PARP Inhibitors Sensitize Ewing Sarcoma Cells to Temozolomide-Induced Apoptosis via the Mitochondrial Pathway

Florian Engert, Cornelius Schneider, Lilly Magdalena Weiβ, Marie Probst, and Simone Fulda

Abstract

Ewing sarcoma has recently been reported to be sensitive to poly(ADP)-ribose polymerase (PARP) inhibitors. Searching for synergistic drug combinations, we tested several PARP inhibitors (talazoparib, niraparib, olaparib, veliparib) together with chemotherapeutics. Here, we report that PARP inhibitors synergize with temozolomide (TMZ) or SN-38 to induce apoptosis and also somewhat enhance the cytotoxicity of doxorubicin, etoposide, or ifosfamide, whereas actinomycin D and vincristine show little synergism. Furthermore, triple therapy of olaparib, TMZ, and SN-38 is significantly more effective compared with double or monotherapy. Mechanistic studies revealed that the mitochondrial pathway of apoptosis plays a critical role in mediating the synergy of PARP inhibition and TMZ. We show that subsequent to DNA damage-imposed checkpoint activation and G2 cell-cycle arrest, olaparib/TMZ cotreatment causes downregulation of the antiapoptotic protein MCL-1, followed by activation of the proapoptotic proteins BAX and BAK, mitochondrial outer membrane permeabilization (MOMP), activation of caspases, and caspase-dependent cell death. Overexpression of a nondegradable MCL-1 mutant or BCL-2, knockdown of NOXA or BAX and BAK, or the caspase inhibitor N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) all significantly reduce olaparib/TMZ-mediated apoptosis. These findings emphasize the role of PARP inhibitors for chemosensitization of Ewing sarcoma with important implications for further (pre)clinical studies.

Introduction

Ewing sarcoma is the second most common pediatric primary bone tumor in children and young adults (1). State-of-the-art treatment options for Ewing sarcoma patients include chemotherapy consisting of vincristine, ifosfamide, doxorubicin, temozolomide (TMZ), actinomycin D, irinotecan, etoposide, and cyclophosphamide (2). Despite aggressive treatment protocols, patients especially with advanced or relapsed diseases still harbor an overall poor survival (2). This highlights the unmet medical need to develop innovative treatment strategies.

Tumor response to cytotoxic therapies including chemotherapy critically relies on intact cell death programs in cancer cells, as chemotherapeutics exert their anticancer effects to a large extent by engaging programmed cell death (3). Apoptosis is mediated via two well-defined pathways, that is the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway, which both eventually lead to activation of caspases as cell death effector molecules (4). Engagement of the mitochondrial pathway results in mitochondrial outer membrane permeabilization (MOMP)

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Simone Fulda, Goethe-University, Komturstr. 3a, 60528 Frankfurt, Germany. Phone: 49-69-67866557; Fax: 49-69-678659157; E-mail: simone.fulda@kgu.de

doi: 10.1158/1535-7163.MCT-15-0587

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Figure 1.
Screening for synergistic drug interactions of PARP inhibitors and chemotherapeutic drugs. A, A4573 and SK-ES-1 cells were treated for 72 hours with indicated concentrations of talazoparib, niraparib, olaparib, or veliparib. Cell viability was assessed by crystal violet assay and is shown as percentage of untreated control. B, A4573 and SK-ES-1 cells were treated for 72 hours with different PARP inhibitors (i.e., talazoparib, niraparib, olaparib, and veliparib) in combination with different cytostatic drugs (i.e., TMZ, SN-38, etoposide, ifosfamide, doxorubicin, vincristine, and actinomycin D). Cell viability was assessed by crystal violet assay. Synergistic, additive, or antagonistic drug interactions were calculated by combination index (CI). CI values were then used to generate a heatmap of drug interactions according to the subclassification provided by the software's manual.
Synergistic Apoptosis by PARP Inhibitors and Temozolomide

A4573

SK-ES-1

Cell viability (% of control)

DNA fragmentation (%)

Colonies (% of control)

Synergistic Apoptosis by PARP Inhibitors and Temozolomide

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to the role of PARP in the repair of the DNA lesions caused by these cytotoxic therapies (9, 13, 16, 17). In addition to impairing single-strand break (SSB) repair, the antitumor effects of PARP inhibitors have recently also been attributed to their ability to trap PARP–DNA complexes, thereby generating cytotoxic lesions (18).

Although different PARP inhibitors were reported to vary in their potency of PARP-DNA trapping (18, 19), they have not yet been systematically tested in combination with a range of anticancer drugs in Ewing sarcoma. In addition, little is yet known on the role of PARP in the repair of the DNA lesions caused by these cytotoxic therapies (9, 13, 16, 17). In addition to impairing single-strand break repair, the antitumor effects of PARP inhibitors have recently also been attributed to their ability to trap PARP–DNA complexes, thereby generating cytotoxic lesions (18).

