Supplementary Online Material

PARP-1 inhibition as a targeted strategy to treat Ewing's sarcoma

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Materials and Methods

Cell Lines and inhibitors

PC3 (ATCC), CADO-ES1 (DSMZ), RD-ES (ATCC), SAOS-2 (ATCC), A-204 (ATCC), COG10 and COG258 (www.COG.org) Ewing's sarcoma cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) in 5% CO_2 cell culture incubator. All cultures were also maintained with 50 units/ml of Penicillin/streptomycin (Invitrogen). The genetic identity of each cell line was confirmed by genotyping samples. Briefly, DNA samples from each cell line were diluted to 0.10ng/µl and analyzed in the University of Michigan DNA sequencing Core using the Profiler Plus PCR Amplification Kit (Applied Biosystems, Foster City, CA) as previously described (1). Olaparib was synthesized to >99% purity by Axon Biochem. EWS-FLI1_3xFlag and EWS-ERG_3xFlag constructs were created by gene synthesis (Invitrogen) and cloned into the pLL_IRES_GFP vector backbone available from the University of Michigan vector core. Virus and stable isogenic cell lines were made as previously described (2). Cells were monitored for GFP expression and construct overexpression was confirmed by Western blot analysis.

Immunoprecipitation

Cells were lysed in Triton X-100 lysis buffer (20mM MOPS, pH 7.0, 2mM EGTA, 5mM EDTA, 30mM sodium fluoride, 60mM β-glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium orthovanadate, 1% Triton X-100, protease inhibitor cocktail (Roche, #14309200)). Cell lysates (0.5-1.0mg) were then pre-cleaned with protein A/G agarose beads (Santa Cruz, # sc-2003) by incubation for 1 hour with shaking at room temperature followed by centrifugation at 2000xg for 1 minute. Lysates were incubated with 100µg/mL ethidium bromide (Sigma) as previously described (3). Then, after adding 2µg anti-ERG antibody (Epitomics, Burlingame, CA), anti-PARP-1 polyclonal (1:1000 in blocking buffer, Santa Cruz Biotech Cat #sc-8007, Santa Cruz, CA or anti-FLI1 (Santa Cruz C19, Cat# sc-356), lysates were incubated at 4°C overnight with shaking prior to addition of 20µL protein A/G agarose beads. The mixture was then incubated with shaking at 4°C for another 4 hours prior to washing the lysate-bead precipitate (centrifugation at 2000xg for 1 minute) 3 times in Triton X-100 lysis buffer. Beads were finally precipitated by centrifugation, resuspended in 20µL of 2x loading buffer and boiled at 80°C for 10 minutes for separation the protein and beads. Samples were then analyzed by SDS-PAGE Western blot analysis as described below.

Immunoblot Analysis

The cell lines were plated in two wells of a 6-well plate at 250,000 cells/mL 24hours prior to harvesting by trypsinization. Pellets were then flash frozen, briefly sonicated and homogenized in NP40 lysis buffer (50 mM Tris-HCl, 1% NP40, pH 7.4, Sigma, St. Louis, MO), and complete proteinase inhibitor mixture (Roche, Indianapolis, IN). Ten micrograms of each protein extract were boiled in sample buffer, size fractionated by SDS-PAGE, and transferred onto Polyvinylidene Difluoride membrane (GE Healthcare, Piscataway, NJ). The membrane was then incubated overnight at 4ºC in blocking buffer [Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk] and incubated for 4 hours at room temperature with the following: anti-DNA-PKcs mouse monoclonal (1:1000 in blocking buffer, BD Biosciences #610805, San Jose, CA), anti-Ku70 mouse monoclonal (1:1000 in blocking buffer, BD Biosciences #611892), anti-Ku80 rabbit polyclonal (1:1000 in blocking buffer, Cell Signaling Cat #2180S, Danvers, MA), anti-PARP-1 polyclonal (1:1000 in blocking buffer, Santa Cruz Biotech Cat #sc-8007, Santa Cruz, CA, anti-ERG (Epitomics, Burlingame, CA), anti-FLI1 (Santa Cruz C19, Cat# sc-356) and anti-GAPDH rabbit polyclonal antibody (1:1000, Millipore, Cat # ABS16). Following a wash with TBS-T, the blot was incubated with horseradish peroxidase-conjugated secondary antibody and the signals visualized by enhanced chemiluminescence system as described by the manufacturer (GE Healthcare).

