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 acinomycin 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-induced 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.
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.
Figure A:

DNA fragmentation (%) of A4573 and SK-ES-1 cells treated with Olaparib, TMZ, SN-38, and combinations thereof.

Figure B:

Cell viability (%) of A4573 and SK-ES-1 cells treated with Olaparib, TMZ, SN-38, and combinations thereof.

Figure C:

Colonies (%) of A4573 and SK-ES-1 cells treated with Olaparib, TMZ, SN-38, and combinations thereof.

Figure D:

Cell viability (%) of A4573 and SK-ES-1 cells treated with Olaparib, TMZ, SN-38, and combinations thereof.
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 anticancer 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 cell death signaling pathways that mediate the synergistic interaction of PARP inhibitors and anticancer drugs. Therefore, in this study, we aimed (i) at examining the effects of different PARP inhibitors in combination with clinically used chemotherapeutics in Ewing sarcoma cells to identify the most potent synergistic drug combinations and (ii) at elucidating the molecular mechanisms of synergy with a specific focus on cell death pathways.

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 mmol/L 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 Sellckem and miraparib from ChemieTek. actinomycin D, doxorubicin, etoposide, TMZ, SN-38, and vincristine were purchased from Sigma, zVAD.fmk from Bachem, bortezomib from Jansen-eckchem and niraparib from ChemieTek. actinomycin D, doxorubicin, etoposide, TMZ, SN-38, and vincristine were purchased from Sigma, zVAD.fmk from Bachem, bortezomib from Jansen-eckchem, and miraparib from ChemieTek. DNA cell viability, colony formation, and mitochondrial membrane potential (MMP)

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, and 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 reseded, 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, BM-B, PUMA (Cell Signaling), BAX NT, PH3, α-tubulin (Millipore), BMF (Novus Biologicals) MCL-1, NOXA, caspase-8 (Enzo Life Science), GAPDH (HyTest), murine BCL-2 (Life Technologies, Inc.). Goat antinouse 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, si1078 and si1079), or a combination of targeting siRNAs (5 nmol/L for BAK, s1880 and s1881; 5 nmol/L for BAX, s1889 and s1890) using Lipoectamine 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 Lipoectamine 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, sterilized, and used for spin transduction at 37°C in the presence of 8

Figure 2.

Olaparib synergizes with TMZ and SN-38 to induce cell death in Ewing sarcoma cells. A and B, A4573 and SK-E5-1 cells were treated for 48 hours with 0.3 μmol/L olaparib and/or 50 μmol/L TMZ and/or 0.6 mmol/L SN-38. Apoptosis was determined by analysis of DNA fragmentation of PI-stained nuclei using flow cytometry (A). Cell viability was measured by MTT assay and is expressed as percentage of untreated control (B). Data are shown as mean ± SD of three independent experiments performed in triplicate; *, P < 0.05; **, P < 0.01; ***, P < 0.001. C, A4573 and SK-E5-1 cells were treated with 0.3 μmol/L olaparib and/or 50 μmol/L TMZ and/or 0.6 mmol/L SN-38 for 24 hours, living cells were counted and subsequently 100 cells/well were reseeded in drug-free medium in a six-well plate for additional 12 days. Colony formation was assessed by crystal violet staining and colonies were counted macroscopically. The number of colonies is expressed as percentage of untreated control (top) and representative images are shown (bottom). Data are shown as mean ± SD of three independent experiments performed in triplicate; *, P < 0.05; **, P < 0.01. D, A4573 and SK-E5-1 cells were treated for 72 hours with 0.3 μmol/L olaparib and/or 10 μmol/L TMZ and/or 0.2 mmol/L SN-38. Cell viability was measured by crystal violet staining and is expressed as percentage of untreated control. Data are shown as mean ± SD of three independent experiments performed in triplicate; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
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A

A4573

SK-ES-1

DNA fragmentation (%)

Time (h)

Control

Olaparib

TMZ

Olaparib/TMZ

Olaparib

TMZ

Olaparib/TMZ

***

**

***

***

B

A4573

SK-ES-1

Olaparib

TMZ

pChk1

Chk1

α-Tubulin

pChk2

Chk2

GAPDH

56 kDa

56 kDa

55 kDa

62 kDa

62 kDa

36 kDa

α-Tubulin

pChk2

Chk2

GAPDH

56 kDa

55 kDa

36 kDa

C

Frequency of cells (%)

Olaparib

TMZ

G2–M

S

G1

D

A4573

SK-ES-1

Olaparib

TMZ

VCR

pH3

GAPDH

17 kDa

36 kDa

17 kDa

36 kDa

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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 SK-ES-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 ≈ 10 000 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 preferentially 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 1:**