Materials and Methods

Cell culture and chemicals

Ewing sarcoma cell lines were kindly provided by C. Roessig (Muenster, Germany) or obtained from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) or American Type Culture Collection (Manassas, VA, USA) in 2013. Cells were maintained in DMEM GlutaMAX-I or RPMI medium (Life Technologies, Inc.), supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1 mM sodium pyruvate (all from Life Technologies, Inc.). Cell lines were authenticated by STR profiles and regularly tested for mycoplasma contamination. Talazoparib, olaparib, and veliparib were obtained from Selleckchem and niraparib from ChemiTek. actinomycin D, doxorubicin, etoposide, TMZ, SN-38, and vincristine were purchased from Sigma, ZVAD.fmk from Bachem, bortezomib from Janssen-Cilag. 4-Hydroperoxy-ilosamidase (active metabolite of ifosfamide) was kindly provided by Baxter and TRAIL receptor-2 agonistic antibody lexatumumab (ETR2) Human Genome Sciences. Chemicals were purchased from Sigma or Carl Roth unless otherwise indicated.

Determination of apoptosis, cell viability, colony formation, and mitochondrial membrane potential (MMP)

Apoptosis was determined by flow cytometric analysis (FACS-Canto II; BD Biosciences) of DNA fragmentation of propidium iodide (PI)-stained nuclei as described previously (20). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s instructions (Roche Diagnostics) or by crystal violet assay using crystal violet solution (0.5% crystal violet, 30% ethanol, 3% formaldehyde). Plates were then rinsed with water and crystal violet incorporated by the cells was resolubilized in a solution containing 1% SDS. Absorbance at 550 nm was measured using a microplate reader (Infinite M100; Tecan). Results are expressed as percentage of cell viability relative to untreated controls. For colony formation assay, cells were treated as indicated for 24 hours. Subsequently, living cells were counted, 100 cells were reseeded, and cultured in drug-free medium for additional 12 days before fixation and staining with 0.5% crystal violet, 30% ethanol, and 3% formaldehyde. Colonies were counted macroscopically.

Western blot analysis

Western blot analysis was performed as described previously (20) using the following antibodies: BAK, BCL-2, BCL-xL (BD Biosciences), pChk1, pChk2, Chk1, Chk2, caspase-3, caspase-9, PARP, BIM, PUMA (Cell Signaling), BAX NT, pH 3, α-tubulin (Millipore), BMF (Novus Biologicals) MCL-1, NOXA, caspase-8 (Enzo Life Science), GAPDH (Hyclone), murine BCL-2 (Life Technologies, Inc.). Goat antimouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used as secondary antibodies and enhanced chemiluminescence (Amersham Bioscience) or infrared dye-labeled secondary antibodies and infrared imaging were used for detection (Odyssey Imaging System, LI-COR Bioscience). Representative blots of at least two independent experiments are shown.

Cell-cycle analysis

Cells were stained with PI as described previously (20). Cell-cycle analysis was performed using FlowJo Software (TreeStar Inc.) following the manufacturer’s instructions.

RNA interference and overexpression

Cells were reversely transfected with 10 nmol/L Silencer Select (Life Technologies, Inc.) control siRNA (10 nmol/L, 4390844), NOXA siRNA (10 nmol/L s10708 and s10709), or a combination of targeting siRNAs (5 nmol/L for BAK, s1880 and s1881; 5 nmol/L for BAX, s1889 and s1890) using Lipofectamine RNAiMAX reagent (Life Technologies, Inc.) and Opti-MEM medium (Life Technologies, Inc.). After 6 hours of incubation with transfection solution, the medium was changed and cells recovered for 48 hours before drug treatment. For transient overexpression, Ewing sarcoma cells were transfected with 4 µg of pCMV-Tag 3B plasmid [empty vector; MCL-1 4A (S64A/S121A/S159A/T163A)], supplied with Lipofectamine 2000 (Life Technologies, Inc.) and selected with 500 µg/ml G418 (Carl Roth). Murine BCL-2 was stably overexpressed by using lentiviral vectors. Briefly, Phoenix cells were transfected with 20 µg of pMSCV plasmid (empty vector, BCL-2) using calcium phosphate transfection. Virus-containing supernatant was collected, sterile-filtered, and used for spin transduction at 37°C in the presence of 8
Synergistic Apoptosis by PARP Inhibitors and Temozolomide

**A**

**A4573**

- Control
- Olaparib
- TMZ
- Olaparib/TMZ

**SK-ES-1**

- Control
- Olaparib
- TMZ
- Olaparib/TMZ

**B**

**A4573**

- Olaparib
- TMZ

- pChk1: 56 kDa
- Chk1: 56 kDa
- α-Tubulin: 55 kDa
- pChk2: 62 kDa
- Chk2: 62 kDa
- GAPDH: 36 kDa

