The Need for a PARP *in vivo* Pharmacodynamic Assay

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Poly(ADP-ribose) polymerases are promising therapeutic targets. In response to DNA damage, poly(ADP-ribose) polymerases-1and 2 (PARP-1, PARP-2) are rapidly activated by DNA strand breaks. Once activated, NAD^+ is consumed for the synthesis of highly negatively charged polymers of ADP-ribose (PAR) on target nuclear proteins that include PARP-1 itself as a major acceptor [1, 2]. These highly branched polymers are in turn rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG). As a consequence of PARP activation, extensive DNA damage can lead to the depletion of NAD^+ in the cell and lead to cell death [3]. An overview of this metabolic response to DNA breakage is shown in Figure 1.

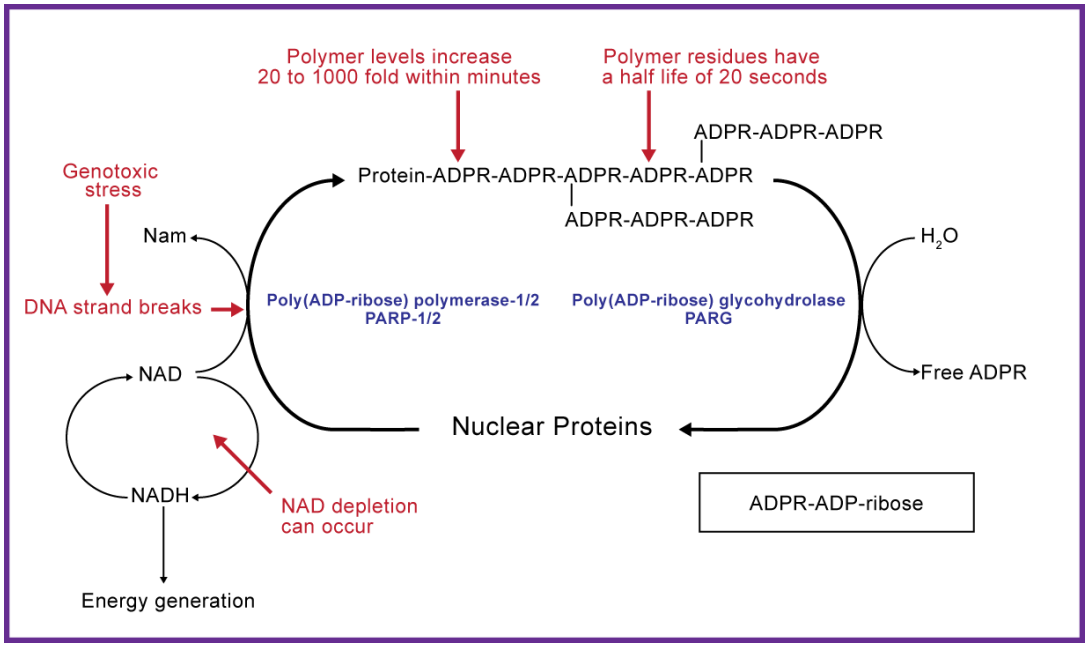

Figure 1:
Relationship between PARP 1 and 2 and PARG in response to DNA damage.
Excess genotoxic stress can lead to the depletion of NAD, resulting in apoptosis or necrosis.

PARP inhibitors are under development by multiple pharmaceutical companies. Figure 2 shows a working model that provides the basis for different approaches for the therapeutic targeting of PARPs that are currently under development by multiple pharmaceutical companies:

1. At relatively low levels of DNA damage, PARP-1, the best understood of the DNA damage responsive PARPs, mediates the repair of single strand breaks in DNA leading to cell recovery (Figure 2, left panel). This has led to the development of PARP inhibitors as chemo- and radio-sensitizing agents for cancer therapy [4].
2. Since the discovery that defects in homologous recombination sensitize cancer cells to PARP inhibitors there has been a renewed attention on PARP 1 as pharmaceutical target for cancer intervention and other diseases. In cells inhibited for PARP-1 activity (Figure 3 steps 3 and 4) single strand breaks (SSBs) are not repaired. Upon collision with replication forks (Figure 3 step 4), SSBs are converted to double strand breaks (DSBs) and subsequently repaired by homologous

recombination (Figure 3, step 5). Exciting new cancer therapies using nontoxic inhibitors of PARP-1 are now under development. In tumors that are deficient in homologous recombination lethal DSBs induced by PARP are not properly repaired (Figure 3 step 6) resulting in cell death [5, 6]. This approach offers the exciting possibility of minimizing or eliminating the toxic side effects that frequently limit the effectiveness of the agent, because the oncologist must avoid exceeding the dose that the individual patient can tolerate. Therefore, specifically targeting tumor cells with nontoxic inhibitors, based on metabolic deficiencies, offers tremendous potential benefits for future patient care.

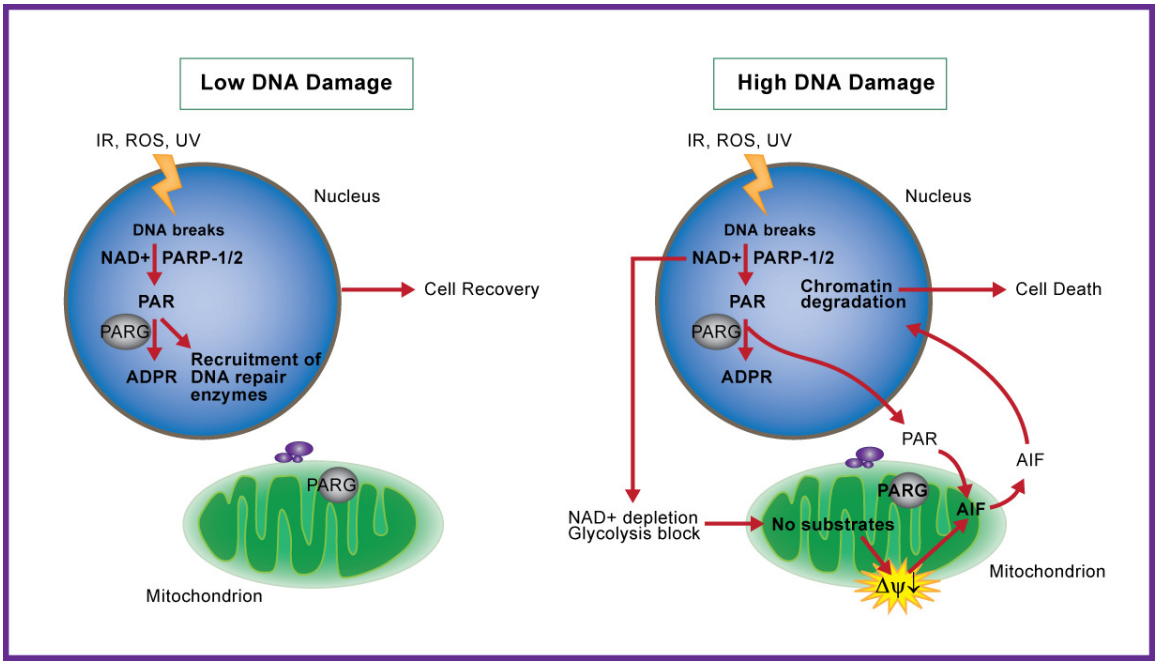

Figure 2:
Illustration of the role of PARP 1 and 2 and PARG in mediating cell death following neurotoxicity or ischemia, reperfusion injury.

The potential for PAR metabolism for targeting neurotoxicity and ischemia-reperfusion injury has been raised by studies demonstrating that PARP-1 knockout animals are extremely resistant to the cell killing effects of these conditions [7-9]. This has led to the discovery that PARP-1 activation is required for mitochondrial Apoptosis Releasing Factor (AIF) release [10] and that by a number of possible mechanisms including NAD and ATP depletion and release of PAR from the nucleus into the cytoplasm can cause mitochondrial AIF release [11] (Figure 2, right panel).

