



Homogeneous Fluorescent Assay

for High Throughput Screening of PARP Inhibitors

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Abstract

Poly (ADP-ribose) polymerase 1 (PARP-1) plays an active role in various DNA repair processes by binding to DNA single-strand breaks and catalyzing the formation of PAR polymers on itself and other proteins resulting in NAD⁺ depletion. PARP inhibitors are potential chemotherapeutic agents for cancer treatment since they sensitize cells to DNA damaging agents. Standard assays for PARP inhibition are not homogeneous or convenient for screening compound libraries. A highly sensitive fluorescent screening assay for screening PARP-1 inhibitors in a purified system is presented.* This one hour endpoint assay is performed in two successive steps requiring only the addition of reaction components. A PARP reaction is first performed followed by a detection step. Inhibitors are identified by an increase in fluorescent signal when PARP mediated NAD⁺ depletion is inhibited. The level of NAD⁺ is coupled to a cycling assay involving alcohol dehydrogenase and diaphorase. Each time NAD⁺ cycles through these coupled reactions, a molecule of highly fluorescent resorufin is generated. Alcohol dehydrogenase reduces NAD⁺ to NADH, while the diaphorase cycles NADH back to NAD⁺ with the production of a highly fluorescent resorufin molecule from the non-fluorescent substrate, resazurin. The number of cycles can be controlled by the time of incubation to adjust assay sensitivity as needed and the reaction terminated by the addition of a stop solution. In addition, the assay can be used to determine IC₅₀ values for PARP inhibitors and detect as low as 10% inhibition of PARP-1 activity.

Introduction

While it has never been shown that Poly ADP ribose polymerase 1(PARP-1) directly acts as a DNA repair protein, it is well documented that PARP-1 binds to single strand breaks and subsequently recruits other proteins in the base excision repair pathway (BER) to the site of damage. After binding to the DNA strand breaks, PARP-1 synthesizes negatively charged poly-(ADP-ribose) (PAR) polymers from NAD⁺ that covalently modify numerous proteins including histones, polymerases, topoisomerases and PARP-1 itself is a target. Subsequently PARP-1 dissociates from the strand break most likely as a result of charge repulsion between the negatively charged DNA backbone and the poly-(ADP-ribose) polymer. The activation of PARP-1 mediates the BER process where direct interaction between BER enzymes such as polymerase β and XRCC1 have been documented and point to a direct role for PARP-1.

Recently a link between PARP-1 and the familial breast cancer genes (BRCA-1 and BRCA-2) has been demonstrated. DNA double-strand break (DSB) repair occurs by homologous recombination which is mediated by the familial breast cancer genes BRCA-1 and BRCA-2. Mutations in these genes greatly increase the risk of developing breast and ovarian cancer. In two separate papers Tutt (1) and Bryant (2) showed that replication induced DSBs that result from PARP-1 inhibition are repaired by homologous recombination (HR). The principal DSB repair mechanism in higher eukaryotes is non Homologous End Joining (NHEJ), which entails the simple ligation of two DNA ends with little or no homology to one another (3-6). However, NHEJ accounts for only a small percentage of the repair of broken (3) replication forks. As a result cells with a defect in either BRCA-1 or BRCA-2 cannot undergo HR and do not repair DSBs that result from PARP-1 inhibition. The implication from these results suggests that nontoxic inhibitors of PARP-1 are excellent drug candidates to treat breast and ovarian cancers that are deficient in BRCA-1 and BRCA-2. Needless to say high throughput screening assays will be required to identify PARP-1 inhibitors in compound libraries.

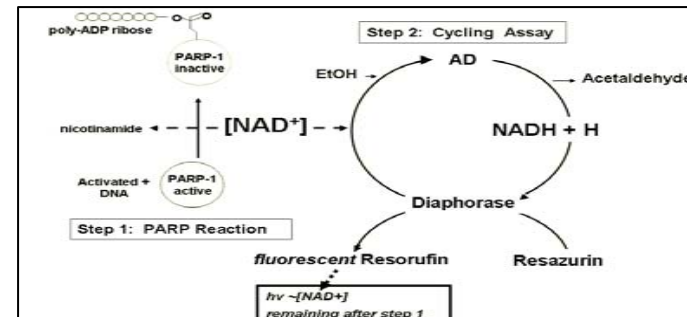
Most commonly, PARP activity is detected with radiolabeled NAD (7,8) but ideally a simple single tube assay is required with inexpensive and non-hazardous/caustic reagents. In 2003, a sensitive chemical quantitation method was described for screening PARP inhibitors that involved incubating the PARP reaction with acetophenone in base, followed by incubation at 110°C with formic acid (9). The assay presented in Figure 1 is a novel cycling assay developed for screening PARP inhibitors with nanomolar sensitivity using reagents that are widely available and can be performed using a multiwell fluorescence plate reader. In addition, assay sensitivity can be increased due to amplification by an enzyme-coupled cycling assay.

