PARP inhibitors sensitize Ewing sarcoma cells to
Temozolomide-induced apoptosis via the mitochondrial
pathway

Florian Engert¹, Cornelius Schneider¹, Lilly Weiß¹-³, Marie Probst¹, Simone Fulda¹-³

¹Institute for Experimental Cancer Research in Pediatrics, Goethe-University,
Komturstr. 3a, 60528 Frankfurt, Germany
²German Cancer Consortium (DKTK), Heidelberg, Germany
³German Cancer Research Center (DKFZ), Heidelberg, Germany

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To whom correspondence and reprint requests should be addressed:
Prof. Dr. Simone Fulda
Institute for Experimental Cancer Research in Pediatrics
Goethe-University
Komturstr. 3a
60528 Frankfurt
Tel.: +49 69 67866557
Fax: +49 69 6786659157
Email: simone.fulda@kgu.de
Abstract

Ewing sarcoma (ES) 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 to 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 co-treatment causes downregulation of the antiapoptotic protein MCL-1, followed by activation of the proapoptotic proteins BAX and BAK, mitochondrial outer membrane permeabilization, activation of caspases and caspase-dependent cell death. Overexpression of a non-degradable MCL-1 mutant or BCL-2, knockdown of NOXA or BAX and BAK, or the caspase inhibitor N-benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) all significantly reduce olaparib/TMZ-mediated apoptosis. These findings emphasize the role of PARP inhibitors for chemosensitization of ES with important implications for further (pre)clinical studies.
Introduction

ES is the second most common pediatric primary bone tumor in children and young adults (1). State-of-the-art treatment options for ES patients include chemotherapy consisting of vincristine, ifosfamide, doxorubicin, temozolomide, 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, i.e. 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) accompanied by the release of mitochondrial intermembrane space proteins such as cytochrome c into the cytosol, which in turn results in caspase activation and apoptosis (5). MOMP is tightly controlled by various factors including proteins of the BCL-2 family. BCL-2 family proteins consist of both antiapoptotic members, e.g. BCL-2, BCL-xL and MCL-1, and proapoptotic molecules such as the multidomain proteins BAK and BAX and BH3-only domain proteins, e.g. BIM, BMF, PUMA and NOXA (6).

Characteristic for ES is the existence of a chimeric fusion protein which is in >85% of cases generated by the translocation of the EWS gene on chromosome 22 and the FLI1 gene on chromosome 11 (11;22)(q24;q12) (7). This fusion protein encodes the chimeric transcription factor EWS-FLI1 that causes upregulation of a range of target...
genes including PARP (7, 8), which promotes transcriptional activation by EWS-FLI1 in a positive feedback loop (9). Elevated PARP levels have been detected in ES specimens (8, 10), indicating that PARP may represent a promising target for therapeutic exploitation in ES. Indeed, ES cells have been shown to be particularly sensitive to PARP inhibition (9, 10). Several PARP inhibitors including talazoparib (BMN-673), niraparib (MK-4827), olaparib (AZD-2281) and veliparib (ABT-888) are currently under investigation (11).

Despite the sensitivity of ES cells to PARP inhibitors in vitro (9, 10, 12), PARP inhibitors as single therapy displayed limited efficacy in xenograft models of ES and in initial clinical trials (12-15), highlighting the need for combination therapies. PARP inhibitors have been documented to potentiate DNA damage-mediated cytotoxicity caused in particular by topoisomerase I poisons, DNA methylating agents or radiation in several cancers including ES, which has been related 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).

While 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 ES. 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 ES 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

ES 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., Darmstadt, Germany), 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 (Munich, Germany) and niraparib from ChemieTek (Indianapolis, IN, USA). Actinomycin D, doxorubicin, etoposide, TMZ, SN-38 and vincristine were purchased from Sigma (Deisenhofen, Germany), zVAD.fmk from Bachem (Heidelberg, Germany), bortezomib from Jansen-Cilag (Neuss, Germany). 4-Hydroperoxylfosfamide (active metabolite of ifosfamide and further designated as ifosfamide) was kindly provided by Baxter (Unterschleißheim, Germany) and TRAIL receptor-2 agonistic antibody lexatumumab (ETR2) Human Genome Sciences (Rockville, MD, USA). Chemicals were purchased from Sigma or Carl Roth (Karlsruhe, Germany) unless otherwise indicated.