**SK-ES-1**

- pChk1: 56 kDa
- Chk1: 56 kDa
- α-Tubulin: 55 kDa

**C**

**A4573**

- Frequency of cells (%)

- Olaparib
- TMZ

- G_{0-1}, G_{2-M}, S

**SK-ES-1**

- Frequency of cells (%)

- Olaparib
- TMZ

- G_{0-1}, G_{2-M}, S

**D**

**A4573**

- Olaparib
- TMZ
- VCR

- pH3: 17 kDa
- GAPDH: 36 kDa

**SK-ES-1**

- Olaparib
- TMZ
- VCR

- pH3: 17 kDa
- GAPDH: 36 kDa
μg/mL polybrene. Transduced Ewing sarcoma cells were selected with 10 μg/mL blasticidin (Carl Roth).

Determination of BAK and BAX activation or MMP

BAK and BAX activation was determined by immunoprecipitation of active conformation by specific antibodies. Briefly, cells were lysed in CHAPS lysis buffer (10 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl; 1% CHAPS). Five hundred micrograms of protein were incubated overnight at 4°C with 8 μg mouse anti-BAX antibody (clone 6A7; Sigma) or 0.5 μg mouse anti-BAK antibody (AB-1; Calbiochem) and 10 μL pan mouse IgG Dynabeads (Dako), washed with CHAPS lysis buffer and analyzed by Western blotting using rabbit anti-BAX NT antibody (Millipore) or rabbit anti-BAX antibody (BD Biosciences). Loss of MMP was assessed by tetramethylrhodamine methyl ester (TMRM+) staining according to the manufacturer's instructions (Life Technologies Inc.).

Statistical analysis

Statistical significance was assessed by Student t-test (two-tailed distribution, two-sample, equal variance) using Microsoft Excel (Microsoft Deutschland GmbH); * P < 0.05; ** P < 0.01; *** P < 0.001. Drug interactions were analyzed by the combination index (CI) method based on that described by Chou (21) using CalcuSyn software (Biosoft). Subclassification of CI values according to CalcuSyn's manual was used (CI < 0.1 very strong synergism, 0.1–0.3 strong synergism, 0.3–0.7 synergism, 0.7–0.85 moderate synergism, 0.85–0.9 slight synergism, 0.9–1.1 nearly additive, 1.1–1.2 slightly antagonistic, 1.2–1.45 moderate antagonistic, and CI > 1.45 antagonism.

Results

Screening for synergistic drug interactions of PARP inhibitors and chemotherapeutic drugs

To investigate the sensitivity of Ewing sarcoma against PARP inhibitors, we initially tested the dose response to four different PARP inhibitors using the Ewing sarcoma cell lines A4573 and SKES-1 and subsequently extended our studies also to two additional Ewing sarcoma cell lines. Talazoparib, niraparib, olaparib, and veliparib showed a differential ability to reduce cell viability of Ewing sarcoma cells with talazoparib being the most active and veliparib being the least active compound (talazoparib IC50 ≈ 10 nmol/L; > niraparib IC50 ≈ 300 nmol/L > olaparib IC50 ≈ 1000 nmol/L > veliparib IC50 ≈ 10000 nmol/L; Fig. 1A, Supplementary Table S1).

To investigate the question whether PARP inhibitors modulate chemosensitivity of Ewing sarcoma cells, we screened the efficacy of several anticancer drugs that are used in the clinic for the treatment of Ewing sarcoma (11) in the presence and absence of PARP inhibitors. We used suboptimal drug concentrations of PARP inhibitors and anticancer drugs that caused up to ≈ 20% reduction of cell viability when used as single agents compared with untreated control (Supplementary Fig. S1). Synergistic, additive, or antagonistic drug interactions were calculated by CI (Supplementary Table S2). CI values were then used to generate a heatmap of drug interactions according to the subclassification provided by the software's manual (Fig. 1B). The strongest synergism was observed for PARP inhibitors in combination with TMZ; the second best synergistic interaction was found for cotreatment with SN-38 (Fig. 1B, Supplementary Fig. S2). We also noted that, among the tested anticancer drugs, talazoparib preferably synergized with TMZ (Fig. 1B, Supplementary Fig. S2). In addition, PARP inhibitors enhanced doxorubicin-, etoposide- or ifosfamide-induced cytotoxicity in Ewing sarcoma cells, although less consistently (Fig. 1B, Supplementary Fig. S2). By comparison, the DNA-binding drug actinomycin D and the vinca alkaloid vincristine exerted little synergistic effects together with PARP inhibitors (Fig. 1B, Supplementary Fig. S2).