* Patent Pending

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Figure 1: PARP Inhibition Assay



During the course of the PARP reaction NAD⁺ is converted to PAR polymer. As PAR polymer is synthesized NAD⁺ is consumed and disappears from the reaction mixture. In the assay free NAD⁺ is converted to NADH in the presence of alcohol and alcohol dehydrogenase (ADH). Subsequently, NADH is converted back to NAD⁺ by the enzyme diaphorase (DPH) with the concomitant reduction of resazurin to form the fluorescent molecule resorufin. The molecule of NAD⁺ can now be recycled through the ADH and DPH to form more resorufin. The accumulation of resorufin is proportional to the level of NAD⁺ not completely converted to PAR polymer.

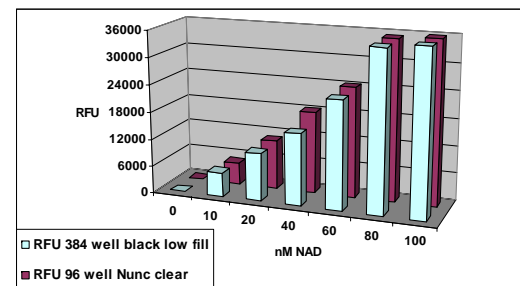
Figure 2: Assay Design

A. Setup

1. Add NAD⁺
2. Add PARP Inhibitors
3. Start 30' rx by addition of PARP Enzyme
4. Start 30' rx by addition of Cycling Mix
5. Add Stop Solution
6. Read fluorescence

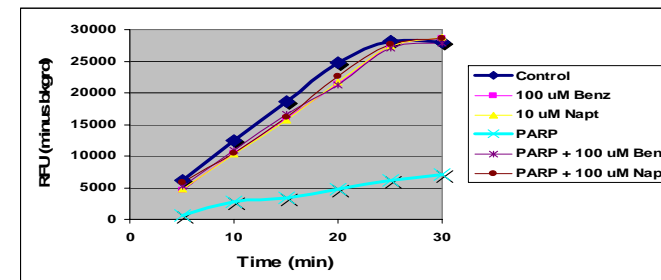


B. Sensitivity



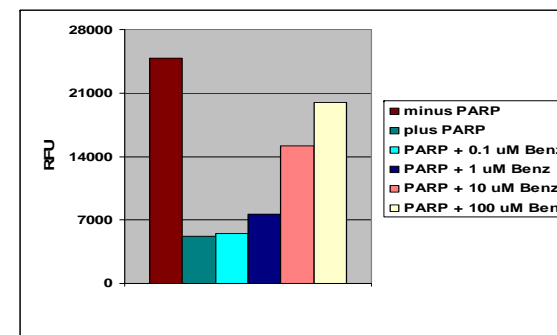
A. This ~1 hr endpoint assay is performed in two successive reactions (PARP and Cycling) requiring only the successive addition of reaction components. Assay components are easily added to 96 well plates using multichannel pipette. The number of Cycles can be controlled by the time of incubation to adjust assay sensitivity. An NAD standard curve is performed with each experiment and the cycling reaction can be monitored in real-time to assure linearity of standard curve or at endpoint through the addition of a Stop Solution. Fluorescent plate reader (544 nm ex/590 nm em) is used to record results. **B.** The NAD standard curve is used to determine relative IC₅₀ values for PARP inhibitors and detect as low as 10 nM NAD remaining in the PARP reaction. The reaction has the potential for scale down to 384 well plates.