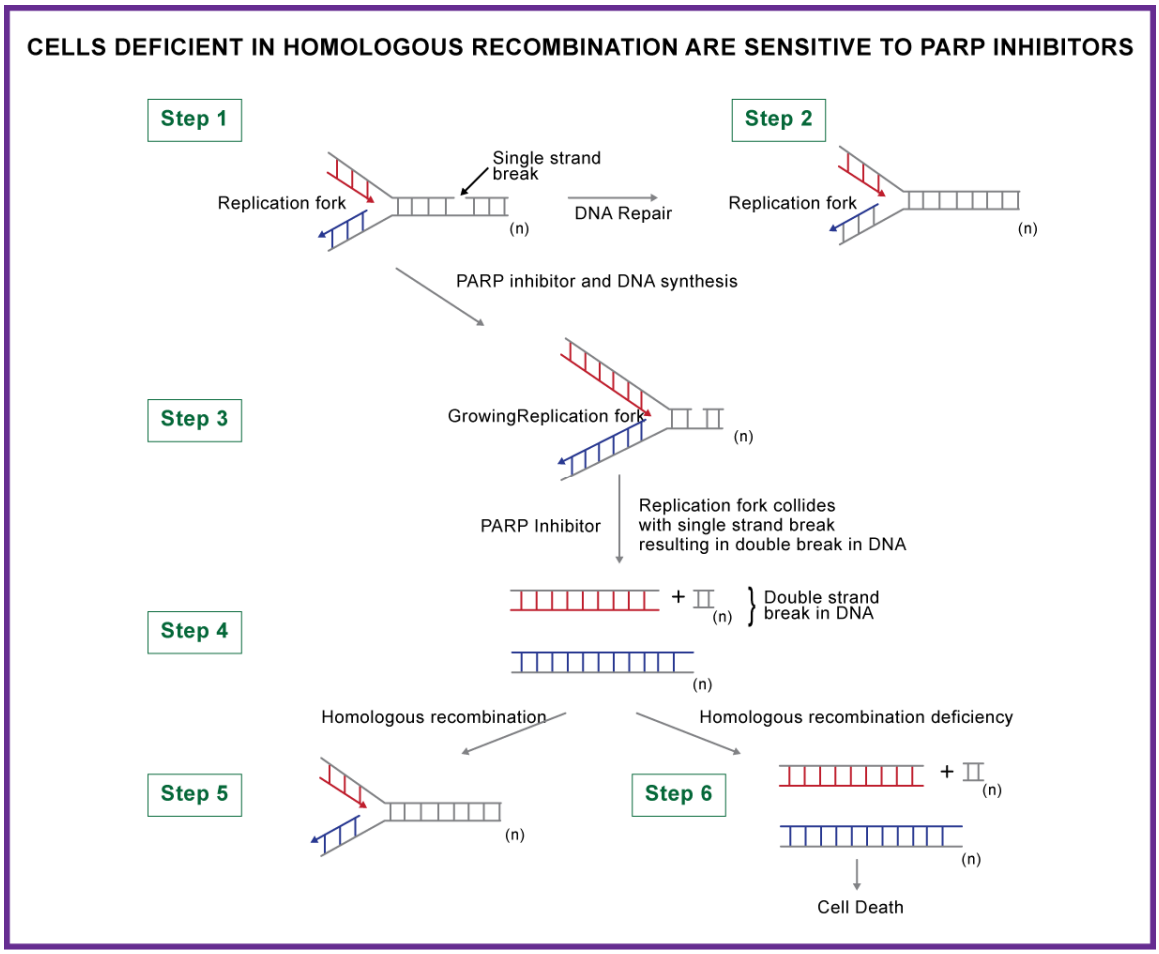

Figure 3:
Illustration of relationship between PARP-1 and homologous recombination.

Poly(ADP-ribose) glycohydrolases are potentially promising therapeutic targets. A number of lines of evidence indicate that PARG should be seriously evaluated as a potential therapeutic target.

