TDP1 and PARP1 Deficiency Are Cytotoxic to Rhabdomyosarcoma Cells

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Abstract

Rhabdomyosarcoma is the most common soft tissue sarcoma in children. Metastatic rhabdomyosarcoma in children has a 5-year event-free survival rate of <30%, and a recent clinical trial with irinotecan, a topoisomerase I inhibitor, failed to improve outcome. Therefore, it was surmised that failure of irinotecan may be the result of overexpression of the DNA repair enzyme tyrosyl-DNA phosphodiesterase (TDP1), which processes topoisomerase I-DNA complexes resulting from topoisomerase I inhibitor treatment. Using human tissue microarrays and gene expression arrays, a marked overexpression of TDP1 protein and mRNA in RMS tumors was observed. Critically, knockdown of TDP1 or inhibition of poly(ADP-ribose) polymerase-1 (PARP-1), an enzyme in the same complex as TDP1, sensitized rhabdomyosarcoma cell lines to analogues of irinotecan. Interestingly, BRCA1/2 mutations or altered expression was not detectable in rhabdomyosarcoma cells; however, TDP1 knockdown and PARP-1 inhibition alone were cytotoxic to a subset of rhabdomyosarcoma cells, suggesting that they harbor genetic lesions in DNA repair components that have synthetic lethal interactions with loss of TDP1 or PARP-1 function. Furthermore, culturing embryonal rhabdomyosarcoma cells in serum/nutrient-restricted medium increased cellular cytotoxicity upon PARP-1 inhibition and was intrinsically cytotoxic to alveolar, though not embryonal rhabdomyosarcoma cells. The results of these studies suggest a compensatory role for TDP1 in rhabdomyosarcoma after topoisomerase-I based therapy and further demonstrate that TDP1 knockdown, PARP-1 inhibition, and dietary restriction have therapeutic validity.

Implications: Selective targeting of TDP1 and/or PARP-1 in rhabdomyosarcoma induces cytotoxicity and sensitizes to DNA damaging agents. Mol Cancer Res; 11(10); 1179–92. ©2013 AACR.

Introduction

Conventional chemotherapeutic agents have targeted general cellular processes such as nucleotide synthesis, DNA replication, and cell division. Because each of these processes is required for homeostasis of normal tissues, these treatments cause significant morbidity and limit the doses of drugs that can be given for a cure. Despite these shortcomings, combinations of cytotoxic agents have improved cure rates for many malignancies such as pediatric acute lymphoblastic leukemia (1).

Pediatric sarcomas and other cancers have not responded as well to these conventional therapies (2). Improvement in cancer-free survival therefore requires a better understanding of cancer biology and the identification of cancer-specific targets (3). During the evolution of human cancers, cancer cells accumulate genetic alterations such that each tumor contains multiple genetic and epigenetic abnormalities (4). As illustrated by imatinib inhibition of BCR-ABL in chronic myelogenous leukemia (5), the inhibition of a single oncogene is sufficient to block cancer cell growth in some cancers. In other cancers, DNA maintenance pathways mutate during the evolution of the cancer (6) and inhibition of remaining DNA repair pathways causes cancer-cell death. This is illustrated by the synthetic lethal interaction of PARP-1 inhibitors with defects of homologous recombination such as those arising from mutations of BRCA1 and BRCA2 (7). Sparing normal tissues, these 2 approaches show the potential for preferential targeting of malignant cells.
The question then arises as to whether such targets might be identifiable for the high-risk pediatric sarcomas (2). Rhabdomyosarcoma is one of the most common soft tissue sarcomas of childhood. Each year approximately 300 children in the United States are diagnosed with this disease and two-thirds are younger than 10 years old (8). The 2 major subtypes are embryonal and the more aggressive alveolar rhabdomyosarcoma (8). Although the overall cure increased from 25% to 70% between the 1970s and the 1990s (8, 9), the 5-year failure-free survival rate is only 65% for children with nonmetastatic alveolar rhabdomyosarcoma or embryonal rhabdomyosarcoma at unfavorable sites (9). Survival is even less for the approximately 30% of children presenting with metastatic rhabdomyosarcoma (10).

The genetic alterations associated with rhabdomyosarcoma include chromosomal translocations and deletions and these correlate with outcome. Many embryonal rhabdomyosarcoma tumors have loss of heterozygosity at the 11p15.5 locus (11) and 70% to 80% of alveolar rhabdomyosarcoma have chromosomal translocations t(2;13)(q11;q14) or t (1;13)(p36;q14) resulting in expression of PAX3/7-FOXO1 chimeric proteins that function as transcription factors (12, 13). The PAX-FOXO1 transcription factor increases expression of the insulin growth factor 1 receptor (IGF-IR; 14) and inhibition of IGF-IR signaling shows promise in impeding rhabdomyosarcoma tumor growth (15).