Olaparib synergizes with TMZ and SN-38 to induce cell death in Ewing sarcoma cells

Because in our screening approach we identified cotreatment of olaparib with TMZ or SN-38 as the most potent combinations, we focused our validation experiments on these combinations. Olaparib acted together with TMZ or SN-38 to increase DNA fragmentation, a typical marker of apoptosis, and to reduce cell viability compared to treatment with either agent alone (Fig. 2A and B). Moreover, we extended our studies to two additional Ewing sarcoma cell lines. Similarly, olaparib cooperated with TMZ or SN-38 to induce apoptosis and to decrease cell viability in TC-32 and TC-71 cells (Supplementary Fig. S3). To explore whether combined treatment with olaparib and TMZ or SN-38 also affects long-term survival of Ewing sarcoma cells, we performed colony assays. Of note, cotreatment of olaparib together with TMZ or SN-38 acted in concert to significantly suppress colony formation of Ewing sarcoma cells compared to treatment with olaparib, TMZ, or SN-38 alone (Fig. 2C). Because TMZ and SN-38 are administered together in clinical protocols as second-line treatment of Ewing sarcoma (22), we also tested whether the addition of olaparib further potentiates the antitumor activity of this chemotherapy regimen. Of note, triple therapy of olaparib, TMZ, and SN-38 was significantly more effective to reduce cell viability of Ewing sarcoma cells compared to treatment with single agents or to cotreatment with TMZ/SN-38 (Fig. 2D). Together, this set of experiments shows that olaparib cooperates together with TMZ or SN-38 to induce apoptosis, to reduce cell viability, and to suppress long-term clonogenic survival of Ewing sarcoma cells. To investigate in more detail the molecular mechanisms underlying the synergism of PARP inhibitors and chemotherapeutic drugs in Ewing sarcoma, we focused the subsequent experiments on TMZ.

Figure 3.

Olaparib/TMZ cotreatment causes G2 arrest prior to cell death. A, A4573 and SK-ES-1 cells were treated with 0.3 μmol/L olaparib and/or 50 μmol/L TMZ for indicated time. Apoptosis was determined by quantification of DNA fragmentation of PI-stained nuclei using flow cytometry. Data are shown as mean ± SD of three independent experiments performed in triplicate; * P < 0.05; ** P < 0.01; *** P < 0.001. B, A4573 and SK-ES-1 cells were treated with 0.3 μmol/L olaparib and/or 50 μmol/L TMZ for 18 hours. Phosphorylation of checkpoint kinases was assessed by Western blotting. Expression of GAPDH or α-tubulin served as loading controls. Representative blots of two independent experiments are shown. C, A4573 and SK-ES-1 cells were treated for 18 hours with 0.3 μmol/L olaparib and/or 50 μmol/L TMZ. DNA was stained with PI and cell-cycle analysis was performed using FlowJo software. Data are shown as mean ± SD of three independent experiments performed in triplicate; * P < 0.05; ** P < 0.01 comparing olaparib/TMZ cotreated to single treated or untreated cells in G2/M phase. D, Ewing sarcoma cells were treated with 0.3 μmol/L olaparib and/or 50 μmol/L TMZ or 2.5 nmol/L vincristine for 18 hours. Expression of mitotic marker pH3 was analyzed by Western blotting. Expression of GAPDH served as loading control. Representative blots of two independent experiments are shown.
Synergistic Apoptosis by PARP Inhibitors and Temozolomide

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Caspase-9

- 47 kDa
- 37 kDa
- 35 kDa

Caspase-3

- 32 kDa
- 19 kDa
- 17 kDa
- 12 kDa

PARP

- 116 kDa
- 89 kDa

Caspase-8

- 55 kDa
- 53 kDa
- 43 kDa
- 41 kDa

GAPDH

- 36 kDa

B

A4573

DNA fragmentation (%)

Ctrl
zVAD.fmk

SK-ES-1

DNA fragmentation (%)

Ctrl
zVAD.fmk

Olaparib/TMZ

Olaparib/TMZ

C

A4573

Loss of MMP (%)

Control
Olaparib
Olaparib/TMZ

Time (h)

0 5 10 15 20 25 30

SK-ES-1

Loss of MMP (%)

Control
Olaparib
Olaparib/TMZ

Time (h)

0 5 10 15 20 25 30

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A4573

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- 43 kDa
- 26 kDa
- 28 kDa
- 23 kDa

- 16 kDa
- 13 kDa

- 25 kDa
- 20 kDa
- 23 kDa
- 11 kDa

- 36 kDa

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Published OnlineFirst October 5, 2015; DOI: 10.1158/1535-7163.MCT-15-0587

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Olaparib/TMZ cotreatment causes G2 arrest prior to cell death

To define the onset of olaparib/TMZ-induced apoptosis we performed a kinetic analysis. Olaparib and TMZ cooperated to trigger apoptosis in a time-dependent manner starting at about 18 hours (Fig. 3A).