**A**

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<tr>
<td>Olaparib</td>
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<tr>
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<tr>
<td>ETR2</td>
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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
- 55 kDa
- 53 kDa
- 43 kDa
- 41 kDa
- 36 kDa

Caspase-8

- 23 kDa
- 25 kDa
- 20 kDa
- 13 kDa
- 11 kDa
- 9 kDa
- 8 kDa

GAPDH

- 37 kDa
- 35 kDa
- 36 kDa

**B**

DNA fragmentation (%)

- Ctrl
- zVAD.fmk

**SK-ES-1**

- 60%
- 50%
- 40%
- 30%
- 20%
- 10%
- 0%

**A4573**

- 60%
- 50%
- 40%
- 30%
- 20%
- 10%
- 0%

**C**

Loss of MMP (%)

- Control
- Olaparib
- TMZ
- Olaparib/TMZ

**A4573**

- 30%
- 25%
- 20%
- 15%
- 10%
- 5%
- 0%

- 18 h
- 21 h
- 24 h

**SK-ES-1**

- 30%
- 25%
- 20%
- 15%
- 10%
- 5%
- 0%

- 18 h
- 21 h
- 24 h

**D**

<table>
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<tr>
<td>TMZ</td>
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MCL-1

- 43 kDa

BCL-2

- 26 kDa

BCL-XL

- 28 kDa

BIMEL

- 23 kDa

BIML

- 16 kDa

BIMS

- 13 kDa

BMF

- 25 kDa

PUMA

- 23 kDa

NOXA

- 11 kDa

GAPDH

- 36 kDa
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 pathway
To explore whether induction of apoptosis involved activation of caspases, 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 investigate whether the observed downregulation of MCL-1 upon olaparib/TMZ cotreatment is due to proteasomal degradation and/or caspase-mediated cleavage, we tested the effects of the proteasome inhibitor bortezomib and/or the pan-caspase inhibitor zVAD.fmk. Although addition of bortezomib significantly rescued the olaparib/TMZ-imposed downregulation of MCL-1 protein levels, addition of zVAD.fmk largely failed to significantly protect against MCL-1 downregulation upon olaparib/TMZ cotreatment (Fig. 5A). This indicates that MCL-1 is degraded via the proteasome upon treatment with olaparib/TMZ.

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
host toxicity may limit the therapeutic window for such combina-
tions. Moreover, triple therapy of olaparib, TMZ, and SN-38 proved to be the best and second
best combinations in Ewing sarcoma cells and (ii) to elucidate the molecular mechan-
isms 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 chemotherapeutic regimens for Ewing sarcoma (22). However, increased host toxicity may limit the therapeutic window for such combina-
tions, 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 ifos-
famide-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 PARP1 binds to and is activated by these base excision repair intermediates, and poly (ADP-ribose)ylation is important for efﬁcient repair (27). Consis-
tently, we show that, among the tested anticancer drugs, talazo-
parib, 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, cata-
lytic PARP inhibitors have shown highly synergistic effects in combination with topoisomerase I inhibitors (28). Consistently, we demonstrate that potent catalytic PARP inhibitors such as nirariparib 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.
cells (13), whereas no cooperative interaction was reported in the past for the combination of etoposide with the first-generation PARP inhibitor NU1025 (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 (IC_{50} \approx 10 \text{nmol/L}) > niraparib (IC_{50} \approx 300 \text{nmol/L}) > olaparib (IC_{50} \approx 1,000 \text{nmol/L}) > veliparib (IC_{50} \approx 10,000 \text{nmol/L}). 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-induced checkpoint activation and G2 cell-cycle arrest, olaparib/TMZ cotreatment causes downregulation of the antipapoptotic 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 proteosomal 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 JNK 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 antipapoptotic 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 chemotherapeutic agents. 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 into the potential of PARP inhibitors in the treatment of Ewing sarcoma.

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**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 of each of different combinations of constructs targeting BAX or BAK 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 < 0.01**. 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 GAPDH or α-tubulin served as loading controls. Representative blots of two independent experiments are shown. BCL-2-overexpressing Ewing sarcoma cells were treated for 48 hours with 0.3 µmol/L olaparib and 50 µmol/L TMZ and apoptosis was determined by quantification of DNA fragmentation of PI-stained nuclei using flow cytometry (E). Data are shown as mean ± SD of three independent experiments performed in triplicate; ***p < 0.001.**
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.

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References

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