Unlike recent studies implicating a role for the EWS–FLI fusion protein in maintaining the PARP-1–mediated DNA damage response in Ewing sarcoma, defects of DNA repair have not been reported in rhabdomyosarcoma despite the genomic rearrangements identified in these tumors (16, 17). Of particular interest is whether the recent failure of the irinotecan trials for metastatic or recurrent rhabdomyosarcoma is attributable to compensatory increased expression of the DNA repair enzyme tyrosyl-DNA phosphodiesterase (Tdp1); resistance of some cancers to topoisomerase-I inhibitors has been attributed to overexpression of Tdp1 (18–21). Such overexpression might also suggest that rhabdomyosarcoma tumors have defective DNA double-strand break repair as expression of the Tdp1-PARP-1 complex is increased in other cancers with such deficits (22–24). To assess whether Tdp1 and PARP-1 might be targets for treatment of rhabdomyosarcoma, we profiled the expression of Tdp1 in human pediatric tumors and analyzed the effect of topoisomerase-I inhibition, TDP1 knockdown, PARP-1 inhibition, and nutrient restriction on rhabdomyosarcoma cell lines.

Materials and Methods

Human subjects

Human tissue and material use for this study was approved by the Institutional Review Board of the University of British Columbia (Vancouver, BC; H09-03301).

TMA construction

We assembled pediatric tumor samples from cases referred to BC Children’s Hospital (Vancouver, BC) into 4 tissue microarray (TMA) blocks. The tumors that were readable (vs. total number of tumor samples) included alveolar rhabdomyosarcoma (n = 18/21), embryonal rhabdomyosarcoma (n = 24/25), ganglioneuroma (n = 12/14), neuroblastoma (n = 23/30), Ewing sarcoma (n = 10/22), medulloblastoma (n = 9/14), and Wilms tumor (n = 24/24). Of the 46 rhabdomyosarcoma tumors, 14 were metastatic. Molecular diagnosis conducted on 31 of 46 rhabdomyosarcoma tumors identified 18 fusion-positive and 13 fusion-negative tumors. Hematoxylin–eosin images of all cores are provided online at http://www.crfi.ca/pallenlab/

Additional TMAs were obtained from the Children’s Oncology Group (COG). These included 34 embryonal rhabdomyosarcoma and 39 alveolar rhabdomyosarcoma tumors derived from individuals enrolled in protocols 9602, 9150, D9902, D9802, and D9803.

Immunohistochemistry

The TMAs were screened for Tdp1 expression according to standard immunohistochemistry protocols. Briefly, 4 μm thick sections were cut from each block and immunostained on a Ventana Discovery XT staining system (Ventana Medical Systems). Sections were deparaffinized in xylene, dehydrated through 3 alcohol changes and transferred to Ventana Wash Solution. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. Antigen retrieval was conducted in Ventana CC1 buffer. Slides were incubated with anti-Tdp1 rabbit antibody (dilution 1:200) for 32 minutes and developed in DAB for 10 minutes. Tissue sections were then counterstained with hematoxylin and mounted. Preimmune serum was used as a negative control in place of immune serum.

Three independent observers scored Tdp1 expression. Any discrepancies between the 3 scores were resolved by C.F. Boerkoel. Images of each tumor were acquired using a Zeiss Axiocam HR camera, and the Zeiss Axiosvision imaging system.

Gene expression arrays

Transcriptome-profiling data on primary rhabdomyosarcoma tumors and 7 normal skeletal muscle specimens generated using Human Genome U133A Arrays (Affymetrix) were obtained from the NCI caArray (trich-00099) and GEO (GSE873, GSE1462) databases. From the available 185 samples in the caArray database, expression data on 147 primary rhabdomyosarcoma were selected for analysis after confirmation of their diagnosis and PAX-FOXO1 translocation status. In addition, expression data on 5 randomly selected samples each of non-rhabdomyosarcoma soft tissue sarcoma (NRSTS), osteosarcoma (OS), and Ewing sarcoma (ES) were obtained from GEO (GSE16088, GSE37372) and NCI caArray (trich-00099) databases. Tdp1 expression was compared among specimens using Partek Genomics Suite Software (Partek) after quantile normalization by robust multichip averaging and median summarization of raw data using one-way ANOVA.

Cell culture

RH30 (alveolar rhabdomyosarcoma, PAX3-FOXO1) and CW9019 (alveolar rhabdomyosarcoma, PAX7-FOXO1) cells were cultured in Dulbecco’s modified Eagle medium...
(DMEM; Gibco BRL Life Technologies) supplemented with 10% heat-inactivated FBS (Hyclone) and 1% antibiotic–antimycotic (Gibco BRL Life Technologies). RD (embryonal rhabdomyosarcoma) cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 1% antibiotic–antimycotic, 4 mL/L t-glutamine (Gibco BRL Life Technologies), 4.5 g/L glucose, and 1.5 g/L NaHCO₃. Human skeletal myoblast cells were cultured using skBM-2 (Lonza) supplemented with 15% FBS, 1% antibiotic–antimycotic and 9 g/L d-glucose (final concentration 10 g/L glucose). A204 (embryonal rhabdomyosarcoma) and Birch (embryonal rhabdomyosarcoma) cells were grown in RPMI-1640 media (Gibco BRL Life Technologies) supplemented with 10% FBS and 1% antibiotic–antimycotic. Rhabdomyosarcoma cell lines were procured from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures or the American Type Culture Collection. The human skeletal muscle cell-line was obtained from Lonza (XM13A1). All cells were grown at 37°C with 5% CO₂ in a humidified environment. Human skeletal muscle cells under nutrient-restricted conditions were cultured in skBM-2 supplemented with 1% heat-inactivated FBS and containing 1 g/L d-glucose. RH30 and CW9019 cells under nutrient-restricted conditions were cultured in glucose-free DMEM supplemented with 1% heat-inactivated FBS and 1.0 g/L glucose. A204 and Birch cells under nutrient-restricted conditions were cultured in glucose-free RPMI supplemented with 1% heat-inactivated FBS and 1.0g/L glucose.