Next, we investigated whether the combination treatment leads to a DNA damage-related stress response. Olaparib and TMZ acted together to trigger phosphorylation of the checkpoint kinases Chk1 and Chk2 at 18 hours pointing to activation of DNA damage response pathways prior to cell death (Fig. 3B). Analysis of cell-cycle distribution using flow cytometry revealed that olaparib/TMZ cotreatment caused a significant increase of cells in G2-M phase compared with cells treated with either drug alone or untreated cells (Fig. 3C, Supplementary Fig. S4). As flow cytometric analysis of cell-cycle distribution does not allow to distinguish between G2 or M phase arrest, we additionally analyzed phosphorylation of histone H3 (pH3) as a specific M-phase marker. Olaparib/TMZ cotreatment did not cause phosphorylation of histone H3 in contrast to the microtubule-interfering drug vincristine that was used as a positive control for the induction of M-phase arrest (Fig. 3D). Together, these experiments show that olaparib and TMZ induce cell-cycle arrest in the G2 phase prior to induction of apoptosis in Ewing sarcoma cells.

Olaparib/TMZ-induced apoptosis is executed via caspase-dependent effector pathways

To explore whether induction of apoptosis involved activation of caspasas, we performed Western blotting. Olaparib and TMZ acted together to trigger cleavage of caspase-9 into active p37/p35 fragments, cleavage of caspase-3 into active p17/p12 fragments, and cleavage of PARP into p89 fragment. By comparison, little cleavage of caspase-8 was observed upon olaparib/TMZ combination treatment in contrast to treatment with the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor 2 agonistic antibody lexatumumab that served as positive control (Fig. 4A). Expression levels of caspase-8 were very low in A4573 cells consistent with previous studies demonstrating that caspase-8 is frequently silenced by epigenetic mechanisms in Ewing sarcoma (23, 24).

To test whether caspase activity is required for the induction of apoptosis, we used the broad-range caspase inhibitor zVAD.fmk. Addition of zVAD.fmk significantly diminished olaparib/TMZ-induced apoptosis compared with cells that were treated with olaparib/TMZ in the absence of zVAD.fmk (Fig. 4B). These experiments show that olaparib and TMZ cooperate to trigger caspase activation and caspase-dependent apoptosis in Ewing sarcoma cells.

Olaparib/TMZ cotreatment downregulates MCL-1 levels

To investigate whether olaparib/TMZ combination treatment engages the mitochondrial pathway of apoptosis, we assessed the MMP. Olaparib and TMZ cooperated to trigger loss of MMP in a time-dependent manner at the onset of apoptosis (Fig. 4C). Because BCL-2 family proteins are key regulators of the mitochondrial pathway, we then asked whether olaparib/TMZ cotreatment causes changes in their expression levels. Of note, cotreatment with olaparib and TMZ resulted in downregulation of MCL-1, whereas expression levels of BCL-2, BCL-xL, BIM, BMF, and PUMA remained largely unchanged (Fig. 4D). We also noted that NOXA levels decreased upon olaparib/TMZ cotreatment (Fig. 4D).

Olaparib/TMZ cotreatment promotes proteasomal degradation of MCL-1

To elucidate the role of MCL-1 in olaparib/TMZ-mediated cell death we overexpressed a phosphomutant variant of MCL-1 (MCL-1 4A), which is resistant to phosphorylation of a phospho-degron and subsequent proteasomal degradation (Fig. 5B). Notably, ectopic expression of nondegradable MCL-1 mutant significantly decreased olaparib/TMZ-induced apoptosis (Fig. 5C). Because NOXA is known to specifically antagonize MCL-1, we also knocked down NOXA by RNAi to further investigate the involvement of the MCL-1/NOXA axis. NOXA silencing significantly reduced olaparib/TMZ-induced apoptosis in Ewing sarcoma cells (Fig. 5D and E). Together, these results indicate that olaparib/TMZ-triggered degradation of MCL-1 contributes to olaparib/TMZ-induced apoptosis.

Olaparib/TMZ cotreatment promotes BAX/BAK activation and MOMP

Next, we explored whether degradation of MCL-1 leads to activation of BAK and BAX, two multidomain proapoptotic BCL-2 proteins that control MOMP. Since activation of BAK and
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MCL-1

GAPDH

- 43 kDa
- 36 kDa

A4573

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SK-ES-1

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<th>DNA fragmentation (%)</th>
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BAX is accompanied by a conformational change that can be detected by specific antibodies, we immunoprecipitated BAK and BAX using conformation-specific antibodies. Combination treatment with olaparib and TMZ stimulated activation of BAK and BAX (Fig. 6A). To explore the functional relevance of BAK and BAX in olaparib/TMZ-induced apoptosis we used a combined siRNA approach to concomitantly silence BAK and BAX (Fig. 6B). Knockdown of BAK and BAX significantly rescued olaparib/TMZ-mediated apoptosis in all different siRNA construct combinations we used (Fig. 6C), emphasizing the importance of BAK and BAX in olaparib/TMZ-induced apoptosis. Overexpression of BCL-2 protects against Olaparib/TMZ-induced apoptosis

To further examine the requirement of the mitochondrial pathway for the synergistic induction of apoptosis by olaparib and TMZ we overexpressed the antiapoptotic protein BCL-2 that is known to block mitochondrial apoptosis (Fig. 6D). Importantly, BCL-2 overexpression significantly decreased olaparib/TMZ-induced apoptosis (Fig. 6E). This underscores that the synergistic induction of apoptosis by olaparib and TMZ is mediated via the mitochondrial pathway of apoptosis.