Determination of apoptosis, cell viability, colony formation and MMP

Apoptosis was determined by flow cytometric analysis (FACSCanto II, BD Biosciences, Heidelberg, Germany) 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, Mannheim, Germany) 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 re-solubilized in a solution containing 1% SDS. Absorbance at 550 nm was measured using a microplate reader (Infinite M100, Tecan, Crailsheim, Germany). 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-\(x_L\) (BD, New Jersey, USA), pChk1, pChk2, Chk1, Chk2, caspase-3, caspase-9, PARP, BIM, PUMA (Cell Signaling, Beverly, MA), BAX NT, pH3, \(\alpha\)-Tubulin (Millipore, Darmstadt, Germany), BMF (Novus Biologicals, Littleton, USA) MCL-1, NOXA, caspase-8 (Enzo Life Science, Lörrach, Germany), GAPDH (HyTest, Turku, Finland), murine BCL-2 (Life Technologies, Inc., Darmstadt, Germany). Goat anti-mouse IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany) were used as secondary antibodies and enhanced chemiluminescence (Amersham Bioscience, Freiburg, Germany) or infrared dye-labeled secondary antibodies and infrared imaging were used for detection (Odyssey Imaging System, LI-COR Bioscience, Bad Homburg, Germany). 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, Oregon, USA) following the manufacturer's instructions.

RNA interference and overexpression

Cells were reversely transfected with 10 nM Silencer Select (Life Technologies, Inc.) control siRNA (10 nM, 4390844), NOXA siRNA (10 nM s10708 and s10709) or a combination of targeting siRNAs (5 nM for BAK, s1880 and s1881; 5 nM for BAX, s1889 and s1890) using Lipofectamine RNAiMAX reagent (Life Technologies, Inc., Darmstadt, Germany) and Opti-MEM medium (Life Technologies, Inc.). After six hours of incubation with transfection solution, the medium was changed and cells recovered for 48 hours before drug treatment. For transient overexpression, ES 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 μg/ml polybrene. Transduced ES 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). 500 µg protein was 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, Darmstadt, Germany) and 10 µl pan mouse IgG Dynabeads (Dako, Hamburg, Germany), washed with CHAPS lysis buffer and analyzed by Western blotting using rabbit anti-BAX NT antibody (Millipore, Darmstadt, Germany) or rabbit anti-BAK 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’s t-Test (two-tailed distribution, two-sample, equal variance) using Microsoft Excel (Microsoft Deutschland GmbH, Unterschleißheim, Germany); *, P < 0.05; **, P < 0.01; ***, P < 0.001. Drug interactions were analyzed by the CI method based on that described by Chou (21) using CalcuSyn software (Biosoft, Cambridge, UK). 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 slight 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 ES against PARP inhibitors we initially tested the dose response to four different PARP inhibitors using the ES cell lines A4573 and SK-ES-1 and subsequently extended our studies also to two additional ES cell lines. Talazoparib, niraparib, olaparib and veliparib showed a differential ability to reduce cell viability of ES cells with talazoparib being the most active and veliparib being the least active compound (talazoparib IC<sub>50</sub> ≈ 10 nM > niraparib IC<sub>50</sub> ≈ 300 nM > olaparib IC<sub>50</sub> ≈ 1000 nM > veliparib IC<sub>50</sub> ≈ 10000 nM) (Fig. 1A, Suppl. Tab. 1).