Anti-Tdp1 serum production

An anti-human Tdp1 serum was generated in rabbits against amino acids 1–152 of the human Tdp1 protein as previously described (25). The specificity of the antiserum was confirmed by immunofluorescent comparison with Tdp1+/+/ and Tdp1+/−/ mouse skeletal muscle (Supplementary Fig. S1).

Immunofluorescence

Immunofluorescence was conducted as described by Hirano and colleagues (25). Rabbit anti-Tdp1 and mouse anti-α-tubulin (DM1A, Sigma) were diluted 1:100 and 1:400, respectively. Images were acquired using a Zeiss Axiovert 200 microscope, a Zeiss AxiocamMR camera, and the Zeiss Axiovision imaging system.

Immunoblot analysis

Immunoblotting was conducted as previously described (25). Antibodies used were anti-human TDP1 (1:1,000), mouse anti-GAPDH monoclonal antibody (1:5,000; Chemicon), mouse anti-α-tubulin (1:1,000; Abcam), rabbit anti-histone H2B (1:1,000; Upstate), rabbit anti-PARP-1 (1:400, Abcam), mouse anti-Poly-ADP Ribosyl Polymer PAR (1:1000; Abcam) and rabbit anti-caspase-3 (1:1,000, Cell signaling).

qRT-PCR

RNA was isolated from each cell line using the Qiagen RNeasy Mini Kit (Qiagen) and 3 μg were reverse transcribed with qScript cDNA supermix (Quanta Biosciences). Quantitative PCR was then done using PerfeCTa Sybergreen Mix (Quanta Biosciences) and the 7500 Applied Biosystems qPCR machine. Data was analyzed using the 7500 software (v2.0.1). The sequences of the primers used for qRT-PCR are listed in Supplementary Table S1.

M TT assay

Cell proliferation and viability were measured by MTT assay as previously described (26). Briefly, 5 × 10⁴ cells were plated in each well of a 96-well plate and cultured in phenol red-free media. MTT solution was prepared by dissolving 5 mg of MTT in 1 mL of 1× PBS. Following the described treatments, the culture media was supplemented with 10% MTT and incubated for 3 hours. This was followed by a 30-minute incubation in 100% dimethyl sulfoxide (DMSO). Spectrophotometry was done at 565 nm using the Wallac VICTOR2 Multilabel Plate Reader (Beckman–Coulter).

TUNEL assay

Briefly, 5 × 10³ cells were seeded per well in 6-well plates and following the described treatments, apoptotic cells were visualized by colorimetric labeling of free 3′OH DNA termini using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit according to the manufacturer’s protocol (EMD Millipore). The number of stained nuclei per 100 total cells was determined for 3 independent replicates to quantify apoptosis.

Homogeneous caspase-3/7 assay

The activity of caspases 3 and 7, which are key effectors of mammalian cell apoptosis, was measured using the Apo-ONE Homogeneous Caspase-3/7 Kit according to the manufacturer’s protocol (Promega). Briefly, 5 × 10³ cells were plated in each well of a 96-well plate and cultured for 24 hours and following the described treatments, fluorescence of the cleaved caspase-3, and -7 substrate was measured at 520 nm using the Wallac VICTOR2 Multilabel Plate Reader (Beckman–Coulter).

Comet assay

DNA damage was assessed by the alkaline comet assay as previously described (25). This assay is unable to distinguish between single-stranded versus double-stranded breaks because the alkaline conditions used also detect single-stranded break sites and convert alkali-labile apurinic and apyrimidinic sites to DNA breaks. As Tdp1 also participates in the repair of apyrimidinic sites (27), the alkaline conversion of these sites to single-stranded breaks allows a greater appreciation of the function of Tdp1. Briefly, rhabdomyosarcoma cell lines were seeded at a density of 3 × 10⁵ cells in a 6-well (9.6 cm²) plate. After 24 hours, the cells were treated for 2 hours (37°C, 5% CO₂) with 4 μmol/L, 8 μmol/L, and 16 μmol/L camptothecin (CPT; Sigma), harvested by trypan- simization, resuspended in 80 μL of 0.5% low melting point agarose and pipetted onto microscope slides precoated with 1% agarose. After addition of a second layer of 1% normal melting point agarose, the cells were immersed in lysis...
PARP inhibition

Supplemental Table S2. The sequences of the shRNAs are listed in Table S1. MOI that gave the best knockdown was used for each cell line.

TDP1 knockdown

TDP1 was knocked down transiently by transfecting 8 × 10^5 cells with 100 nmol/L of pooled siRNAs (Dharmacon) in a 24-well plate using Lipofectamine 2000 (Invitrogen). Knockdown was confirmed by immunoblotting and qRT-PCR. The sequences of the siRNAs are listed in Supplemental Table S1.

For stable knockdown of TDP1, 1 to 2 × 10^6 cells were plated in each well of a 96-well plate and cultured overnight (37°C, 5% CO2). The culture medium was replaced for 4 to 18 hours with serum-free culture medium, 3 μg/mL polybrene and lentivirus carrying the SMARTvector 2.0 shRNA (Dharmacon). The infection was carried out at a multiplicity of infection (MOI) of 0.3, 1, 2, or 5. Stably infected cells were selected with 0.5 μg/mL puromycin and ultimately the MOI that gave the best knockdown was used for each cell line. Knockdown of TDP1 was verified by immunoblotting and qRT-PCR. The sequences of the shRNAs are listed in Supplemental Table S2.