Discussion

PARP is currently considered as a promising target for therapeutic exploitation in Ewing sarcoma. Therefore, the two aims of this study were (i) to examine the effects of different PARP inhibitors in combination with a range of commonly used chemotherapeutics to identify the best synergistic combinations in Ewing sarcoma cells and (ii) to elucidate the molecular mechanisms of synergy with a specific focus on cell death pathways.

Here, we report that different PARP inhibitors synergized in particular together with TMZ and also with SN-38 to reduce cell viability and to trigger cell death in Ewing sarcoma cells. Calculation of CI values confirmed that the combinations of PARP inhibitors together with TMZ or SN-38 were the best and second best combinations among the tested chemotherapeutics. Furthermore, triple therapy of olaparib, TMZ, and SN-38 proved to be significantly more effective to reduce cell viability of Ewing sarcoma cells compared to cotreatment with TMZ/SN-38 or single-agent treatment. These findings may have important clinical implications, because topoisomerase I poisons and DNA methylating agents such as TMZ are used in second-line chemotherapy regimens for Ewing sarcoma (22). However, increased host toxicity may limit the therapeutic window for such combinations, because there is preclinical and clinical evidence for enhanced normal tissue toxicity upon cotreatment with PARP inhibitors and TMZ or topoisomerase I inhibitors (12, 16, 25). PARP inhibitors also enhanced doxorubicin-, etoposide-, or ifosfamide-induced cytotoxicity in Ewing sarcoma cells, although less consistently. By comparison, the cytotoxic antibiotic actinomycin D and the vinca alkaloid vincristine did hardly act in a synergistic or additive manner together with PARP inhibitors and also showed some antagonistic effects, depending on the drug concentrations. Antagonistic interactions of olaparib together with vincristine have previously been described in Ewing sarcoma cells and have been linked to vincristine’s mechanism of action which is independent of DNA damage (13). In line with the results of our systematic testing approach that identified TMZ and SN-38 as the most suitable anticancer drugs for combinations with PARP inhibitors in Ewing sarcoma, cooperative interaction of PARP inhibitors together with TMZ or SN-38 has previously been described for Ewing sarcoma cells in vitro and in preclinical Ewing sarcoma models in vivo (9, 12, 13, 16, 26).

The differential ability of chemotherapeutic drugs to synergize with PARP inhibitors has been attributed to the type of DNA lesions generated by the anticancer agents that differentially require PARP1 for DNA repair or affect binding of PARP1 to the DNA (27). In combination with PARP-trapping PARP inhibitors such as talazoparib, the DNA-methylating agent TMZ elicits its cytotoxicity through N3 and N7 methyl adducts that induce base excision repair. Single-strand breaks generated during base excision repair are cytotoxic via PARP trapping, as PARP binds to and is activated by these base excision repair intermediates, and poly (ADP-ribose)ylation is important for efficient repair (27). Consistently, we show that, among the tested anticancer drugs, talazoparib, a potent PARP-trapping agent, preferentially synergizes with TMZ, confirming that PARP inhibitors with high PARP-DNA-trapping activity are especially effective together with TMZ (28). By comparison, TMZ as single agent induces cytotoxicity mainly through O6 methylation followed by futile cycles of mismatch repair, pointing to different mechanisms of cytotoxicity for TMZ monotherapy or in combination with PARP inhibitors. Topoisomerase I inhibitors such as SN-38 cause DNA SSBs and covalent topoisomerase I-DNA complexes, which trigger PARP activation and require PARP1 for repair (29). Accordingly, catalytic PARP inhibitors have shown highly synergistic effects in combination with topoisomerase I inhibitors (28). Consistently, we demonstrate that potent catalytic PARP inhibitors such as niraparib and olaparib cause synergistic cytotoxicity together with SN-38. Olaparib has previously been reported to act in concert with doxorubicin, melphalan, and carboplatin in Ewing sarcoma.
Synergistic Apoptosis by PARP Inhibitors and Temozolomide
cells (13), whereas no cooperative interaction was reported in the past for the combination of etoposide with the first-generation PARP inhibitor NL11025 (30).