To investigate the question whether PARP inhibitors modulate chemosensitivity of ES cells, we screened the efficacy of several anticancer drugs that are used in the clinic for the treatment of ES (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 to untreated control (Suppl. Fig. 1). Synergistic, additive or antagonistic drug interactions were calculated by combination index (CI) (Suppl. Tab. 2). 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 co-treatment with SN-38 (Fig. 1B, Suppl. Fig. 2). We also noted that, among the tested anticancer drugs, talazoparib preferentially synergized with TMZ (Fig. 1B, Suppl. Fig. 2). In addition, PARP inhibitors enhanced doxorubicin-, etoposide- or ifosfamide-induced cytotoxicity in ES cells, although less consistently (Fig. 1B, Suppl. Fig. 2). By comparison, the DNA-
binding drug actinomycin D and the vinca alkaloid vincristine exerted little synergistic effects together with PARP inhibitors (Fig. 1B, Suppl. Fig. 2).

**Olaparib synergizes with TMZ and SN-38 to induce cell death in ES cells.**

Since in our screening approach we identified co-treatment 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, 2B). Moreover, we extended our studies to two additional ES 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 (Suppl. Fig. 3). To explore whether combined treatment with olaparib and TMZ or SN-38 also affects long-term survival of ES cells, we performed colony assays. Of note, co-treatment of olaparib together with TMZ or SN-38 acted in concert to significantly suppress colony formation of ES cells compared to treatment with olaparib, TMZ or SN-38 alone (Fig. 2C). Since TMZ and SN-38 are administered together in clinical protocols as second-line treatment of ES (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 ES cells compared to treatment with single agents or to co-treatment 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 ES cells. To investigate in more detail the molecular mechanisms underlying the synergism of PARP inhibitors
and chemotherapeutic drugs in ES, we focused the subsequent experiments on TMZ, since this drug yielded the most pronounced synergistic interaction.

**Olaparib/TMZ co-treatment 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 co-treatment caused a significant increase of cells in G2/M phase compared to cells treated with either drug alone or untreated cells (Fig. 3C, Suppl. Fig. 4). 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 co-treatment 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 ES cells.
Olaparib/TMZ-induced apoptosis is executed via caspase-dependent effector pathways.

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 ES (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 to 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 ES cells.

Olaparib/TMZ co-treatment downregulates MCL-1 levels.

To investigate whether olaparib/TMZ combination treatment engages the mitochondrial pathway of apoptosis, we assessed the mitochondrial membrane potential (MMP). Olaparib and TMZ cooperated to trigger loss of MMP in a time-dependent manner at the onset of apoptosis (Fig. 4C). Since BCL-2 family proteins are key regulators of the mitochondrial pathway, we then asked whether
olaparib/TMZ co-treatment causes changes in their expression levels. Of note, co-treatment with olaparib and TMZ resulted in downregulation of MCL-1, while 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 co-treatment (Fig. 4D).

**Olaparib/TMZ co-treatment promotes proteasomal degradation of MCL-1.**

To investigate whether the observed downregulation of MCL-1 upon olaparib/TMZ co-treatment 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. Whereas 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 co-treatment (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 (Figure 5B). Notably, ectopic expression of non-degradable MCL-1 mutant significantly decreased olaparib/TMZ-induced apoptosis (Figure 5C). Since 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 ES cells (Fig. 5D, 5E). Together, these results indicate that olaparib/TMZ-triggered degradation of MCL-1 contributes to olaparib/TMZ-induced apoptosis.
**Olaparib/TMZ co-treatment 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 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 ES. 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 ES 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 ES 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 ES cells compared to co-treatment with TMZ/SN-38 or single agent treatment. These findings may have important clinical implications, since topoisomerase I poisons and DNA methylating agents such as TMZ are used in second-line chemotherapy regimens for ES (22).