PARP inhibition

PARP inhibitors AG-014699 (Rucaparib), AZD-2281 (Olaparib), and ABT-888 (Velparib) were obtained from Selleck Chemicals. The LD_{50} for each inhibitor was determined by seeding each wild-type rhabdomyosarcoma cell line at 5 × 10^4 cells per well in 96-well microtiter plates. After 24 hours, the PARP inhibitors were added to the culture medium to a final concentration of 0.1 μmol/L, 0.5 μmol/L, 1 μmol/L, or 10 μmol/L. Cell viability and proliferation were measured in triplicate every 24 hours for 96 hours by the MTT assay.

Tdp1 cleavage assay

Cells were lysed and their nuclear contents extracted as previously described (28). Briefly, cells were pelleted and lysed in 1 mL of lysis buffer. The cells were placed on ice for 10 minutes and vortexed every 2 minutes to allow complete lysis. The nuclei were pelleted for 4 minutes at 2,000 rpm.

Following removal of the supernatant, nuclei were pelleted for 4 minutes at 2,000 rpm at 4°C for 1 hour to isolate nuclear proteins. The lysate was clarified for 10 minutes at 11,000 rpm to obtain nuclear extracts. The supernatant containing nuclear proteins were diluted 1:10 in reaction buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.01% Brij-35, and pH 7.5).

Tdp1 enzymatic activity was determined by cleavage of an artificial substrate (Dean and colleagues; manuscript in preparation). Briefly, the assay was run at room temperature, in 96-well format with a final volume of 100 μL per well. A final concentration of 50 nmol/L DNA substrate was used (5'-/56-TAMN/AGT CAC TAA AAG ACT T/3BHQ_1/-3'). Kinetic analysis was conducted using a Varioskan Plate Reader (Thermo Scientific) at Ex557/Em582 for the TAMRA fluorophore. To establish Tdp1 cleavage activity, 20 reads were recorded at a kinetic interval of 45 seconds per read. A 2.5 nmol/L of purified Tdp1 was used in the control samples. Purified Tdp1 was isolated as previously described.

Statistical analysis

Microsoft Excel was used to compute the group means and SDs for all treatment and control groups from cell viability data derived from the MTT, caspase, and apoptosis assays. The GraphPad Prism software was used for two-way ANOVA to compare the differences in growth and viability under varying treatment conditions over time. The F-ratio was used to test the validity of interactions between treatment groups over the course of the experiment. Statistical significance between individual groups was established by the Student t test and a P value of less than 0.05 was judged significant. The Bonferroni correction was applied to all comparisons of replicate means for the duration of the experiments.

Sequencing of TDP1, BRCA1 and BRCA2

Genomic DNA was isolated from rhabdomyosarcoma cell lines using the Gentra Puregene Blood Kit (Qiagen). RNA was extracted from rhabdomyosarcoma cells using the Qiagen RNeasy Kit (Qiagen) and cDNA synthesized using the qScript cDNA SuperMix. PCR amplification of the TDP1 coding exons was conducted as previously described (29). PCR amplification of the BRCA1 cDNA and of the BRCA2 exons was conducted using the primers listed in Supplementary Table S4 and HotStar Taq Plus (Qiagen) with the following conditions: Denaturation stage: 95°C for 5 minutes, cycling stage (40 cycles): 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute. End stage: 72°C, 10 minutes, hold at 4°C. All amplicons were sequenced at Macrogen and the sequences were aligned and analyzed using Sequencher 4.3 (Gene Codes).

Results

Tdp1 is highly expressed in rhabdomyosarcoma tumors

To determine whether Tdp1 is expressed in rhabdomyosarcoma and other pediatric solid tumors, we immunohistochemically screened a pediatric tumor microarray consisting of 24 embryonal rhabdomyosarcoma, 18 alveolar rhabdomyosarcoma, 12 ganglioneuroma, 23 neuroblastoma, 10 Ewing sarcoma, 9 medulloblastoma, and 24 Wilms tumor readable tissue cores. This showed that Tdp1 was expressed with the highest frequency in rhabdomyosarcoma tumors (Supplementary Fig. S2). To confirm this expression in rhabdomyosarcoma tumors, we obtained 34 embryonal rhabdomyosarcoma and 39 alveolar rhabdomyosarcoma...
tumors from the COG and observed Tdp1 expression respectively in 97% and 100% of the tumors. In comparison with adjacent noncancerous tissue, Tdp1 also seemed to have increased expression in the cancer cells (Fig. 1A–C).

To confirm TDP1 mRNA expression in rhabdomyosarcoma tumors, we screened an independent soft tissue sarcoma cohort that included 20 rhabdomyosarcoma (specifically, 10 PAX-FKHR-positive aRMS, 5 PAX-FKHR-negative aRMS, and 5 eRMS), 9 fusion-negative RMS, 69 eRMS, and 5 spindle RMS. The boxes represent median with interquartile range; whiskers extend 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter. Dots represent outliers.

