The Ewing Family of Tumors Relies on BCL-2 and BCL-X₇ to Escape PARP Inhibitor Toxicity

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Abstract

Purpose: It was recently demonstrated that the EWSR1-FLI1 t(11;22)(q24;q12) translocation contributes to the hypersensitivity of Ewing sarcoma to PARP inhibitors, prompting clinical evaluation of olaparib in a cohort of heavily pretreated Ewing sarcoma tumors. Unfortunately, olaparib activity was disappointing, suggesting an underappreciated resistance mechanism to PARP inhibition in patients with Ewing sarcoma. We sought to elucidate the resistance factors to PARP inhibitor therapy in Ewing sarcoma and identify a rational drug combination capable of rescuing PARP inhibitor activity.

Experimental Design: We employed a pair of cell lines derived from the same patient with Ewing sarcoma prior to and following chemotherapy, a panel of Ewing sarcoma cell lines, and several patient-derived xenograft (PDX) and cell line xenograft models.

Results: We found olaparib sensitivity was diminished following chemotherapy. The matched cell line pair revealed increased expression of the ant apoptotic protein BCL-2 in the chemotherapy-resistant cells, conferring apoptotic resistance to olaparib. Resistance to olaparib was maintained in this chemotherapy-resistant model in vivo, whereas the addition of the BCL-2/X₇ inhibitor navitoclax led to tumor growth inhibition. In 2 PDXs, olaparib and navitoclax were minimally effective as monotherapy, yet induced dramatic tumor growth inhibition when dosed in combination. We found that EWS-FLI1 increases BCL-2 expression; however, inhibition of BCL-2 alone by venetoclax was insufficient to sensitize Ewing sarcoma cells to olaparib, revealing a dual necessity for BCL-2 and BCL-X₇ in Ewing sarcoma survival.

Conclusions: These data reveal BCL-2 and BCL-X₇ act together to drive olaparib resistance in Ewing sarcoma and reveal a novel, rational combination therapy that may be put forward for clinical trial testing.

Introduction

The Ewing family of tumors (EFTs), consisting of primitive neuroectodermal tumor (PNET) and Ewing sarcoma, is a malignancy of predominantly bone. These cancers are diagnosed most often in children and adolescents. Great strides have been made in treating localized disease by using intensive neoadjuvant and adjuvant chemotherapy regimens, increasing the 5-year survival from about 10% to approximately 75%. However, there is a 30% survival rate for patients with Ewing sarcoma presenting with metastasis or that relapse following systematic chemotherapy (1).

The EWSR1-FLI1 t(11;22)(q24;q12) translocation event is found in approximately 90% of EFTs. Since the identification of EWSR1-FLI1 in Ewing sarcoma, it has become clear that the resultant fusion oncogene is the vital driving event in these tumors (2–7). The molecular consequence of juxtaposing the EWSR1 and FLI1 genes is a EWS-FLI1 fusion protein where EWS potently increases the ability of transcription factor FLI1 to activate or suppress target genes.

Unfortunately, FLI1 is currently undruggable, and effective targeted therapies for treating Ewing sarcoma remain elusive. Recent findings have highlighted the role of EWS-FLI1 in inducing a wide range of changes throughout the epigenome, affecting both histone marks and enhancers (5–9), leading to simultaneous enhanced expression of tumor oncogenes and reduced expression of tumor suppressors (6). However, these studies have yet to reveal specific, druggable targets with associated clinically available therapies.

Brenner and colleagues (10) and the Genomics of Drug Sensitivity in Cancer (GDSC), a high-throughput drug screening platform (11), both demonstrated in 2012 that EWSR1-FLI1–translocated Ewing sarcoma displays hypersensitivity to PARP inhibition; this has since been replicated by several other groups (12–14). These data have provided a promising
drug target for EWTs, with corresponding FDA-approved PARPi inhibitors (15).

However, in the initial clinical study of olaparib in Ewing sarcoma, no objective responses were observed in 12 evaluable patients (15). Although there were no objective responses, 4 of 12 patients achieved stable disease, with 2 of the 4 achieving minor responses (tumor shrinkage of 9% and 12%), indicating a modest level of efficacy by PARP inhibition in these patients.

Based on the hypersensitivity of Ewing sarcoma to PARP inhibition in vitro and olaparib activity in patients with Ewing sarcoma, we sought to identify intrinsic resistance mechanisms to PARP inhibitor (PARPi) therapy as well as a rational drug combination that could overcome these mechanisms. We and others have shown that a low apoptotic response, even in the presence of growth arrest, mitigates response to targeted therapies (16–21). We therefore hypothesized that mitigated responses of PARP inhibition may be due to loss of apoptotic potential of EWTs, which could prove particularly true in the chemorefractory population. This hypothesis was further supported by the fact that deficient DNA damage repair is thought to contribute to, if not define, PARPi sensitivity in Ewing sarcoma (12), as well as the established role of antiapoptotic BCL-2 family proteins in protecting cancer cells from DNA damage–induced apoptosis (17, 22) and their inverse correlation of expression to cytotoxic agent sensitivity (23).

