μg/mL) and nicked DNA (at 75 μg/mL) in PARP assay buffer was added, bringing the final concentration of PARP-1 to 2.5 μg/mL, that of DNA to 15 μg/mL, that of NAD<sup>+</sup> to 1 μM, and that of compound to 10 μM. The plate was incubated at room temperature for 20 min and the amount of NAD<sup>+</sup> present was then determined by the addition of 10 μL of an aqueous 2 M KOH solution and 10 μL of a 20% acetophenone (in EtOH) solution. The plate was then incubated at 4 °C for 10 min. Then 45 μL of 88% formic acid was added, yielding final concentrations of 222 mM KOH, 2.2% acetophenone, and 44% formic acid. The plate was incubated in an oven set at 110 °C for 5 min. The plate was allowed to cool and then read on a Criterion Analyst AD (Molecular

Devices) with an excitation of 360 nm and an emission of 445 nm. 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 10  $\mu$ M) and NAD<sup>+</sup> (1  $\mu$ M) in PARP assay buffer (total volume of 25  $\mu$ L) 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 (see below).

Other control wells were also analyzed; these contained either (1) NAD<sup>+</sup> with 0.2  $\mu$ L of DMSO transferred into them or (2) NAD<sup>+</sup> and PARP-1 with 0.2  $\mu$ L of DMSO transferred into them. The amount of PARP-1 inhibition was determined by first subtracting out any intrinsic fluorescence of the test compounds. Second, the average values of the control wells containing only NAD<sup>+</sup> were set as 100% inhibition, while the control wells containing NAD<sup>+</sup> and PARP-1 were set as 0% inhibition. Third, the values of the test compounds were converted to a percentage of PARP-1 inhibition and plotted.

#### Determination of IC<sub>50</sub> values for PARP inhibitors

To determine IC<sub>50</sub> values of the PARP inhibitors, 20  $\mu$ L of a 250 nM solution of NAD<sup>+</sup> in PARP assay buffer, 10  $\mu$ L of activated DNA at a concentration of 50  $\mu$ g/mL (in PARP assay buffer), and 10  $\mu$ L of the inhibitors at varying concentrations (in PARP assay buffer) were added into the wells of a 96-well plate. The reaction was initiated by adding 10  $\mu$ L of PARP-1 at a concentration of 10  $\mu$ g/mL (in PARP assay buffer), bringing the final concentration to 2  $\mu$ g/mL PARP-1, 10  $\mu$ g/mL DNA, and 100 nM NAD<sup>+</sup> with varying concentrations of inhibitors in a total volume of 50  $\mu$ L. The plate was incubated for 15 min at room temperature and the amount of NAD<sup>+</sup> was then determined by the fluorescence method as described above for the calibration curve. The average value of control wells containing only NAD<sup>+</sup> was set as 0% PARP activity, while the average value of control wells containing NAD<sup>+</sup> and PARP (but no inhibitor) was set as 100% PARP activity. Any intrinsic fluorescence exhibited by the PARP inhibitors was subtracted out and the values obtained from the various concentrations of inhibitors were converted to a percentage of PARP activity and plotted. All data points in Fig. 7 were determined in quadruplicate.

#### Data analysis

Graphs were analyzed using Table Curve 2D. NAD<sup>+</sup> standard curves were fitted with a least squares linear model and inhibitor curves were fitted with a logistic dose response curve.

## Results and discussion

To develop a nonradiometric method of measuring PARP activity, we sought protocols in which the reaction by-product (nicotinamide) or the starting material (NAD<sup>+</sup>) could be conveniently quantitated via either a UV/visible or a spectrofluorometric readout. It is well recognized that ideal enzyme assays quantitate one of the products of a given enzymatic transformation; however, multiple enzyme assays have been developed for systems in which product formation cannot be conveniently monitored. In these cases, it is possible to assess enzymatic activity based on the amount of substrate remaining after the enzymatic reaction. Thus, in the case of the reaction catalyzed by PARP, quantitation of the NAD<sup>+</sup> remaining after the PARP-catalyzed reaction appeared as an attractive alternative to the standard PARP assay in which activity is determined by the amount of radioactivity transferred to PARP through the use of <sup>3</sup>H-NAD<sup>+</sup>.