Figure 1. Tdp1 expression in rhabdomyosarcoma (RMS) tumors. Photomicrographs showing immunohistochemical detection of Tdp1 in alveolar RMS (aRMS; A), embryonal RMS (eRMS; B), and unaffected human skeletal muscle tissue (C). Scale bar = 10 μm. D, graph of the TDP1 transcript intensity in soft-tissue sarcomas as measured by Affymetrix array. NRSTS, Non-Rhabdomyosarcoma Soft Tissue Sarcoma; OS, osteosarcoma; and ES, Ewing sarcoma. E, Tukey box plots representing Tdp1 expression intensities across 7 normal skeletal muscle samples, 44 PAX3-FKHR RMS, 17 PAX7-FKHR RMS, 9 fusion-negative RMS, 69 eRMS, and 8 spindle RMS. The boxes represent median with interquartile range; whiskers extend 1.5 times the interquartile distance or to the highest or lowest point, whichever is shorter. Dots represent outliers.
192.2; Mann–Whitney $P < 0.001$, data not shown). Thus, although TDPI is highly expressed in rhabdomyosarcoma in general ($P < 0.001$), it is most significantly overexpressed in fusion-positive rhabdomyosarcoma ($P < 0.001$) compared to normal skeletal muscle. This suggests a correlation between rhabdomyosarcoma subtypes and TDPI expression.

Tdp1 is highly expressed in rhabdomyosarcoma cell lines

To validate further expression of Tdp1 in rhabdomyosarcoma cells, we studied rhabdomyosarcoma cell lines RD, A204, Birch, RH30, and CW9019. Immunoblotting and qRT-PCR indicated increased expression of TDP1 mRNA and protein (Fig. 2A and B) in rhabdomyosarcoma cell lines relative to skeletal muscle. Immunoblotting for Tdp1 protein confirmed that Tdp1 was most abundant in the cell lines that most highly express TDP1 mRNA (CW9019 and RH30). For each cell line, immunofluorescence staining of Tdp1 showed both nuclear and cytoplasmic expression (Fig. 2C), and sequencing of all TDP1 exons detected no mutations within the exons or canonical splice sites (data not shown).

Figure 2. Tdp1 expression in alveolar rhabdomyosarcoma (aRMS) and embryonal RMS (eRMS) cell lines. A, graph showing the level of TDP1 mRNA in rhabdomyosarcoma cell lines relative to unaffected skeletal muscle and standardized to GAPDH mRNA levels ($n = 3$). B, immunoblot showing Tdp1 protein expression in rhabdomyosarcoma cell extracts. GAPDH was used as a loading control. C, photomicrographs showing immunofluorescent detection of Tdp1 expression in the nucleus and cytoplasm of cultured embryonal (A204, RD, and Birch) and alveolar (CW9019 and RH30) rhabdomyosarcoma cells. $n$, number of replicates. Scale bar = 10 μm.
**TDP1** knockdown preferentially increases CPT sensitivity of rhabdomyosarcoma cell lines compared with control skeletal myoblasts

As Tdp1 expression is elevated in rhabdomyosarcoma cells relative to skeletal muscle, this might explain the unresponsiveness of rhabdomyosarcoma to treatment with the irinotecan, an analogue of camptothecin (CPT; ref. 18). Consistent with this, siRNA knockdown of **TDP1** by 70% to 85% decreased the viability of all CPT-treated rhabdomyosarcoma cell lines except RD (Supplementary Fig. S3A and Fig. 3A). In contrast, siRNA **TDP1** knockdown of nearly 90% (Supplementary Fig. S3A) did not significantly decrease the viability of CPT-treated control skeletal myoblasts. As complete loss of functional Tdp1 dramatically

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**Figure 3.** **TDP1** knockdown increases the sensitivity of rhabdomyosarcoma cell lines to CPT treatment. A, graphs showing the percent proliferation of rhabdomyosarcoma cells and control myoblasts over a 96-hour period during which the cells (i) received no CPT, (ii) were treated with 1 μmol/L CPT, or (iii) were treated with 1 μmol/L CPT after transient **TDP1** knockdown (n = 4). B, graphs showing the results of an alkaline comet assay, which analyzes the number of DNA breaks (tail moment). The assay was performed after 2 hours of culture under the conditions stated above. Each dot represents a single cell/comet. A total of 100 cells were scored for each treatment. Error bars, mean ± SD; **, P < 0.01; ***, P < 0.001.
increased the sensitivity of mouse embryonic fibroblasts to CPT (Supplementary Fig. S3B), we hypothesized that residual Tdp1 activity provided this resistance of skeletal myoblasts to CPT and indeed observed 50% residual activity among myoblasts with 85% knockdown of TDP1 mRNA (Supplementary Fig. S4).

To determine whether the increased sensitivity of rhabdomyosarcoma cells to CPT arose from accumulation of DNA breaks, we used alkaline comet assays to compare 1 μmol/L CPT treatment alone and 1 μmol/L CPT treatment plus knockdown of TDP1. The combined treatment induced 5-fold more DNA strand breaks in A204, Birch, and CW9019 cells and 15-fold more in RH30 cells over cells treated with CPT alone (Fig. 3B). In contrast, combined treatment did not significantly increase the amount of DNA breaks over CPT treatment alone in control myoblasts.