Materials and Methods

Cell lines

A673 (ATCC CRL-1598) and HEK293T cells were cultured in DMEM (Gibco) with 10% FBS (Seradigm) and 1 μg/mL penicillin and streptomycin. CHLA9 and CHLA10 cells were grown in DMEM with 20% FBS, 1 μg/mL penicillin and streptomycin, and 1% Insulin-Transferrin-Selenium 100X (Gibco). SK-ES-1 (ATCC HTB-86) was grown in DMEM/F12 (Corning) with 15% FBS and 1 μg/mL of penicillin and streptomycin. ES4 and EW16 cells were grown in RPMI1640 (Lonza Group) with 10% FBS and 1 μg/mL of penicillin and streptomycin. Routine mycoplasma testing was performed on all cell lines. CHLA9 and CHLA10 were obtained from the Children’s Oncology Group (COG) Cell Culture and Xenograft Repository, special thanks to Dr. C. Pat Reynolds, Texas Tech University Health Sciences Center. ES4, EW16, HEK293T, A673 (ATCC CRL-1598), and SK-ES-1 (ATCC HTB-86) were obtained from either the Molecular Center Therapeutics laboratory at Massachusetts General Hospital which performs routine testing of cell lines using short tandem repeat and SNP analysis, or the American Type Culture Collection (ATCC).

Antibodies and reagents

Primary antibodies used for Western blotting were as follows: GAPDH (sc-3233) and FLI1 (sc-365294) from Santa Cruz Biotechnology; cleaved PARP1 (5625), BCL-2 (4223), BCL-XL (2764), MCL-1 (5453), PARP1 (9532), γH2AX (9781), and BIM (2933) from Cell Signaling Technology. Secondary antibodies used were mouse IgG (GE Healthcare Life Sciences; NXA931) and rabbit IgG (GE Healthcare Life Sciences; NA934). IgG (sc-2027) for immunoprecipitation was from Santa Cruz Biotechnology. Olaparib (AZD-2281) was from AbbVie, and A-1331852, navitoclax (ABT-263), and venetoclax (ABT-199) were kindly provided by AbbVie Inc.

Animal experiments

For the SK-ES-1 xenograft model, 5 × 10^6 cells were injected s.c. into the flank of 6- to 8-week-old Nus/Nu mice. For the CHLA10 xenograft model, 5 × 10^6 cells were injected s.c. into the flank of 6- to 8-week-old Nod/Scid gamma (NSG) mice. The patient-derived xenograft (PDX) models were obtained from Crown Bioscience, and 5 × 10^6 cells were injected into the flank of NSG mice s.c. Treatment began when tumors reached approximately 150 to 200 mm^3, and mice were randomized into treatment cohorts. Tumor size and mouse weight were measured 3 days per week with a digital scale and calipers, where tumor volume was calculated as length × width^2 × 0.52. Navitoclax and olaparib were administered by oral gavage. Navitoclax was dissolved in 60% Phosal 50 PG, 30% PEG400, and 10% ethanol, for a final dosage of 80 mg/kg of body weight. Olaparib was dissolved in 10% hydroxypropyl-β-cyclodextrin for a final dosage of 100 mg/kg of body weight. All drugs were administered once per day, 5 days/week. For pharmacodynamics studies, tumor-bearing mice were treated with drug for 3 days, and tumors were harvested on the 3rd day 2 hours after the final treatment. Tumors were flash frozen in liquid nitrogen. For the blood toxicity study, NSG mice were treated with no drug (no Rx), navitoclax, olaparib, or the combination, at the same doses as above. At 3 and 7 days, mice were exsanguinated, and blood was sent to IDEXX BioResearch for testing. The recovery cohort was treated for 7 days and allowed 24 hours of recovery from treatment before exsanguination. All animal experiments were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC protocol #AD10001048).

Dataset analysis

The online database for publically accessible drug sensitivity data (www.cancerr2genie.org) was used to generate Fig. 3C. The cancer cell line encyclopedia (CCLE; ref. 24) was used to analyze expression between FLI1 and BCL-2 (Supplementary Fig. S7D).

Statistical considerations

For gene expression analyses and the complete blood count analyses, significance was determined using the nonparametric Mann–Whitney U test. Differences were considered statistically different if P < 0.05. All other analyses were performed using the Student t test and considered statistically different if P < 0.05. Asterisks indicate levels of significance: ns, P ≥ 0.05; *, P < 0.05; **, P < 0.01; ****, P < 0.001; and ****, P < 0.0001.