1. The activities of PARPs and PARG are closely coordinated as cells respond to DNA damage [12] (Figure 2).
2. Disruption of this coordinate regulation of PARPs and PARG inhibits DNA repair and sensitizes cells to the cell killing effects of genotoxic stress [13].
3. Both PARP and PARG inhibitors also show promise for the treatment of shock and ischemia, reperfusion injury [14, 15] and PARG inhibitors demonstrate protective effects that compare favorably with PARP inhibitors [16].

4 The activity of PARG is almost certainly required for the release of PAR from the nucleus leading to the release of AIF (Figure 2).

Measurement of PAR in clinical samples. To facilitate development of PARP and PARG targeted therapeutics, validated assays to measure *in vivo* response to inhibitors in concurrence with preclinical studies are desirable. Currently therapeutic development is directed to PARP, and therefore, assays should measure response of PARP at the molecular level to treatment with candidate therapeutics. A rapid assay used in conjunction with clinical trials to measure PARP activity would be highly valuable.

While numerous companies are pursing the development of PARP targeted therapeutics, until recently there has not been a commercially available validated assay to access the effect of PARP inhibitors in vivo. Previously approaches relied on radiometric assays to determine PARP activity in cell extracts. At best these approaches are indirect and depend upon retention of a PARP inhibitor by the enzyme through multiple in vitro isolation steps. To circumvent this problem Trevigen has developed a PARP pharmacodynamic assay that measures the *in vivo* intracellular concentration of PAR. Now investigators can obtain evidence of drug action on PARP in both *in vivo* and *in vitro* settings.

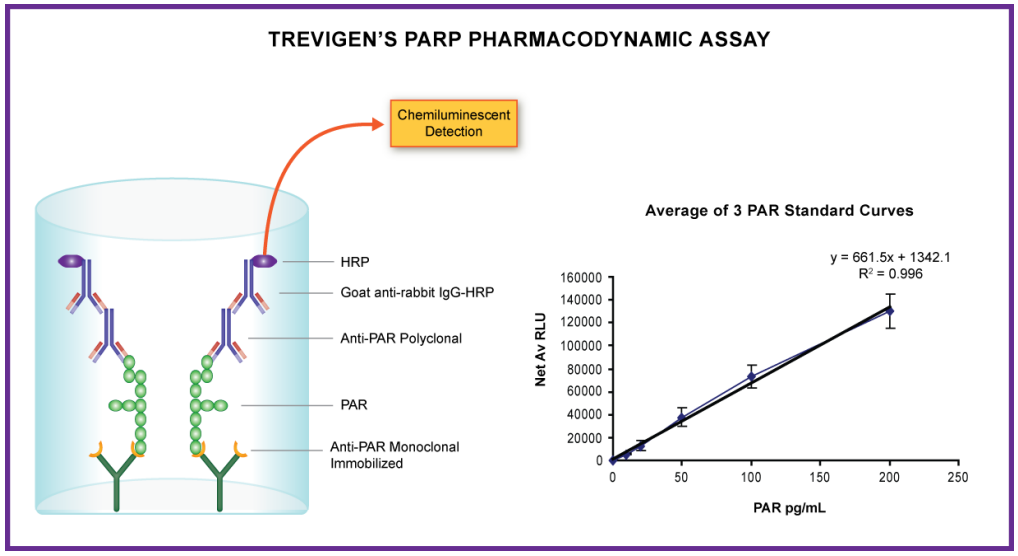

Figure 4:
Illustration of PARP pharmacodynamic assay.

Using a capture ELISA format, levels of poly-ADP-ribose (PAR) present in cells are easily determined. In this assay (Figure 1 , left panel), free PAR and PAR associated proteins are captured by a monoclonal antibody and subsequently quantified using a PAR directed rabbit polyclonal antibody. A typical standard curve using purified PAR polymer supplied with the kit is shown in Figure 4, right panel.