To determine whether this differential sensitivity of rhabdomyosarcoma cells versus control myoblasts was attributable to different proliferation rates that gave rise to differences in the likelihood of collision between the replication apparatus and DNA-TopoI complexes, we analyzed the consequences of TDP1 knockdown on the breast cancer cell line MDA-231. The proliferation rate of MDA-231 cells was 16 hours (Supplementary Fig. S5A); a rate that is comparable with that of CW9019 cells (Fig. 3A) and approximately 3-fold faster than normal myoblasts (48 hours; Fig. 3A). TDP1 expression in MDA-231 cells was comparable with that of the myoblasts (Supplementary Fig. S5B). A 1 μmol/L CPT treatment of MDA-231 cells after 75% TDP1 knockdown did not significantly increase the amount of DNA breaks compared with CPT treatment alone (Supplementary Fig. S5C and S5D).

**PARP-1 expression is increased in rhabdomyosarcoma cell lines and its inhibition preferentially increases the CPT sensitivity of rhabdomyosarcoma cell lines compared with control skeletal myoblasts**

As PARP-1 and Tdp1 are in the same DNA-repair complex, we hypothesized that PARP-1 inhibition also sensitizes rhabdomyosarcoma cells to CPT. As observed for Tdp1, rhabdomyosarcoma cells expressed more PARP-1 mRNA and protein than control skeletal myoblasts (Supplementary Fig. S6A and S6B). The administration of the PARP-1-specific inhibitor AG-014699 (Rucaparib) reduced the amount of PARP-1 (poly-ADP ribosyl polymers, PAR) products in all cell lines (Fig. 4, insets). As shown by comet assay (Supplementary Fig. S7), treatment with 10 μmol/L rucaparib increased the number of DNA breaks in all rhabdomyosarcoma cell lines exposed to 1 μmol/L CPT by 4 to 5-fold compared with treatment with 1 μmol/L CPT alone. In contrast, the same treatment on control myoblasts only increased the sensitivity to 1 μmol/L CPT by 2-fold.

To ascertain whether inhibition of PARP-2 in conjunction with inhibition of PARP-1 would be more effective than inhibition of PARP-1 alone, we tested inhibitors targeting both PARP-1 and PARP-2. In contrast with Tdp1 and PARP-1, PARP-2 mRNA and protein expression are unaltered or decreased in most rhabdomyosarcoma cells lines compared with control myoblasts (Supplementary Fig. S6A and S6C). As measured by comet assay, AZD-2281 (Olaparib) and ABT-888 (Veliparib), which inhibit both PARP-1 and PARP-2, sensitized rhabdomyosarcoma cells less to CPT than did Rucaparib (Supplementary Fig. S7). As Rucaparib ($K_i = 1.4$ nmol/L) is a more potent inhibitor of PARP-1 than are Olaparib ($K_i = 5$ nmol/L), and Veliparib ($K_i = 5.2$ nmol/L), we hypothesize that the difference in effectiveness of these 3 compounds is the differential inhibition of PARP-1.

**TDP1 knockdown and PARP-1 inhibition alone and together are more cytotoxic to rhabdomyosarcoma cells than to control skeletal myoblasts**

We observed that either TDP1 knockdown or PARP-1 inhibition impaired proliferation of the rhabdomyosarcoma cell lines (Fig. 4A), as measured using the MTT assay. About 70% to 90% siRNA knockdown of TDP1 reduced proliferation of A204, Birch, and RH30 by 30%, 50%, and 30% respectively, but had no effect on control myoblasts (Fig. 4A). Of the PARP-1 inhibitors tested, Rucaparib was most detrimental to rhabdomyosarcoma cell proliferation (Supplementary Table S3). Treatment with 10 μmol/L Rucaparib for 96 hours led to a 50% or greater decrease in proliferation of 4 of 5 rhabdomyosarcoma cell lines compared with untreated cells, but only a 10% decrease in the proliferation of control myoblasts (Supplementary Table S3 and Fig. S4A). Excepting Birch, this reduction in proliferation is 2-fold more than that observed with TDP1 knockdown alone (Fig. 4A), suggesting that either PARP-1 inhibition is more effective than TDP1 knockdown or that PARP-1 inhibition is affecting pathways essential for rhabdomyosarcoma cell survival other than those involving Tdp1.

Investigating whether TDP1 knockdown and PARP-1 inhibition are additive, we found that the combination of 70% to 90% knockdown of TDP1 and 10 μmol/L Rucaparib decreased A204, Birch, RH30, and CW9019 cell proliferation more than either treatment alone; this treatment had no effect on control myoblasts (Fig. 4A). Over the course of 96 hours, A204, Birch, and RH30 cell numbers declined by 70% to 80%, and as measured by caspase-3/7 expression and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)-positive staining, this toxicity correlated with induction of apoptosis (Fig. 4B and C), implying that cytotoxicity was responsible for the decline in proliferation. In addition, suggestive of TDP1 knockdown and PARP-1 inhibition targeting the same pathway, constitutive TDP1 knockdown (Supplementary Fig. S3C and S3D) resulted in the selection of cells that were resistant to 10 μmol/L rucaparib treatment, whereas cells constitutively expressing a nontargeting shRNA remained sensitive to PARP-1 inhibition (Supplementary Fig. S8A).