It is known that *N*-alkylpyridinium compounds can be converted to fluorescent molecules through reaction with a ketone followed by heating in excess acid [29]. These methods have been developed largely to quantitate *N*-methylnicotinamide (NMN), a nicotinic acid metabolite [30–33]. More recently, an optimized protocol for NMN determination using acetophenone as the ketone and acidifying with formic acid has been reported [34–36]. To adapt this method to the determination of PARP enzymatic activity, we chose to quantitate the amount of NAD<sup>+</sup> remaining after the PARP reaction by treating the reaction mixture with acetophenone in base, followed by incubation at 110 °C with formic acid (Fig. 3). Indeed, we found that the product of this reaction had a strong fluorescence emission at 444 nm. We have assigned the structure of this product as compound **1** (Fig. 3), based on analogy with other reactions of *N*-alkyl pyridines with ketones and taking into account mass spectral data that we have obtained. The excitation and emission spectra of **1** are shown in Fig. 4.

A calibration curve using various amounts of NAD<sup>+</sup> was produced for the reaction in Fig. 3. As shown in Fig. 5, this method is highly sensitive and can detect NAD<sup>+</sup> solutions as low as 10 pM, and the fluorescence is linear over the range of 1 nM to 100  $\mu$ M. Importantly, controls indicate that nicotinamide (the by-product in the PARP reaction) does not react under the assay conditions, and NAD<sup>+</sup> and nicotinamide have no significant intrinsic fluorescence emission at 444 nm.

As there is substantial interest in the development of small-molecule inhibitors of the PARP enzymes, the chemical quantitation of NAD<sup>+</sup> was applied to PARP-1 in the context of inhibitor evaluation. Specifically, we were interested in utilizing this new method to screen large collections of compounds for inhibition of the

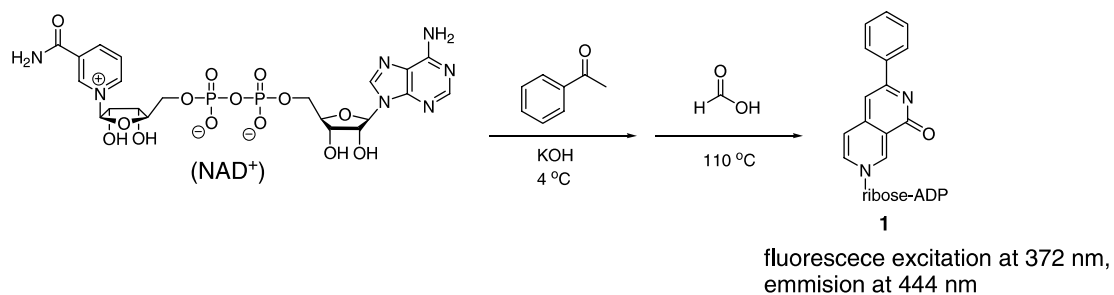


Fig. 3. Quantitation of NAD<sup>+</sup>. NAD<sup>+</sup> is converted into the highly fluorescent compound **1** upon reaction with acetophenone.