Validation of assay performance was determined by obtaining blood from two donors on three separate days and performing assays on three different days. Included in the assays are Jurkat cell extracts containing 22 and 88 pg/ml of PAR used as positive controls. The data in Figure 5 demonstrates that the assay provides reproducible results over of this study. The R^2 value for the average of three standard curves was 0.998 and the Jurkat positive control samples were 22 ± 1 and 88 ± 6.

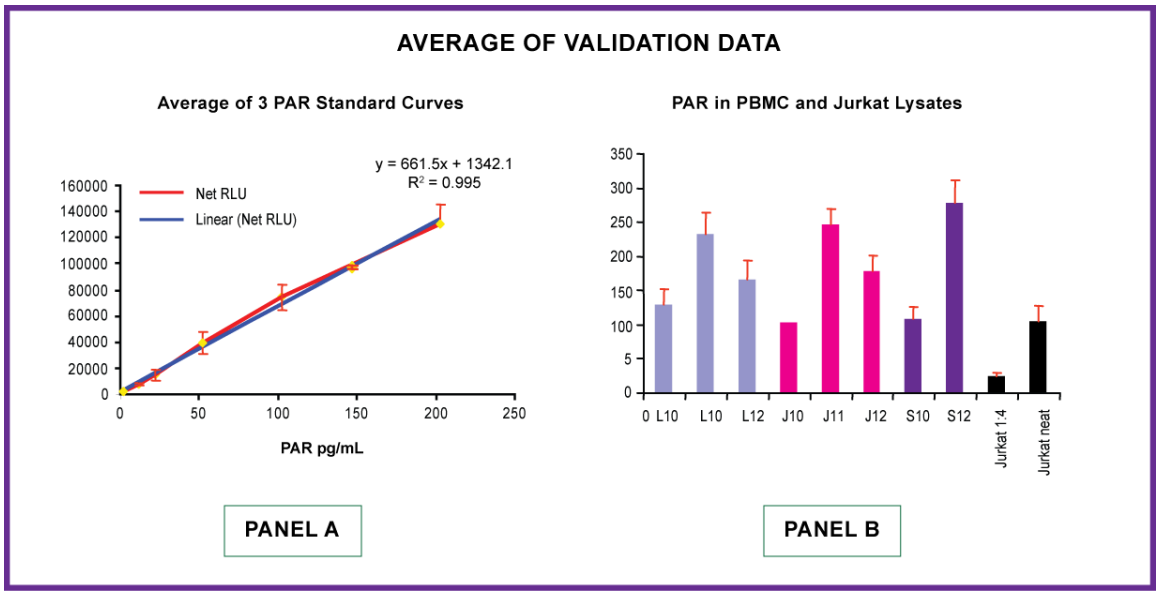

Figure 5:
PARP Pharmacodynamic assay validation data.

To verify the specificity of the assay Jurkat cells were treated with the PARP inhibitor PJ34 for one hour prior to lysate preparation alternatively lysates were treated with poly-ADP-ribose glycohydrolase (PARG) to degrade PAR. Data in figure 6 reveal a suppression of PAR signal in treated cells compared to untreated Jurkat cell populations.