**Rhabdomyosarcoma cell lines do not harbor pathogenic BRCA1 or BRCA2 mutations**

On the basis of the effect of PARP-1 inhibitors on cancer cells with BRCA1 or BRCA2 mutations or other defects of
DNA double-strand break repair (7, 30), we postulated that the cytotoxicity of TDP1 knockdown and PARP-1 inhibition on rhabdomyosarcoma cells could arise from loss of functional BRCA1 or BRCA2. However, qRT-PCR did not detect a significant loss of BRCA1 or BRCA2 mRNA and sequencing of the BRCA1 cDNA and BRCA2 exons detected no pathogenic mutations (Supplementary Tables S4 and S5, Supplementary Fig. S8B). Therefore, BRCA1 and BRCA2 expression did not account for the sensitivity of rhabdomyosarcoma cells to PARP-1 inhibition.

In the absence of BRCA1 and BRCA2 mutations, reversion of such mutations does not explain the PARP-1 inhibitor resistance of rhabdomyosarcoma cells with constitutive TDP1 knockdown. As a result, no pathogenic mutations (Supplementary Tables S4 and S5, Supplementary Fig. S8B). Therefore, BRCA1 and BRCA2 expression did not account for the sensitivity of rhabdomyosarcoma cells to PARP-1 inhibition.

Serum and glucose restriction is cytotoxic to Birch and CW9019 cell lines
Despite the absence of deleterious BRCA1 and BRCA2 mutations, the sensitivity of rhabdomyosarcoma cells to Tdp1 knockdown and PARP-1 inhibition suggests they harbor a defect of DNA repair. Therefore, as glucose and
serum restriction increase cellular oxidative stress and error-prone DNA repair in mammalian cells (32), we tested whether this was cytotoxic to rhabdomyosarcoma cells. As measured by MTT, caspase-3/7 and TUNEL assays, glucose, and serum restriction were cytotoxic to Birch and CW9019 but not to other rhabdomyosarcoma cell lines or control skeletal myoblasts (Fig. 5A–C).

Serum and glucose restriction sensitzes rhabdomyosarcoma cells to PARP-1–mediated cytotoxicity compared with control skeletal myoblasts

Although PARP-1 inhibition in normal tissues can protect tissues from serum starvation by maintaining cellular energy and activating survival pathways (33), recent findings that serum and glucose restriction sensitizes cancer cells to genotoxic agents led us to hypothesize that such restriction increases sensitivity of rhabdomyosarcoma cells to PARP-1 inhibitors (34). Indeed, serum and glucose restriction increased the cytotoxicity of 10 μmol/L rucaparib on the alveolar rhabdomyosarcoma cell lines RH30 and CW9019 but not the embryonal rhabdomyosarcoma cell lines or control myoblasts (Fig. 5A–C).

Rhabdomyosarcoma cells resistant to Tdp1 knockdown and PARP-1 inhibition, are sensitive to serum and glucose restriction

To determine whether nutrition restriction was a potential complementary therapy to TDP1 knockdown and PARP-1 inhibition, we determined whether serum and glucose restriction were cytotoxic to rhabdomyosarcoma

Figure 5. PARP-1 inhibition sensitizes rhabdomyosarcoma cells to nutrient restriction. A, graphs showing the number of rhabdomyosarcoma cells and control myoblasts over a 96-hour period during which the cells were subjected to (i) normal cell culture media (4.5g/L glucose, 10% FBS), (ii) 10 μmol/L rucaparib treatment, (iii) nutrient restriction (1g/L glucose, 1% FBS; NR), and (iv) combination of 10 μmol/L rucaparib treatment and nutrient restriction (n = 4). B, graph of caspase-3/7 cleavage for all cell lines under conditions 1 through 4 above (n = 3). C, graph of the number of terminally apoptotic cells as measured by DNA strand break assay for all cell lines under conditions 1 through 4 above (n = 3). NR, nutrient restriction. Error bars, mean ± SD among treatments 2, 3, and 4. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
cells resistant to 10 μmol/L PARP-1 inhibition and constitutive \( TDP1 \) knockdown. We found that serum and glucose restriction did cause apoptosis of A204, Birch, RH30, and CW9019 cells resistant to PARP-1 inhibitors, whereas control myoblasts were minimally affected (Fig. 6A–C).

**Discussion**

We have shown that Tdp1 and PARP-1 are highly expressed in rhabdomyosarcoma tumors and that as predicted from the functions of Tdp1 and PARP-1 in DNA repair, knockdown of \( TDP1 \) and inhibition of PARP-1 sensitized rhabdomyosarcoma cells to CPT treatment. Suggestive of an intrinsic defect of DNA repair in rhabdomyosarcoma cell lines, \( TDP1 \) knockdown and PARP-1 inhibition alone or in combination also caused apoptosis of rhabdomyosarcoma cells. In addition, stressing the DNA repair and replication system through glucose and serum restriction reduced viability of some rhabdomyosarcoma cells and enhanced sensitivity to PARP-1 inhibitors. Remarkably, each of these manipulations preferentially affected tumor cells compared with control skeletal myoblasts.

Tdp1 and PARP-1 are members of a DNA single-strand break repair complex that also includes XRCC1, Ligase III, and PNKP (35). Within this complex, Tdp1 processes topoisomerase-I–DNA complexes and blocked 3'0-termini (27). Tdp1 mutant cell lines and knockout mice have increased sensitivity to several anticancer agents (25, 36). These observations have consequently led to the suggestion that Tdp1 overexpression in tumor cells confers resistance to...
anticancer agents, particularly the CPT analogues (19). The increased expression of Tdp1 in rhabdomyosarcoma compared with unaffected skeletal muscle and the increased sensitivity of rhabdomyosarcoma cells to CPT after TDP1 knockdown or PARP-1 inhibition suggest a possible answer for the failure of irinotecan to show efficacy against rhabdomyosarcoma (18).