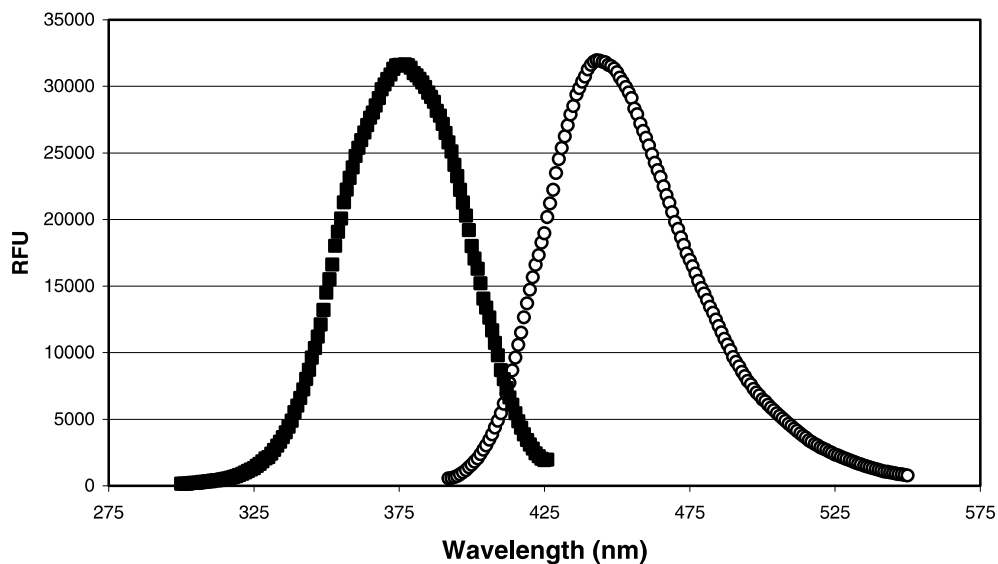


Fig. 4. Fluorescence excitation (closed squares) and emission (open circles) spectra of compound **1**.

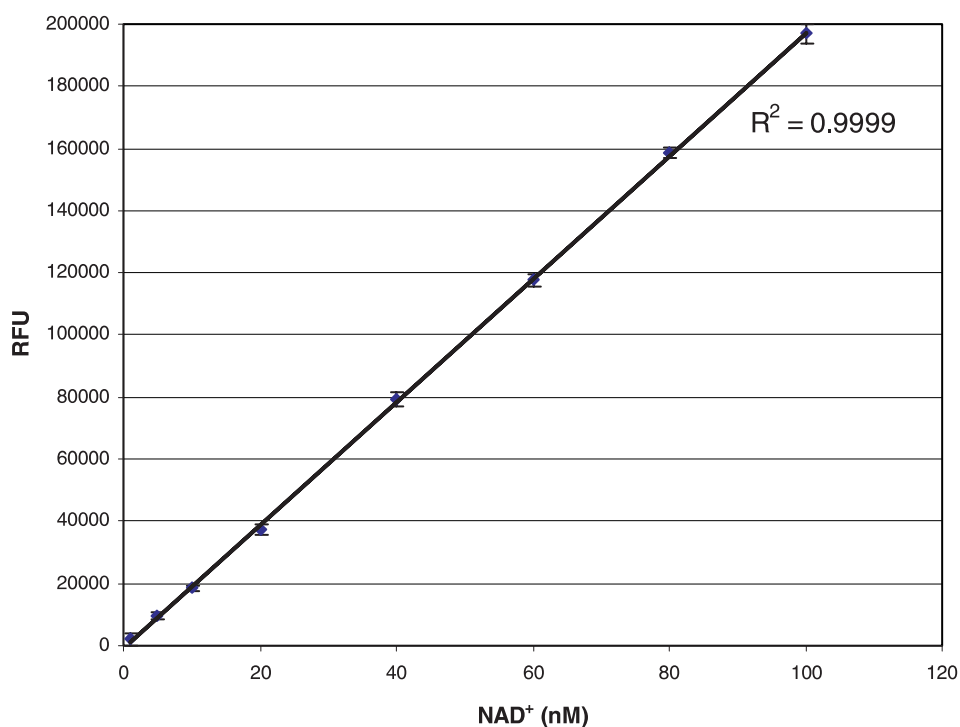


Fig. 5. NAD<sup>+</sup> calibration curve. The quantitation of NAD<sup>+</sup> was found to be linear up to concentrations of 100  $\mu$ M.