Soft Agar Colony Formation

A solid layer of 1% agarose was used to coat each well of a 96-well plate. After allowing two hours to cool, five hundred prostate or Ewing's cells were suspended in a 33% agarose (10% solution), 33% media with 2x FBS and 33% cell suspension in quadruplicate wells of a 96-well plate. Cells were treated with or without Olaparib in an upper layer of media. Every three days, the media was exchanged for fresh Olaparib containing media. After 3-weeks in culture, images were taken on a 10x objective lens and colonies were counted for each condition. This assay was run in three times.

Chemosensitivity Assays

Five thousand cells were plated in each well of a 96-well plate in sets of ten. Cells were then treated with a single dose of Olaparib as indicated for 72 hours. WST assays (Roche) were performed according to company protocol. Briefly, 10% WST was added to each well, the plates were incubated at 37C with 5% CO_2 for 4 hours and the OD was measured.

small RNA interference

Knockdown of specific genes was accomplished by RNA interference using commercially available siRNA duplexes for EWS, ERG, FLI1, DNA-PKcs or PARP-1 (Dharmacon, Lafayette, CO). At least 4 independent siRNAs were screened for knockdown efficiency against each target and the best siRNA was selected. In each case, two independent siRNAs were used. We were unable to identify a 3'-FLI1 siRNA with >80% knockdown efficiency, therefore, these siRNA transfections were run 3 times. Transfections were performed with OptiMEM (Invitrogen) and oligofectamine (Invitrogen) as previously described (4).

Quantitative Real-Time PCR Assays

Total RNA was isolated from cell lines transfected with siRNA or as indicated (Qiagen). Quantitative PCR (QPCR) was performed using SYBR Green dye on an Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster City, CA) as described (5). The relative quantity of the target gene was completed for each sample using the ΔΔCt method by the comparing mean Ct of the gene to the average Ct of the geometric mean of two housekeeping genes, GAPDH and RPL19 (6). All oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). The primer sequences for the transcript analyzed are provided in **Supplementary Table 1** unless used previously (2).

Basement Membrane Matrix Invasion Assays

For invasion assays, the cell lines were transfected with siRNA, drug or negative controls as indicated. Forty-eight hours post-treatment, cells were seeded onto the basement membrane matrix (EC matrix, Chemicon, Temecula, CA) in the chamber insert with 8.0μM pores of a 24-well culture plate in serum free media. Cells were attracted to the lower chamber by the addition of complete media as a chemoattractant. After 48 hours incubation at 37˚C with 5% CO_2, the non-invading cells and EC matrix were gently removed with a cotton swab. Invasive cells, which were located on the lower side of the membrane, were stained with crystal violet, air dried and photographed. To quantify the relative number of invaded cells, colorimetric assays were performed by treating the inserts with 150μl of 10% acetic acid (v/v) and measuring absorbance of each condition at 560nm using a spectrophotometer (GE Healthcare).

Xenografts

Five week-old male SCID mice (CB.17. SCID), were purchased from Charles River, Inc. (Charles River Laboratory, Wilmington, MA). RD-ES cells (2×10^6 cells/injection), PC3-luciferase-LACZ (1×10^6 cells) or PC3-luciferase-EWS-FLI (1×10^6 cells) stable cells were resuspended in 100μl of saline with 50% Matrigel (BD Biosciences, Becton Drive, NJ) and were implanted subcutaneously into the left and right

flank regions of the mice. Mice were anesthetized using a cocktail of xylazine (80-120 mg/kg, IP) and ketamine (10mg/kg, IP) for chemical restraint before tumor implantation. All tumors were staged for two weeks before starting the drug treatment. At the beginning of the third week, mice with RD-ES tumors (10 tumors per treatment group, average size 150-200 mm^3) were treated with Olaparib (100mg/kg, IP twice daily five times per week) and/or Temozolamide (50mg/kg daily five times per week, IP). Similarly, at the beginning of the third week, mice with PC3-luciferase-LACZ or PC3-luciferase-EWS-FLI were treated with Olaparib (100mg/kg, IP twice daily five times per week). Olaparib was obtained from Axon Medchem (Groningen, The Netherlands). We chose 100mg/kg/twice daily because, in mice, this dose corresponds to the maximum clinically achievable dose for which minimal side effects were observed in humans during the initial Phase I trial of Olaparib (Center for Drug Evaluation and Research, 2002; Fong et al., 2009; Reagan-Shaw et al., 2008). Temozolamide was obtained from Selleck Chemicals (Houston, Texas). Growth in tumor volume was recorded weekly by using digital calipers and tumor volumes were calculated using the formula $(\pi/6)$ $(L \times W^2)$, where L = length of tumor and W = width. Loss of body weight during the course of the study was also monitored weekly; all changes in mouse weights occurred within tolerable limits as set by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. At the end of the xenograft studies, mice were sacrificed, and mouse lungs were subsequently fixed in 10% formalin via gavage through a tracheal incision. Lungs were subsequently harvested, maintained in formalin, and subsequently embedded in paraffin. The number of metastases per lung was quantitated via light microscopy by a pathologist (S.A.T.). All procedures involving mice were approved by the UCUCA at the University of Michigan and conform to their relevant regulatory standards.