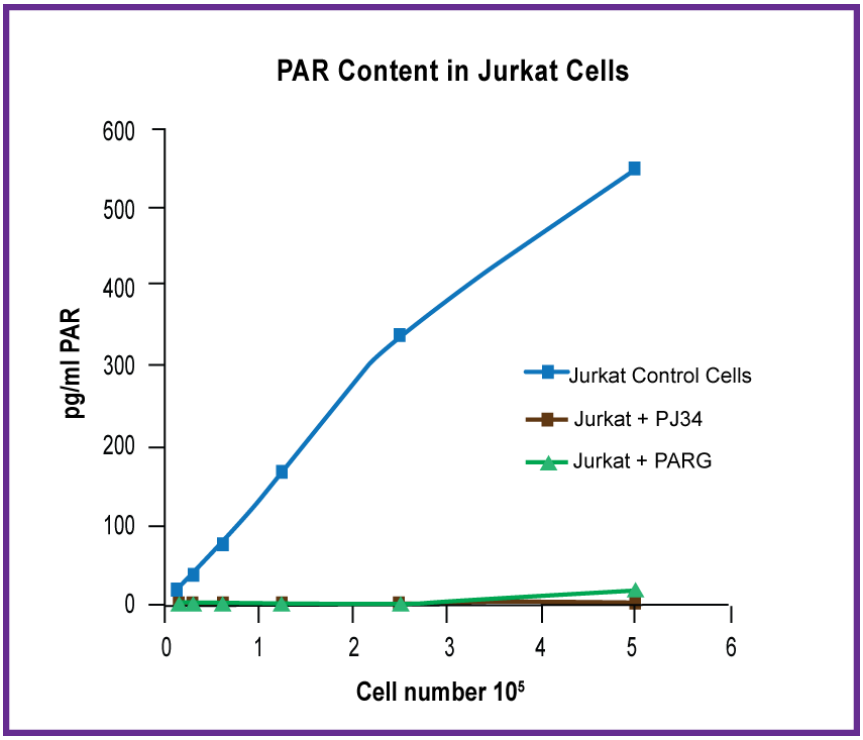

Figure 6:
Illustration of assay specificity for PAR.

In many instances the most cost efficient way to perform the assay will be to batch samples before conducting the assay. The simplest approach is to prepare cell extracts from frozen cell pellets. Figure 7 shows the stability of PAR made from frozen cell pellets compared to extracts prepared from fresh cells. Data indicates that cells can be stored in liquid nitrogen prior to lysate preparation without noticeable changes to PAR levels. In these experiments (Figure 7) lymphocytes were prepared from two donors. From each donor half the lymphocytes were frozen in liquid nitrogen as cell pellets and the other half was use to prepare lysates. After storage for 72 hours lysates were prepared from the frozen pellets. PAR levels were determined in both sets of lysates.

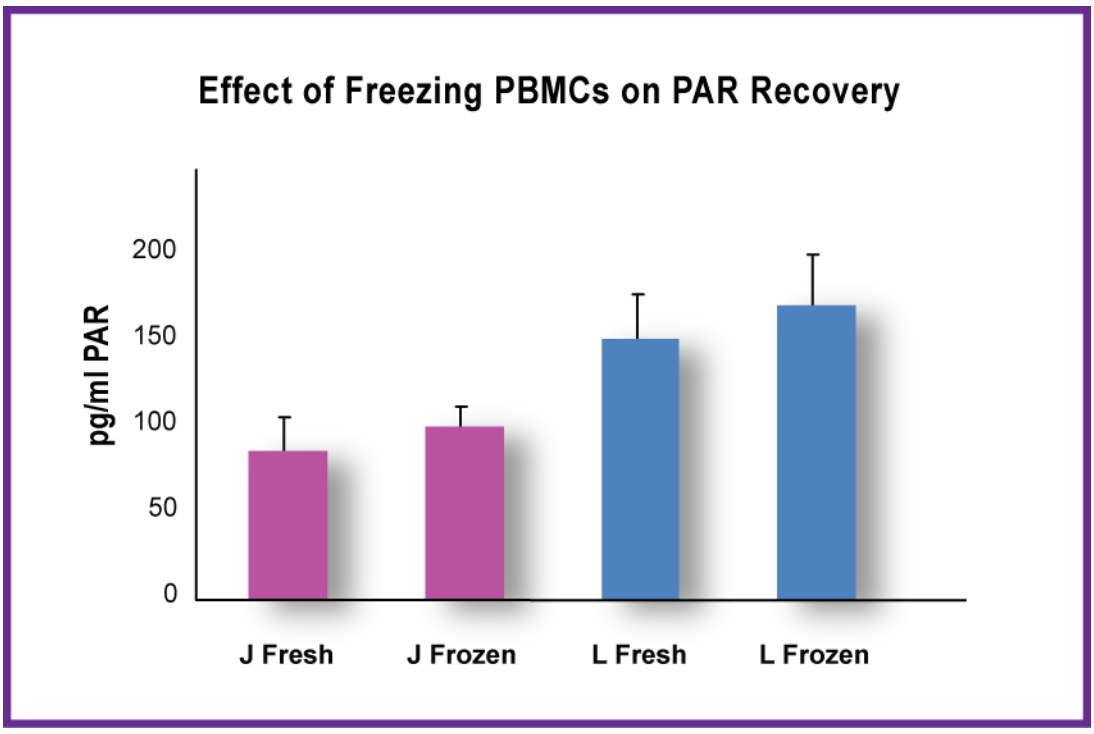

Figure 7:
Comparison of PAR content in extracts prepared from frozen and fresh cells.