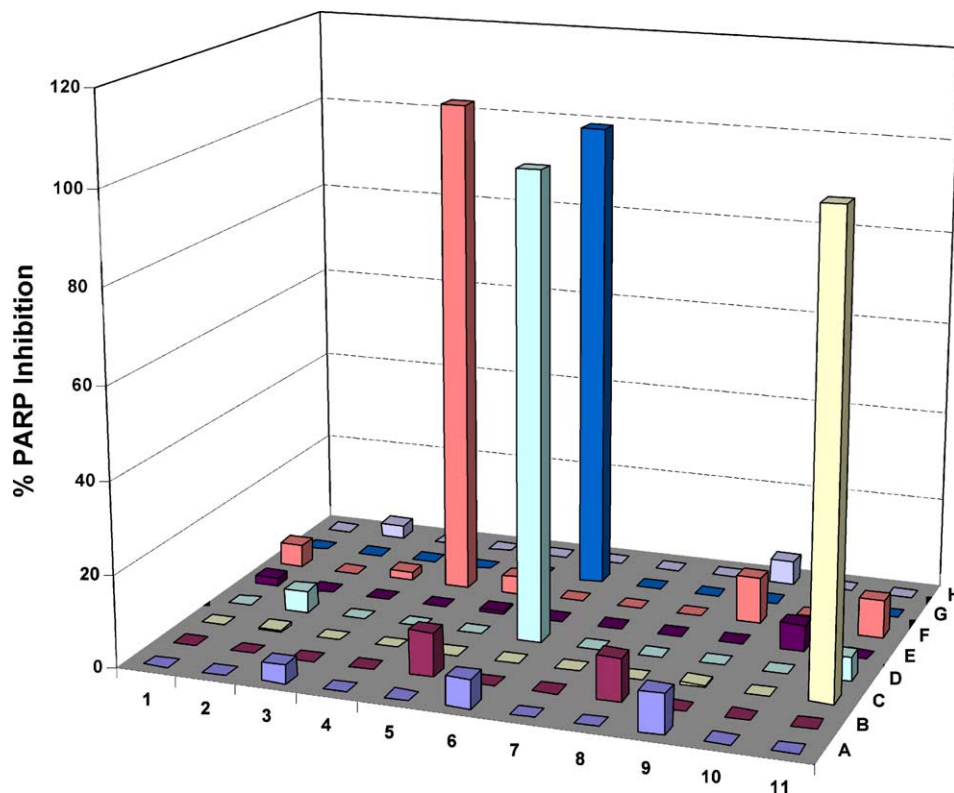


Fig. 6. Screening compounds for PARP-1 inhibition in a 384-well plate. Eighty-eight compounds were placed in the wells of a 384-well plate at 10  $\mu$ M, each in quadruplicate. Four of the compounds were known PARP-1 inhibitors. Activated DNA and NAD<sup>+</sup> were added, followed by PARP-1. After 20 min, the amount of NAD<sup>+</sup> remaining was quantitated as described in the text, and each compound was assigned a percentage PARP inhibition based on the controls. Wells 4F, 6D, 6G, and 11C correspond to 6(5H)-phenanthridinone, 4-amino-1,8-naphthalimide, DPQ, and benzamide, respectively. The average standard deviation for these samples was 5.3%.

