Supporting Information

Ma et al. 10.1073/pnas.1118078109

SI Materials and Methods

Chemicals. Poly(ADP ribose) polymerase (PARP) inhibitors 4-amino-1,8-naphthalimide (4-AN), 3,4-dihydro-5[4-(1-piperindinyl) butoxy]-1(2H)-isoquinoline (DPQ), 1,5-dihydroxyisoquinoline, 5-hydroxy-1(2H)-isoquinoline, 1,5-isoquinolinediol (IQD), 4-[(1-methyl-1H-pyrrol-2-yl)methylene]-1,3(2H,4H)-isoquinolinedione (BYK204165), and 3-aminobenzamide (3-AB) were from Sigma-Aldrich; 4-[(3-[(4-cyclopropylcarbonyl)piperazin-4-yl]carbonyl)-4-fluorophenyl]methyl(2H)phthalazin-1-one (Olaparib) and 4-iodo-3-nitrobenzamide (Iniparib) were from Selleck Chemicals; and N-(5,6-dihydro-6-oxo-2-phenanthridinyl)-2-acetamide hydrochloride (PJ34), 8-hydroxy-2-methylquinazoline-4-one (NU1025), 1,5,7,8-tetrahydro-2-methyl-4H-thiopyrano[4,3-d]pyrimidin-4-one (DR 2313), and the DNA-dependent protein kinase (DNA-PK) inhibitor 2-(4-morpholinyl)-4H-naphthol[1,2-b]pyran-4-one (NU7026) were from Tocris Bioscience.

Cell Culture and Synchronization. Raji, LCL35, and LCL35-derived shRNA cell lines were cultured in RPMI medium 1640 containing 10% (vol/vol) FBS and antibiotics. Cells were grown under humidified conditions at 37 °C in a 5% (vol/vol) CO_2 atmosphere. Synchronization of cells in G1 phase was done by serum starvation. Cell populations enriched for G2/M cells were obtained by treating cells with nocodazole (100 ng/mL) for 24 h.

shRNA Depletion of PARP1. Depletion of PARP1 expression in LCL35 cells was obtained from stably integrated PARP1 shRNA using MISSION shRNA lentiviral plasmids from Sigma-Aldrich (TRCN0000007932). The target sequence introduced was 5′-CCGGCGACCTGATCTGGAACATCAACTCGAGTTG-ATGTTCCAGATCAGGTCGTTTTT-3′. In vitro transfections were done using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols.

In Vitro PARylation Assay. The inhibitory efficiency of PARP inhibitors was analyzed by an in vitro poly(ADP ribosyl)ation (PARylation) assay using a commercially available ELISA kit (HT Universal PARP Assay Kit; Trevigen). The assay measures the incorporation of biotinylated poly(ADP ribose) onto histone proteins. PARP inhibitors were incubated in reaction buffer containing recombinant PARP1 enzyme, NAD, and activated DNA substrates. The activity of PARP inhibitors was determined by the percent reduction of colorimetric readout relative to no inhibitor.

RT-PCR. Total RNA was extracted from cells using the RNeasy Mini Kit in the presence of DNase (QIAGEN). cDNAs were generated from 1 μg of purified RNA using TaqMan reverse-transcription reagents (Applied Biosystems). Then, TaqMan RT-PCR was performed using specific and prevalidated primers for selected targets on an HT7900 System (Applied Biosystems). Relative quantification values were calculated using β2-microglobulin gene for normalization. PARP1 prevalidated primer (Hs0024320_m1) was from Applied Biosystems.

Western Blot Analysis. Cells were lysed in radioimmunoprotein assay buffer (Thermo Fisher Scientific), and the protein concentration was measured with a protein assay kit (Bio-Rad). Equal amounts of whole-cell extracts (30–50 μg) were separated on bis-Tris NuPAGE (4–12%, wt/vol) gels and transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked with 5% (wt/vol) skim milk in PBS containing 0.1% Tween 20 for 1 h at room temperature, and then probed with appropriate primary antibodies overnight at 4 °C or for 2 h at room temperature. Anti-PARP1 antibody (C-2–10; Novus Biologicals) and anti-actin antibody (C-11; Santa Cruz Biotechnology) were used at a concentration of 1:1,000 and 1:2,500, respectively. Results were visualized by chemiluminescence following incubation with the appropriate HRP-linked secondary antibodies (Santa Cruz Biotechnology).