Synergy assay

Cells were seeded at 1 × 10^3 cells in a 96-well plate. Twenty-four hours after seeding, cells were treated with varied concentrations...
of navitoclax (0 to 2 μmol/L) and olaparib (0.1 to 10 μmol/L) for 72 hours, followed by measurement of cell viability by CellTiter-Glo. Percent viability was constrained to a maximum of 100. The percentage over the bliss score was calculated as previously described (25).

Results
A chemotherapy-naive and chemotherapy-resistant cell line pair respond differently to olaparib
Olaparib performed poorly in patients with Ewing sarcoma (15) whose tumors were heavily pretreated and chemotherapy-resistant. We therefore utilized a pair of cell lines established from the same patient prior to and following chemotherapy treatment, at tumor relapse: CHLA9 cells were derived from the chemotherapy-naive PNET, positive for the EWS-FLI1 translocation, whereas the CHLA10 cell line was established after 4 cycles of induction chemotherapy which included cisplatin, doxorubicin, cyclophosphamide, and etoposide (26). We first assessed whether sensitivity to olaparib was different in the 2 cell lines. We found that the chemotherapy-naive cells were more sensitive to olaparib, as evidenced by a 5-day crystal violet viability assay (Fig. 1A, left), 72-hour dose–response curve (Fig. 1A, right), and IC50 curve (Supplementary Fig. S2A) compared with the chemotherapy-resistant CHLA10 cells (Fig. 1A, left and right), despite both cell lines reportedly expressing high levels of PARP1 (23, 27) and olaparib inducing similar growth arrest in both the CHLA9 and CHLA10 cells (Supplementary Fig. S2B). In addition, similar levels of DNA damage were observed following olaparib treatment in both cell lines as evidenced by γH2AX immunofluorescence staining (Supplementary Fig. S3A and S3B). Furthermore, we confirmed the CHL10 cells were more resistant to chemotherapy compared with the CHLA9 cells (Supplementary Fig. S4A and S4B).

Chemotherapy-resistant CHLA10 cells do not undergo cell death in response to olaparib
Because the lack of robust apoptotic responses can underlie resistance to both chemotherapy and targeted therapies, and the apoptotic response following many chemotherapies and targeted therapies is largely governed by the BCL-2 family of proteins (16, 20, 28–30), we first explored the relationship between antiapoptotic BCL-2 family expression and olaparib response in the CHLA9 and CHLA10 models. Expression of BCL-2 was upregulated (P < 0.05) in the CHLA10 cells compared with the CHLA9 cells (Fig. 1B; Supplementary Fig. S4C), whereas expressions of other key BCL-2 family members were not altered (Fig. 1B).

The increase in BCL-2 prompted us to evaluate BCL-2 expression in pretreatment and postchemotherapy biopsy samples from 2 patients with Ewing sarcoma treated at our cancer center. Interestingly, we did not detect an increase in BCL-2 expression in these specimens, in contrast to the cell line pair; however, BCL-XL expression was markedly higher in chemotherapy-resistant tumors (Fig. 1C) relative to the matched chemotherapy-naive samples. These data indicate that BCL-XL is overexpressed in Ewing sarcoma patients’ tumors that have undergone chemotherapy, and our findings in models of EWFTs implicate BCL-2 as a cooperating partner with BCL-XL in resisting apoptosis. Together, these data indicated to us that both BCL-2 and BCL-XL may be imperative in Ewing sarcoma survival. We then moved to chemical interrogation of the cells with specific BCL-2 family inhibitors. Surprisingly, despite the increase in BCL-2, we found the BCL-2-specific inhibitor venetoclax (31) was unable to effectively sensitize CHLA10 cells to olaparib (Supplementary Fig. S5A). Because increased expression of BCL-XL is sufficient to induce resistance to venetoclax (32–34), we next tested the dual BCL-2/BCL-XL inhibitor navitoclax (35, 36) to determine if this agent sensitizes the CHLA10 cells to olaparib. Although venetoclax showed little potentiation of olaparib (Supplementary Fig. S5A and S5B), navitoclax sensitized CHLA10 cells to olaparib treatment compared with venetoclax (P < 0.05), leading to a near-complete loss of cell viability (Fig. 1D), and mild synergy (Supplementary Fig. S5C). Impressively, at low doses of olaparib (1 μmol/L) where there was no single-drug efficacy in the CHLA9 cells, the addition of navitoclax led to substantial loss of cell viability (Supplementary Fig. S5D). Similar to venetoclax, the BCL-XL–selective inhibitor A-1331852 (37) was not effective at sensitizing CHLA10 cells to olaparib (Supplementary Fig. S5E).