However, increased host toxicity may limit the therapeutic window for such combinations, since 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 ES 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 ES 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 ES, cooperative interaction of PARP
inhibitors together with TMZ or SN-38 has previously been described for ES cells in
vitro and in preclinical ES 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-ribos)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 ES 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 ES cells with decreasing potency in the order of talazoparib (IC50 ≈ 10 nM) > niraparib (IC50 ≈ 300 nM) > olaparib (IC50 ≈ 1000 nM) > veliparib (IC50 ≈ 10000 nM). These results are in line with previous studies showing differential antitumor activity of PARP inhibitors as single agents in various tumors including ES (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 co-treatment 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 co-treatment 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 co-treatment 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, while 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 post-mitotic 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, since knockdown of NOXA significantly reduced olaparib/TMZ-mediated cell death. Moreover, we show that expression of a non-degradable 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 co-treatment is required for the synergistic induction of apoptosis, since genetic silencing of BAX and BAK significantly protects ES 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, since 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 ES. Despite the fact that PARP inhibitors as single agents exerted promising cytotoxicity against ES cells in vitro (10), they exerted limited efficacy in xenograft models of ES and did not elicit objective responses in patients with ES in early clinical trials (12-15). This underscores the need for combination strategies with PARP inhibitors, for example together with chemo- or radiotherapy. Phase I clinical trials combining PARP inhibitors (i.e. olaparib, niraparib or talazoparib) together with TMZ for the treatment of ES are currently under way (i.e. NCT01858168, NCT02044120, NCT02116777). Our present study emphasizes the role of PARP inhibitors for chemosensitization of ES. 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 co-treatment 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.

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References


Figure legends

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 CI. CI values were then used to generate a heatmap of drug interactions according to the subclassification provided by the software’s manual.

Figure 2: Olaparib synergizes with TMZ and SN-38 to induce cell death in ES cells.

A and B. A4573 and SK-ES-1 cells were treated for 48 hours with 0.3 µM olaparib and/or 50 µM TMZ and/or 0.6 nM 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-ES-1 cells were treated with 0.3 µM olaparib and/or 50 µM TMZ and/or 0.6 nM SN-38 for 24 hours, living cells were counted and subsequently 100 cells/well were re-seeded 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 (upper panels) and representative images are shown (lower panels). Data are shown as mean +/- SD of three independent experiments performed in triplicate; *, P < 0.05; **, P < 0.01.

D. A4573 and SK-ES-1 cells were treated for 72 hours with 0.3 µM olaparib and/or 10 µM TMZ and/or 0.2 nM 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.

**Figure 3: Olaparib/TMZ co-treatment causes G2 arrest prior to cell death.**

A. A4573 and SK-ES-1 cells were treated with 0.3 µM olaparib and/or 50 µM 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 < 0.01; ***, P < 0.001.

B. A4573 and SK-ES-1 cells were treated with 0.3 µM olaparib and/or 50 µM 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 µM olaparib and/or 50 µM 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 co-treated to single treated or untreated cells in G2/M phase.
D. ES cells were treated with 0.3 µM olaparib and/or 50 µM TMZ or 2.5 nM 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.

Figure 4: Olaparib and TMZ cooperate to trigger caspase activation and mitochondrial perturbations.

A. A4573 and SK-ES-1 cells were treated with 0.3 µM olaparib and/or 50 µM TMZ for 24 hours. Cleavage of caspase-9, caspase-3, caspase-8 and PARP was analyzed by Western blotting. Arrowheads indicate active cleavage fragments, expression of GAPDH served as loading control; asterisk denotes unspecified protein bands. SK-ES-1 cells treated for two hours with 2 µg/ml lexatumumab (ETR-2) served as positive control for caspase activation. Representative blots of two independent experiments are shown.

B. A4573 and SK-ES-1 cells were treated for 48 hours with 0.3 µM olaparib and/or 50 µM TMZ in the presence or absence of 50 µM zVAD.fmk. 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 < 0.001

C. A4573 and SK-ES-1 cells were treated with 0.3 µM olaparib and/or 50 µM TMZ for indicated times and loss of MMP in the living cell population was determined by flow cytometry using TMRM fluorescent dye. Data are shown as mean +/- SD of three independent experiments performed in triplicate; *, P < 0.05; **, P < 0.01.

D. A4573 and SK-ES-1 cells were treated with 0.3 µM olaparib and/or 50 µM TMZ for 18 hours. Expression of BCL-2 family proteins was analyzed by Western blot,
expression of GAPDH served as loading control. Representative blots of two independent experiments are shown.

Figure 5: Olaparib/TMZ co-treatment promotes proteasomal degradation of MCL-1.