For the rhabdomyosarcoma cell lines RD, A204, Birch, RH30, and CW9019, this markedly elevated expression of Tdp1 is not explicable as a compensatory reaction to a TDP1 hypomorphic allele or to aberrant Tdp1 subcellular localization. As many cancers compensate for defects of DNA repair, which are permissive for rapid tumor evolution, by overexpression of other DNA repair pathways (37, 38), we had wondered whether rhabdomyosarcoma cells might also have a defect of DNA repair and whether the overexpression of Tdp1 was a compensatory survival mechanism. Finding that TDP1 knockdown and PARP-1 inhibition preferentially decreased rhabdomyosarcoma cell viability over that of control myoblasts, we can now hypothesize that rhabdomyosarcoma tumors have a damaged DNA repair process that is at least partially compensated for by processes in which Tdp1 and PARP-1 are involved.

Tdp1 and PARP-1 are involved in repairing both single- and double-strand DNA breaks (36, 39, 40). However, PARP-1 has in addition been shown to participate in the processes of DNA replication and transcription (41, 42). The role of PARP-1 in these latter functions might partially explain why PARP-1 inhibition had a more deleterious effect on rhabdomyosarcoma cells than did TDP1 knockdown. Alternatively, PARP-1 inhibition might have reduced DNA-repair activity more than TDP1 knockdown as the knockdown alone left more than 10% expression of Tdp1. Supporting the last explanation, 85% knockdown of TDP1 diminished Tdp1 cleavage activity by 50% in myoblasts and was insufficient to induce CPT hypersensitivity, a property of tissues and organisms fully deficient for Tdp1 (25, 35, 36).

Even though TDP1 knockdown was insufficient to cause hypersensitivity of control myoblasts to CPT, it was sufficient to decrease the viability of rhabdomyosarcoma cells and to increase their sensitivity to CPT. These observations raise the possibility of selectively targeting Tdp1 and PARP-1 to sensitize cancer but not noncancerous cells. In addition, Tdp1 knockdown or PARP-1 inhibition alone were cytotoxic to a subset of cells in all rhabdomyosarcoma cell lines analyzed. However, because some cells were resistant to these treatments, rhabdomyosarcoma cells have or rapidly develop inhibitor resistance and that BRCA1 expression is relevant to the resistance to PARP-1 inhibition, then there must be other mutations of DNA repair in the rhabdomyosarcoma cell lines to complement the reduced TP53BP1 expression. In addition, the presence or development of cells resistant to TDP1 knockdown and PARP-1 inhibition suggest that targeting Tdp1 and PARP-1 in rhabdomyosarcoma needs to be part of a multimodal therapy.

Targeting the altered energy metabolism common among solid tumors is a potential component of multimodal therapy (45). Altered tumor metabolism arises because although solid tumor cells require a high metabolic rate to support their rapid proliferation (46), they come to rely on inefficient anaerobic metabolism as they outgrow their blood supply. In a recent study, glucose deprivation in glioblastoma cells triggered mitochondria-derived reactive oxygen species (ROS) production that caused ROS-mediated cell death (47). As Tdp1 and PARP-1 contribute to the base excision repair processes targeting oxidatively damaged nuclear and mitochondrial DNA (48–50), the complementation between inhibition of Tdp1 or PARP-1 and glucose deprivation has a logical basis. Further studies are required; however, to test this hypothesis as was recently done for other cell lines (49, 51). Nonetheless, the observation does suggest that nutritional restriction is a consideration for treatment of rhabdomyosarcoma.

A final therapeutic consideration arising from our study is that none of the 5 rhabdomyosarcoma cell lines responded identically to any one therapy, let alone all therapies. This suggests that the genetic and epigenetic defects intrinsic to each rhabdomyosarcoma tumor are likely different. In the understanding of these differences resides the ability to target therapies to the tumor and minimally harm normal tissues. The 3 potential therapeutic approaches identified herein all preferentially affected rhabdomyosarcoma cell lines compared with control myoblasts and thus provide direction for tumor stratification, analysis of genomic studies of rhabdomyosarcoma tumors and a basis for future preclinical trials.

In summary, we have shown that TDP1 knockdown and PARP-1 inhibition increase the sensitivity of rhabdomyosarcoma cells to CPT analogues and have a direct toxic effect on some rhabdomyosarcoma cell types. We also find that glucose and serum restriction can be cytotoxic to rhabdomyosarcoma cells and sensitize them to PARP-1 inhibition. Although further study is needed to understand the molecular mechanisms underlying the effects of these therapies on rhabdomyosarcoma cells and to determine whether they have relevance in vivo, these or similar therapies have the potential to improve outcomes for individuals with rhabdomyosarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The contents of this manuscript have not been published, in whole or in part, prior to or simultaneous with our submission of the manuscript to Molecular Cancer Research. The necessary ethics committee approval was secured for the study reported.


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**References**


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## TDP1 and PARP1 Deficiency Are Cytotoxic to Rhabdomyosarcoma Cells

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