Detection of DNA Strand Breaks by Pulsed-Field Gel Electrophoresis. Control and ionizing radiation (IR)-treated cells were collected and mixed with low-melting agarose [2% (wt/vol) stock in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), final concentration 0.6%] to prepare agarose-embedded DNA plugs. The cells in the plugs were then digested with proteinase K (10 mM Tris, pH 8.0, 100 mM EDTA, 1.0% *N*-lauroylsarcosine, 0.2% sodium deoxycholate, 1 mg/mL proteinase K) for 24 h at 30 °C. Pulsed-field gel electrophoresis (PFGE) was performed using a CHEF-Mapper XA System (Bio-Rad). The parameters for CHEF gel separation of human chromosomes and Epstein–Barr virus (EBV) in a 1% agarose gel were 6 V/cm for 24 h with a 10–90 s switch time ramp and 120 °C switch angle (14 °C). Subsequently, the DNA was analyzed by Southern blotting. Hybridization was carried out with a probe specific for EBV. Autoradiographs were digitized and densitometric analysis was performed using Kodak MI software (version 5.0; Carestream Health).

Quantitation of Single-Strand Breaks and Double-Strand Breaks. Quantitation of single-strand breaks (SSBs) and double-strand breaks (DSBs) was based on changes in the amounts of supercoiled and linear forms of EBV, respectively. After PFGE and Southern blotting, three bands were detected, representing the three forms of EBV: relaxed circular EBV in the well, supercoiled circular EBV (containing no SSB or DSB), and linear, full-length EBV (containing one DSB). The average number of SSBs or DSBs in EBV was calculated based on Poisson distribution:

$$P = \frac{X^A}{A!e^X}.$$

P is the probability of EBV molecules with A number of SSBs or DSBs; X is the expected average number of SSBs in EBV; Y represents the expected average number of DSBs. For the supercoiled band, $A = 0$ (because one or more SSBs will relax the supercoil). For the linear EBV band, $A = 1$ (one DSB at any position of the circular EBV converts it into a linear molecule). Therefore,

$$\text{supercoil}: P = \frac{1}{e^X}$$

$$\text{linear}: P = \frac{Y}{e^Y}.$$

The estimation of DSBs is based on the following equations:

$$P_0 = \frac{Y_0}{e^{Y_0}},\ P_r = \frac{Y_r + Y_0}{e^{(Y_r+Y_0)}},\ \text{and}\ P_{nr} = \frac{nY_r + Y_0}{e^{(nY_r+Y_0)}}.$$

P_0 and Y_0 correspond to values for DSBs at base level when there is no damage. P_r and the derived $(Y_r + Y_0)$ correspond to values

for total DSBs after a dose r. P_{nr} represents the value when the dose is increased n times that or r. Assuming that DSB formation is linear with dose for IR as demonstrated in other studies, P_0 and P_r at a high dose can be approximated by the following two equations:

$$Y_r \approx LN(nP_r/P_{nr})/(n-1) \text{ (when } y_r \gg y_0) \text{ and}$$
$$Y_0 = Y_r/(e^{Y_r} \bullet P_r/P_0 - 1).$$

The Newton–Raphson iteration method is used to calculate the Y_r at different IR doses.

The change in SSBs as measured by loss of the amount of supercoiled DNA after two different doses or at two time points during repair can be calculated with the following equation: $\Delta X = X_1 - X_2 = \ln(P_2/P_1)$. Although DSBs can contribute to loss of supercoiled DNA, their contribution is considered small relative to the induction of SSBs.