In addition to the identification of synergistic PARP inhibitor-based combination therapies, our study demonstrates marked differences in the single-agent cytotoxicity of the four PARP inhibitors in Ewing sarcoma cells with decreasing potency in the order of talazoparib (> niraparib > olaparib > veliparib) (13, 14). These results are in line with previous studies showing differential antitumor activity of PARP inhibitors as single agents in various tumors including Ewing sarcoma (18, 28). The antitumor activity of PARP inhibitors has been shown to depend on both catalytic PARP inhibition and PARP-DNA trapping (18). Consistent with our findings showing that talazoparib exerts the highest single-agent cytotoxicity of the tested PARP inhibitors, the potency of talazoparib has been attributed to its high efficiency at trapping PARP-DNA complexes (19).

Our molecular studies revealed that the mitochondrial pathway of apoptosis plays a critical role in mediating the synergy of concomitant PARP inhibition (using olaparib as a prototypic PARP inhibitor) and TMZ used as a prototypic DNA-damaging agent. This is of particular importance, as to our knowledge the downstream signaling events mediating synergistic cell death induction by PARP inhibition together with TMZ have so far remained largely elusive. We show that subsequent to DNA damage-imposed checkpoint activation and G2 cell-cycle arrest, olaparib/TMZ cotreatment causes downregulation of the antiapoptotic protein MCL-1, activation of the proapoptotic proteins BAX and BAK, MOMP, proteolytic activation of caspases, and caspase-dependent cell death. This conclusion is underscored by the following independent pieces of experimental evidence.

First, olaparib/TMZ cotreatment triggers activation of the DNA damage response, indicated by increased phosphorylation of the checkpoint kinases Chk1 and Chk2. In response to DNA damage, ATM and ATR phosphorylate and thereby activate Chk1 and Chk2, which control the G2–M checkpoint (31). Consistently, olaparib/TMZ cotreatment results in cell-cycle arrest in G2 prior to cell death induction. Second, our rescue experiments showing that addition of the proteasome inhibitor bortezomib rescues cells from olaparib/TMZ-imposed downregulation of MCL-1 protein indicate that MCL-1 is degraded via the proteasome upon treatment with olaparib/TMZ. MCL-1 expression is tightly regulated at multiple levels, involving ubiquitination of MCL-1 that targets it for proteasomal degradation (32). Four different E3 ubiquitin-ligases that mediate MCL-1 ubiquitination have been identified, i.e., Mule, SCF/β-TrCP, SCF/Fbw7, and Trim17 (32). Mule is considered to be responsible for constitutive MCL-1 degradation, whereas ubiquitination of MCL-1 by SCF/β-TrCP, SCF/Fbw7, and Trim17 is mediated via phosphorylation of a phosphodegron site by several kinases, for example by INK and GSK3 in interphase or postmitotic cells (32).

Furthermore, binding of NOXA to MCL-1 has been reported to induce MCL-1 degradation by the proteasome (33), which may also explain the downregulation of NOXA that we observed upon prolonged treatment with olaparib/TMZ. During the initial induction phase of apoptosis, constitutive expression of NOXA turned out to be necessary for olaparib/TMZ-induced cell death, because knockdown of NOXA significantly reduced olaparib/TMZ-mediated cell death. Moreover, we show that expression of a nondegradable phosphomutant variant of MCL-1 significantly reduces olaparib/TMZ-mediated apoptosis, emphasizing that degradation of MCL-1 contributes to olaparib/TMZ-induced apoptosis.

Third, rescue experiments demonstrate that activation of BAX and BAK upon olaparib/TMZ cotreatment is required for the synergistic induction of apoptosis, because genetic silencing of BAX and BAK significantly protects Ewing sarcoma cells from olaparib/TMZ-induced apoptosis. Fourth, the importance of the mitochondrial pathway of apoptosis is underscored by overexpression of the antiapoptotic protein BCL-2, which is known to block mitochondria-mediated apoptosis, because BCL-2 overexpression inhibits olaparib/TMZ-induced apoptosis. Fifth, olaparib and TMZ act together to trigger cleavage of caspases into their active fragments. The requirement of caspases for the induction of cell death is demonstrated by using the broad-range caspase inhibitor zVAD.fmk, which significantly rescues olaparib/TMZ-induced apoptosis.

Our study has important implications for the development of PARP inhibitor-based therapies in Ewing sarcoma. Despite the fact that PARP inhibitors as single agents exerted promising cytotoxicity against Ewing sarcoma cells in vitro (10), they exerted limited efficacy in xenograft models of Ewing sarcoma and did not elicit objective responses in patients with Ewing sarcoma in early clinical trials (12–15). This underscores the need for combination strategies with PARP inhibitors, for example together with chemoradiotherapy. Phase I clinical trials combining PARP inhibitors (i.e., olaparib, niraparib, or talazoparib) together with TMZ for the treatment of Ewing sarcoma are currently under way (i.e., NCT01858168, NCT02044120, NCT02116777). Our present study emphasizes the role of PARP inhibitors for chemosensitization of Ewing sarcoma. By showing that PARP inhibitors synergistically induce apoptosis together with several DNA-damaging agents, most notably TMZ and also with SN38, it provides new insights showing that talazoparib exerts the highest cytotoxicity of the four tested PARP inhibitors, the potency of talazoparib has been attributed to its high efficiency at trapping PARP-DNA complexes (19).