Figure 3: PARP Inhibition and Cycling Controls



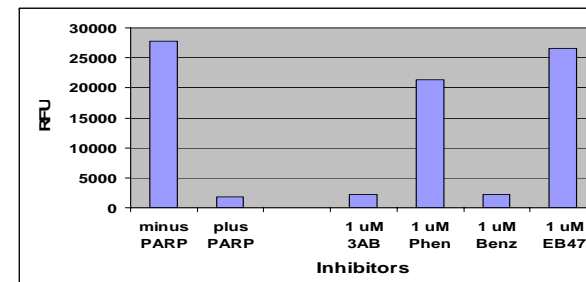
The cycling assay was performed with 100 nM NAD resulting in a linear increase in resorufin fluorescence up to 20 minutes. If 12.6 nM PARP is incubated with NAD prior to addition of the cycling reaction an approximate 5 fold decrease in resorufin fluorescence was observed indicating PARP activity. However, when 100 μ M Benzamide or 10 μ M Naphthalimide are present with PARP a decrease in resorufin fluorescence is not observed indicating the inhibition of PARP activity. Minimal inhibition of the cycling reaction was observed in the absence of PARP.

Figure 4: Inhibitor Titration



At a given concentration of inhibitor, PARP inhibition can be monitored based on the final NAD concentration. PARP reactions were incubated with varying concentrations of Benzamide for 30 min and cycling reaction monitored at 20 min. PARP inhibition increases with Benzamide concentration - 10% (1 μ M), 44% (10 μ M), and 66% (100 μ M). The results of the assay indicate an IC₅₀ of 10-50 μ M comparable to reported literature values.

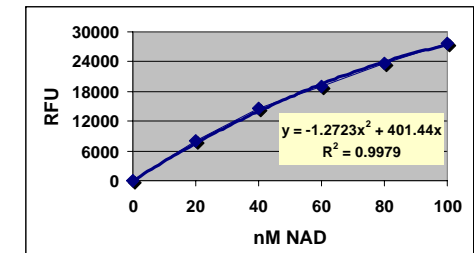
Figure 5: 1 μ M Inhibitor Screen



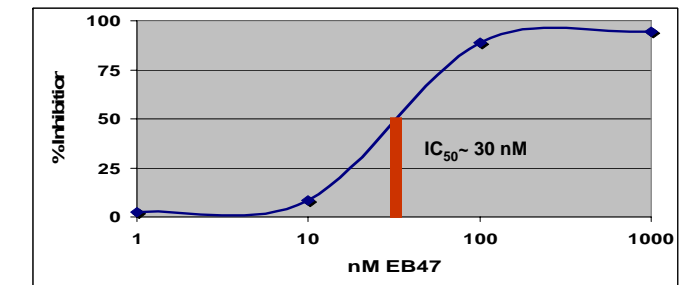
At 1 μ M, Phenanthridinone and EB47 show significant inhibition of PARP activity (maximal signal) with minimal inhibition observed with 3-aminobenzamide and benzamide (low signal).

Figure 6: Relative IC₅₀ values

A. Standard Curve



B. Inhibition Curve



C. Inhibitor Data

PARP Inhibitors	Observed Ki*	Published Ki
3-aminobenzamide	51 \pm 10 μ M	33 μ M
4-amino-1,8-naphthalimide	23 \pm 7 nM	153-180 nM
6(5H)-phenanthridinone	408 \pm 130 nM	305 nM
Benzamide	21 \pm 5 μ M	1-22 μ M
PJ34	139 \pm 55 nM	20 nM
EB47	25 \pm 6 nM	45 nM

* Average of several independent experiments

A. NAD standard curve is performed with each assay. A trend line is generated using a two order polynomial with the Y intercept forced through zero.

B. A quadratic equation is setup from the Standard Curve to calculate the amount of NAD remaining (also % inhibition). The NAD remaining (% inhibition) is plotted on the Y axis versus the EB47 inhibitor concentration. The IC₅₀ is estimated from the curve at 50% inhibition. The inhibitor EB47 results in an estimated value of 30 nM at 50% inhibition.

C. Relative IC₅₀ values determined for six inhibitors from multiple experiments. The assay is designed for the screening of PARP inhibitors using a NAD concentration below the Km of PARP. Once potential inhibitors are identified, more accurate Ki values using Michaelis-Menton kinetics can be determined.

Conclusions

• A highly sensitive fluorescent screening assay for PARP-1 inhibitors is performed in two successive steps requiring only the addition of reaction components.

• Inhibitors are identified by an increase in fluorescent signal.

• The assay can be used to determine IC₅₀ values for PARP inhibitors and detect as low as 10% inhibition of PARP-1 activity