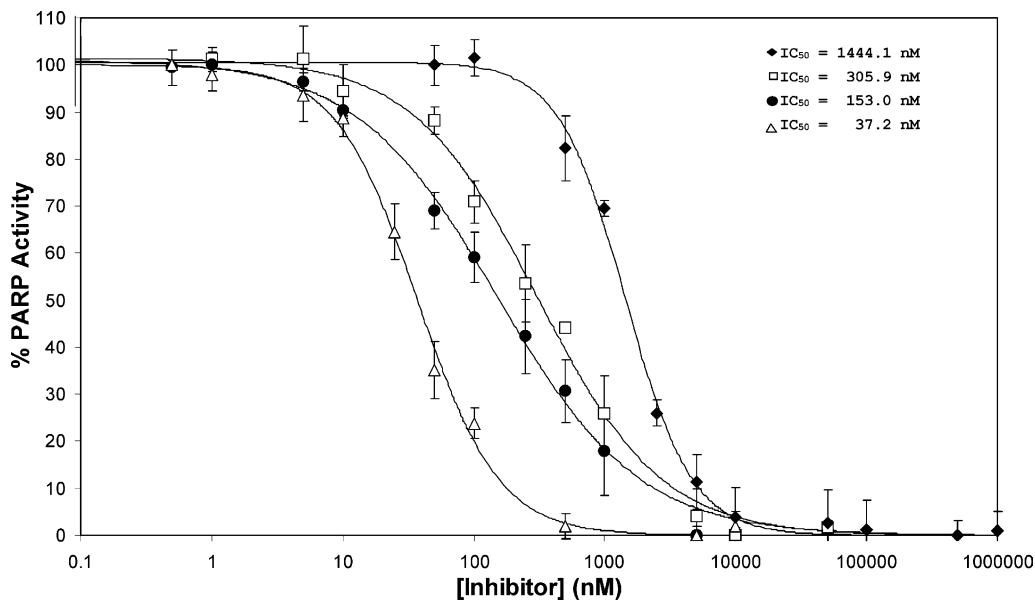


Fig. 7. Evaluation of PARP-1 inhibitors with the NAD<sup>+</sup> quantitation assay. Dose response curves were generated for benzamide (closed diamonds), 6(5H)-phenanthridinone (open squares), 4-amino-1,8-naphthalimide (closed circles), and DPQ (open triangles) with  $r^2$  values of 0.997, 0.995, 0.996, and 0.997, respectively.

Table 1. Excellent correlation was obtained between this  $\text{NAD}^+$  quantitation method and the literature values [28,37–39]. Obviously, putative inhibitors that contain *N*-alkyl pyridinium moieties would give a large background in this assay and should be evaluated via another method. However, such compounds are typically not potent PARP inhibitors [22].

A few additional comments are in order about the  $\text{IC}_{50}$  determination. To ensure that initial rate remained constant in this assay, the PARP reactions were allowed to proceed only to 8–15% completion. Even at this low level of conversion, the signal-to-noise level was more than adequate, and the assay was found to be highly reproducible; each data point in Fig. 7 was determined in quadruplicate, and the error bars are shown on the graph. In addition, only very low levels of  $\text{NAD}^+$  were utilized in this assay (100 nM). This low concentration, coupled with the minimal volume required by the 96- or 384-well format, allowed extremely small quantities of PARP to be utilized. Although PARP-1 is commercially available, it is quite expensive, and thus it is very useful to minimize the quantity of PARP-1 consumed, especially if large compound collections are to be screened or multiple  $\text{IC}_{50}$  values are to be determined. However,  $\text{NAD}^+$  concentrations up to 100  $\mu\text{M}$  can also be used if one desires a direct correlation with other assay methods that typically utilize micromolar amounts of  $\text{NAD}^+$  substrate.

We have thus developed a highly sensitive, convenient, simple assay for the evaluation of inhibitors of a medically important class of enzymes, the PARP family. Although we have tested this method only with PARP-1, this assay should be readily applicable to the other members of the PARP class as it relies on the quantitation of  $\text{NAD}^+$ . Because it does not require radiation or specialized reagents (such as biotinylated  $\text{NAD}^+$ ), this assay is an excellent alternative to methods known in the literature for the evaluation of PARP inhibitors. In addition, if laboratories interested in adopting this method do not have access to fluorescence plate readers, compound **1** also has a characteristic UV signal with a maximum absorbance at 378 nm that can be quantitated, albeit with a reduction in sensitivity relative to the fluorescence (the absorbance of **1** at 378 nm is linear from 1 to 100  $\mu\text{M}$ ). Application of this method to the identification of novel inhibitors of the PARP family of enzymes will be reported in due course.

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