Figure 6. Olaparib/TMZ cotreatment promotes BAX/BAK activation and MOMP. A, A4573 and SK-ES-1 cells were treated with 0.3 μmol/L olaparib and/or 50 μmol/L TMZ for 21 hours. Active conformations of BAX or BAK were immunoprecipitated using active conformation-specific antibodies and were analyzed by Western blotting. Expression of total BAX or BAK and GAPDH served as loading controls. Representative blots of two independent experiments are shown. B and C, A4573 and SK-ES-1 cells were transiently transfected with 10 nmol/L nonsilencing siRNA or 5 nmol/L each of different combinations of constructs targeting BAK or BAX and expression of BAX and BAK was analyzed by Western blotting (B). GAPDH served as loading control. Representative blots of two independent experiments are shown. Transiently transfected Ewing sarcoma cells were treated for 24 hours with 0.3 μmol/L olaparib and/or 50 μmol/L TMZ and apoptosis was determined by quantification of DNA fragmentation of PI-stained nuclei using flow cytometry (C). Data are shown as mean ± SD of three independent experiments performed in triplicate; *P < 0.05. D and E, A4573 and SK-ES-1 cells were transfected with a murine BCL-2 construct or empty vector and BCL-2 expression was analyzed by Western blotting (D). Expression of NOXA turned out to be necessary for olaparib/TMZ-induced apoptosis as shown by the following independent pieces of experimental evidence: First, olaparib/TMZ cotreatment triggers activation of the DNA damage response, indicated by increased phosphorylation of the checkpoint kinases Chk1 and Chk2. In response to DNA damage, ATM and ATR phosphorylate and thereby activate Chk1 and Chk2, which control the G2–M checkpoint (31). Consistently, olaparib/TMZ cotreatment results in cell-cycle arrest in G2 prior to cell death induction. Second, our rescue experiments showing that addition of the proteasome inhibitor bortezomib rescues cells from olaparib/TMZ-imposed downregulation of MCL-1 protein indicate that MCL-1 is degraded via the proteasome upon treatment with olaparib/TMZ. MCL-1 expression is tightly regulated at multiple levels, involving ubiquitination of MCL-1 that targets it for proteasomal degradation (32). Four different E3 ubiquitin-ligases that mediate MCL-1 ubiquitination have been identified, i.e., Mule, SCF/β-TrCP, SCF/Fbw7, and Trim17 (32). Mule is considered to be responsible for constitutive MCL-1 degradation, whereas ubiquitination of MCL-1 by SCF/β-TrCP, SCF/Fbw7, and Trim17 is mediated via phosphorylation of a phosphodegron site by several kinases, for example by INK and GSK3 in interphase or postmitotic cells (32). Furthermore, binding of NOXA to MCL-1 has been reported to induce MCL-1 degradation by the proteasome (33), which may also explain the downregulation of NOXA that we observed upon prolonged treatment with olaparib/TMZ. During the initial induction phase of apoptosis, constitutive expression of NOXA turned out to be necessary for olaparib/TMZ-induced cell death, because knockdown of NOXA significantly reduced olaparib/TMZ-mediated cell death. Moreover, we show that expression of a nondegradable phosphomutant variant of MCL-1 significantly reduces olaparib/TMZ-mediated apoptosis, emphasizing that degradation of MCL-1 contributes to olaparib/TMZ-induced apoptosis.

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insights into cotreatment rationales for distinct PARP inhibitors and chemotherapeutic drugs and sets the ground for further (pre)clinical evaluation of PARP inhibitor-based combination regimens with cytotoxic chemotherapy.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**
Conception and design: F. Engert, C. Schneider, S. Fulda
Development of methodology: F. Engert, L.M. Weiß
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Engert, C. Schneider, M. Probst
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Engert, C. Schneider, M. Probst, S. Fulda

**Acknowledgments**
The authors thank Human Genome Sciences (Rockville, MD) for kindly providing TRAIL-R2 agonistic antibody and C. Hugenberg for expert secretarial assistance.

**Grant Support**
This work has been partially supported by a grant from the BMBF (to S. Fulda).

Received July 17, 2015; revised September 22, 2015; accepted September 23, 2015; published OnlineFirst October 5, 2015.

**References**

Molecular Cancer Therapeutics

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Mol Cancer Ther  Published OnlineFirst October 5, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-15-0587

Supplementary Material  Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2015/10/02/1535-7163.MCT-15-0587.DC1

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