**A.** A4573 and SK-ES-1 cells were treated for 18 hours with 0.3 µM olaparib and/or 50 µM TMZ and/or 50 µM zVAD.fmk and/or 5 ng/ml bortezomib. Expression of MCL-1 was analyzed by Western blotting, expression of GAPDH served as loading control. For further evaluation Western blots were quantified using ImageJ® software and changes in MCL-1 protein levels are given as fold change in comparison to untreated control. Data are shown as mean +/- SD of three independent Western blots; *, P < 0.05; **, P < 0.01.

**B** and **C.** A4573 and SK-ES-1 cells were transfected with non-degradable phospho-defective mutant of MCL-1 (MCL-1 4A) or empty vector (EV). Expression of MCL-1 was analyzed by Western blotting, expression of GAPDH served as loading control, arrow indicates exogenously expressed MCL-1 (B, representative blots of two independent experiments are shown). Cells were treated for 48 hours with 0.3 µM olaparib and 50 µM 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; **, P < 0.01.

**D** and **E.** A4573 and SK-ES-1 cells were transiently transfected with 10 nM non-silencing siRNA or two different constructs targeting NOXA. Expression of NOXA was analyzed by Western blotting, α-Tubulin served as loading control, representative blots of two independent experiments are shown (D). Transiently transfected ES cells
were treated for 24 hours with 0.3 µM olaparib and 50 µM TMZ and 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 < 0.01.

**Figure 6: Olaparib/TMZ co-treatment promotes BAX/BAK activation and MOMP.**

**A.** A4573 and SK-ES-1 cells were treated with 0.3 µM olaparib and/or 50 µM TMZ for 21 hours. Active conformations of BAK or BAX were immunoprecipitated using active conformation-specific antibodies and were analyzed by Western blotting. Expression of total BAK or BAX 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 nM non-silencing siRNA or 5 nM each of different combinations of constructs targeting BAK or BAX and expression of BAK and BAX was analyzed by Western blotting (B). GAPDH served as loading control. Representative blots of two independent experiments are shown. Transiently transfected ES cells were treated for 24 hours with 0.3 µM olaparib and/or 50 µM 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 GAPDH or α-Tubulin served as loading controls. Representative blots of two independent experiments are shown. BCL-2-overexpressing ES cells were treated for 48 hours with 0.3 µM olaparib and 50 µM 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.
Fig. 1

A

A4573

- Talazoparib
- Niraparib
- Olaparib
- Veliparib

SK-ES-1

- Talazoparib
- Niraparib
- Olaparib
- Veliparib

B

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CI-Values:
- < 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: Slight antagonistic
- 1.2-1.45: Moderate antagonistic
- >1.45: Antagonism
- Not calculated
Fig. 2

A4573

***

SK-ES-1

***

DNA fragmentation [%]

Olaparib

TMZ

SN-38

0

20

40

60

80

100

- 

+

- 

+

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Fig. 3

A

A4573

SK-ES-1

B

A4573

SK-ES-1

C

A4573

SK-ES-1

D

A4573

SK-ES-1
Fig. 4

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B

A4573

DNA fragmentation [%]

Control

Olaparib

TMZ

Olaparib/TMZ

SK-ES-1

DNA fragmentation [%]

Control

Olaparib

TMZ

Olaparib/TMZ

C

A4573

Loss of MMP [%]

Control

Olaparib

TMZ

Olaparib/TMZ

SK-ES-1

Loss of MMP [%]

Control

Olaparib

TMZ

Olaparib/TMZ

D

A4573

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SK-ES-1

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**Fig. 5**

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**C**

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**A4573**

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**SK-ES-1**

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Fig. 6

(A) A4573

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Bak active

BAK

- 24 kDa

BAX

- 21 kDa

GAPDH

- 36 kDa

(B) A4573

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Bak active

BAK

- 24 kDa

BAX

- 21 kDa

GAPDH

- 36 kDa

(C) A4573

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DNA fragmentation [%]

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GAPDH

- 36 kDa

(E) A4573

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DNA fragmentation [%]
Molecular Cancer Therapeutics

PARP inhibitors sensitize Ewing sarcoma cells to Temozolomide-induced apoptosis via the mitochondrial pathway

Florian Engert, Cornelius Schneider, Lilly Weiß, et al.

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