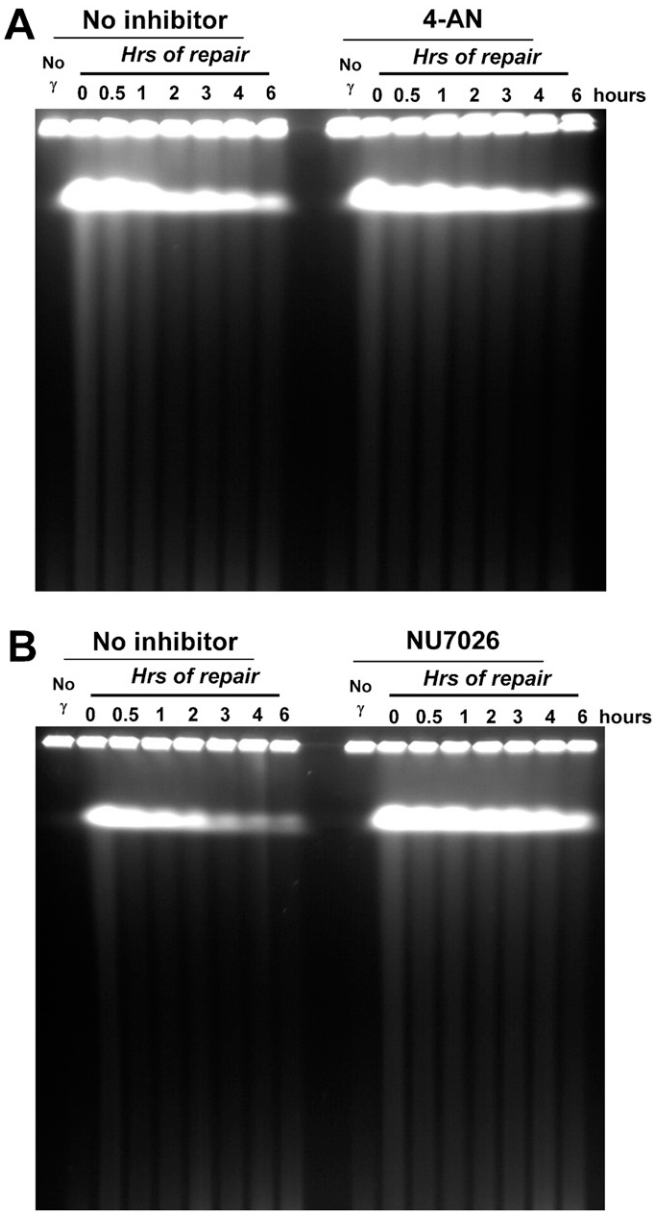

Fig. S1. Radiation-induced human genome fragmentation and repair profile determined by ethidium bromide staining. Nocodazole-arrested G2 Raji cells were γ-irradiated at a dose of 100 Gy in ice-cold medium. (*A*) The PARP inhibitor 4-AN (10 μM) or (*B*) DNA-PK inhibitor NU7026 (10 μM) was added immediately after radiation and the cells were kept on ice for 15 min to allow uptake of the inhibitors and then mixed with prewarmed complete medium and incubated to allow repair. Cells were collected at the indicated times and processed for PFGE analysis. Fragmentation and repair were visualized by ethidium bromide staining.

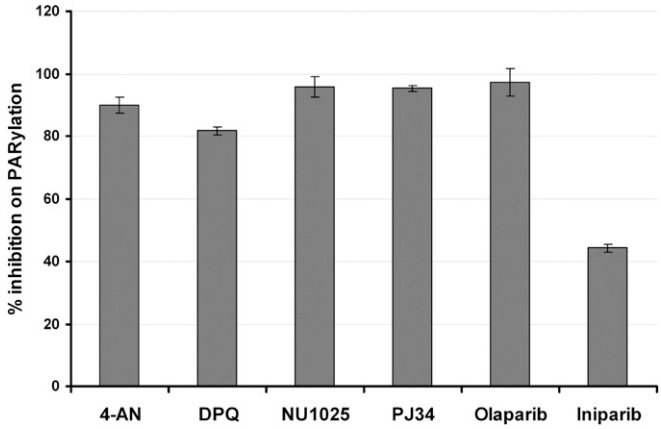

Fig. S2. The inhibition efficiency of PARP inhibitors on PARylation in vitro. PARylation was measured by ELISA based on in vitro recombinant PARP enzyme-catalyzed incorporation of biotinylated poly(ADP ribose) onto histone proteins. PARP inhibitor concentrations were DPQ, 20 μM; NU1025, 50 μM; PJ34, 10 μM; 4-AN, 10 μM; Olaparib, 20 μM; Iniparib, 100 μM. The percent inhibition was calculated based on the colorimetric readout relative to that without inhibitor. Each data point represents the mean value and SD from triple repeats.

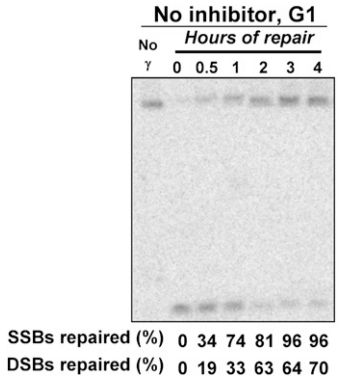

Fig. S3. Repair of IR-induced breaks as in G1 cells. Raji G1 cells were obtained by serum starvation, and then γ-irradiated at a dose of 100 Gy and incubated in complete medium at 37 °C to allow repair. Cells were collected at the indicated times and processed for PFGE and Southern blot analysis. The repair efficiencies of the SSBs and DSBs are indicated at the bottom.