Immunofluorescence

Cells were seeded at 25,000 cells/mL in 4-well chamber slide 24 hours prior to the addition of siRNA or drug as indicated. Merbarone (Sigma) was dissolved in DMSO and stored at 4°C. Cells were then washed once in PBS, fixed for 15 minutes in 100% methanol, washed in PBS, permeabilized for 5 minutes in PBS containing 0.2% Triton-X, washed twice in PBS, blocked for 30 minutes in PBS containing 0.5% donkey serum (Sigma, St. Louis, MO), held for 45 minutes in PBS containing 0.5% donkey serum and primary antibody: anti-γ-H2A.X mouse monoclonal (Millipore Cat #05-636) washed 3 times with PBS, held for 30 minutes in PBS containing 0.5% donkey serum and secondary antibody: Alexa Fluor 488-anti-Mouse (Invitrogen). Cells were then stained with DAPI for 5 minutes and slides were mounted 12 hours prior to analysis using Vectashield (Vector laboratories, Burlingame, CA). Images were taken using 100x oil lens on an Olympus Confocal microscope at the University of Michigan microscopy imaging lab.

COMET Assay

Ewing's sarcoma cell lines were seeded at 250,000 cells/mL in a 6-well plate 24 hours prior to treatment with siRNA, drug or vehicle control. After 48 hours, cells were trypsinized, harvest by centrifugation and re-suspend in PBS. Cell counts were then normalized to 1 x 10^5 cells/mL. Suspended cells (25µL) were then mixed with 250µL 1.0% ultrapure low melting point agarose (Invitrogen) made in 1x Tris-Borate buffer. The agarose-cell mixture was then dropped onto slides allowed to solidify at 4°C in the dark for 20 minutes before immersion in COMET assay lysis solution (Trevigen, Gaithersburg, MD) at 4°C in the dark for 45 minutes. Excess buffer was then removed and slides were submerged in freshly prepared neutral solution (Tris Base 60.57g, Sodium Acetate 204.12g, dissolve in 450 ml of dH2O. Adjust to pH = 9.0 with glacial acetic acid.) at room temperature in the dark for 40 minutes. Slides were then washed twice by immersing in 1 X TBE buffer prior to neutral electrophoresis at 20 volts for 60 minutes unless otherwise indicated. Slides were then fixed in 70% ethanol for 5 minutes. Following air drying of the agarose, slides were stained with SYBR Green dye (Invitrogen) and images were collected with a 10x and 40x objective lens. COMET tail moments were then assessed using COMETscore.v1.5 (AutoCOMET.com, Sumerduck, VA) image processing software as described by the manufacturer with greater than 100 cells analyzed in triplicate experiments.

Promoter Reporter Luciferase Assays

Cells were transfected with PARP1, emptry vector (R01) or GAPDH promoter reporter constructs (Switchgear Genomics, Menlo Park, CA) using Fugene 6.0 (Roche) according to standard protocol. Luciferase activity was then assessed using the Lightswitch Assay kit available (Switchgear Genomics) according to the manufacturer's instructions.

Oncomine expression analysis

The expression signatures from Ewing's sarcoma patients (7) and prostate cancer patients (8, 9) were downloaded from the Oncomine and used to generate scatter plots. Correlation coefficients were determined using Oncomine (10-13).

Statistical Analysis

All statistical assays were two-tailed t-test unless otherwise indicated. On all plots and graphs the standard error of the mean is shown. * $P < 0.05$. For xenograft experiments, comparison of tumor volume growth rates between treatment groups was achieved via likelihood ratio tests. Volume measurements were modeled using linear mixed-effects models incorporating a random intercept for each mouse.

Contributions

J.C.B., F.Y.F. and A.M.C. designed experiments and wrote the manuscript. J.C.B., F.Y.F., S.H., S.P., S.G., L.M.B., M.L. and J.R.P. performed experiments. S.A.T. conducted pathological assessment of tumor metastasis. R.J.L. performed statistical analysis.

Supplemental References

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