PAR levels obtained from either frozen cell pellets or the corresponding fresh cells were not significantly different, demonstrating that samples can be collected and stored and subsequently assayed at a later time without deleteriously affecting the results.

In summary, Trevigen has developed a robust assay for determining PARP activity *in vivo*. This is the first commercially available assay to that permits one to access modulation of PARP 1 at the molecular level in response. The assay measures PAR levels *in vivo*. Cellular PAR levels are regulated by the dynamic interaction of PARP and PARG. The development of PARG inhibitors has lagged behind those for PARP. This is in part due to the fact that only recently has PARG been recognized as an important therapeutic target. There is a possibility that this assay will also serve as a tool to monitor the effect on inhibitors on PARG.

References:

1. Schreiber, V., et al., *Poly(ADP-ribose): novel functions for an old molecule.* Nat Rev Mol Cell Biol, 2006. **7**(7): p. 517-28.
2. Pleschke, J.M., et al., *Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins.* J Biol Chem, 2000. **275**(52): p. 40974-80.
3. Berger, N.A., *Poly(ADP-ribose) in the cellular response to DNA damage.* Radiat Res, 1985. **101**(1): p. 4-15.
4. Curtin, N.J., *PARP inhibitors for cancer therapy.* Expert Rev Mol Med, 2005. **7**(4): p. 1-20.
5. Bryant, H.E., et al., *Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase.* Nature, 2005. **434**(7035): p. 913-7.
6. Farmer, H., et al., *Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy.* Nature, 2005. **434**(7035): p. 917-21.
7. Eliasson, M.J., et al., *Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia.* Nat Med, 1997. **3**(10): p. 1089-95.
8. Hong, S.J., T.M. Dawson, and V.L. Dawson, *Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling.* Trends Pharmacol Sci, 2004. **25**(5): p. 259-64.
9. Koh, D.W., T.M. Dawson, and V.L. Dawson, *Mediation of cell death by poly(ADP-ribose) polymerase-1.* Pharmacol Res, 2005. **52**(1): p. 5-14.
10. Yu, S.W., et al., *Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor.* Science, 2002. **297**(5579): p. 259-63.
11. Andrabi, S.A., et al., *Poly(ADP-ribose) (PAR) polymer is a death signal.* Proc Natl Acad Sci U S A, 2006. **103**(48): p. 18308-13.
12. Meyer-Ficca, M.L., et al., *Poly(ADP-ribose) polymerases: managing genome stability.* Int J Biochem Cell Biol, 2005. **37**(5): p. 920-6.
13. Gao, H., et al., *Altered poly(ADP-ribose) metabolism impairs cellular responses to genotoxic stress in a hypomorphic mutant of poly(ADP-ribose) glycohydrolase.* Exp Cell Res, 2007. **313**(5): p. 984-96.
14. Cuzzocrea, S., et al., *PARG activity mediates intestinal injury induced by splanchnic artery occlusion and reperfusion.* Faseb J, 2005. **19**(6): p. 558-66.
15. Cuzzocrea, S. and Z.Q. Wang, *Role of poly(ADP-ribose) glycohydrolase (PARG) in shock, ischemia and reperfusion.* Pharmacol Res, 2005. **52**(1): p. 100-8.
16. Lu, X.C., et al., *Post-treatment with a novel PARG inhibitor reduces infarct in cerebral ischemia in the rat.* Brain Res, 2003. **978**(1-2): p. 99-103.