Consistent with these findings, we found the CHLA9 cells underwent marked cell death in response to olaparib, as measured by cleaved PARP1 (Fig. 1E); in contrast, there was a near absence of a cell death response in the olaparib-treated CHLA10 cells (Fig. 1E). However, the addition of navitoclax led to marked cleavage of PARP1 in the presence of olaparib in the CHLA10 cells (Fig. 1E), despite the lack of modulation of BCL-2, BCL-XL, or the related MCL-1 (38) by olaparib (Fig. 1F). These data indicate that EWFTs can lose their sensitivity to olaparib following chemotherapy treatment and relapse, underscored by their inability to undergo cell death, and can be rescued by the addition of navitoclax. This was further supported by the observation that, at low concentrations of olaparib where sensitive CHLA9 cells do not yet respond to single-agent olaparib, navitoclax also sensitizes to olaparib (Supplementary Fig. S5D). Of note, the CHLA9 cells have functional p53, whereas the CHLA10 cells have nonfunctional p53 (39). It is well established that functional p53 is capable of binding to and antagonizing the antiapoptotic functions of BH3 proteins such as BCL-2 and BCL-XL (40, 41). In order to rule out p53 as the cause of inherent resistance to olaparib-induced apoptosis in the CHLA10 cells when compared with the CHLA9 cells, we used siRNA to knockdown TP53 in the CHLA9 cells and found no difference in olaparib sensitivity (Supplementary Fig. S5F, left), consistent with a previous report on p53 and PARPi sensitivity (2). To further support the role of BCL-2 and/or BCL-XL overexpression in apoptotic resistance to olaparib treatment, we overexpressed BCL-2 or BCL-XL in the CHLA9 cells. Here, we saw a significant increase in resistance to olaparib treatment in cells overexpressing BCL-2 or BCL-XL compared with the GFP controls (P < 0.0001; Supplementary Fig. S6A). Together, these data reveal a striking interplay between BCL-2/XL inhibition and PARPi inhibition in the EWFTs.

Navitoclax and olaparib cooperate to inhibit tumor growth in a CHLA10 mouse model
We next grew CHLA10 tumors in NSG mice and evaluated single-agent olaparib, navitoclax, and the combination of olaparib and navitoclax to see if the in vitro results would translate in vivo. Consistent with the cell culture experiments (Fig. 1D and E), we found the CHLA10 tumors were not sensitive to olaparib and minimally sensitive to navitoclax as a monotherapy compared to untreated mice (Fig. 1G). However, the combination of olaparib and navitoclax demonstrated robust inhibition of tumor growth (Fig. 1G). This contrasted with the venetoclax/olaparib...
Chemotherapy-resistant CHLA10 cells are sensitized to olaparib with the inhibition of BCL-2 and BCL-XL. A, (left) Crystal violet staining of CHLA9 and CHLA10 cells after a 5-day treatment of 5 mM olaparib compared to a no-treatment control (no Rx). Right, 72-hour CellTiter Glo of CHLA9 and CHLA10 using the indicated concentrations of olaparib. B, Western blot analysis with the indicated antibodies in chemotherapy-naive and chemotherapy-resistant paired lines. C, Two cases of Ewing sarcoma with available paired primary and recurrent metastatic tissues were immunostained for BCL-2 and BCL-XL. In both cases, similar expression of BCL-2 was noted in primary. For BCL-XL, however, increased expression was noted in both recurrences compared with the primary tumors. Case 1: Primary: Archival sections of the untreated biopsy of the primary tumor (patella), which was localized at presentation. Recurrence: Lung metastasis 5 years subsequent, after systemic chemotherapy (VAC-IE) and localized radiotherapy to the patellar primary site. Case 2: Primary: Archival sections of the biopsy of the untreated primary tumor (thoracic spine), which was metastatic (rib, lung, bone marrow) at presentation. Recurrence: Bone metastasis (right humerus) 8 months subsequent, after systemic chemotherapy (VAC-IE) and localized radiotherapy to the primary and multiple metastatic sites. D, Crystal violet staining showing olaparib-resistant CHLA10 cells after 5-day treatment of 5 mM olaparib, 1 mM navitoclax, or the combination of 5 mM olaparib + 1 mM navitoclax compared to a no-treatment control (no Rx). E, Western blot analysis of apoptosis indicated by an increase in cleaved PARP1 in CHLA9 and CHLA10 cells after 24-hour treatment of 5 mM olaparib, 1 mM navitoclax, or 5 mM olaparib + 1 mM navitoclax compared to a no-treatment control (no Rx). F, Western blot analysis of the indicated antibodies in the CHLA9 and CHLA10 cell lines after 24-hour treatment of 5 mM olaparib, 1 mM navitoclax, or 5 mM olaparib + 1 mM navitoclax compared to a no-treatment control (no Rx). G, CHLA10 xenografts treated daily with olaparib (100 mg/kg), navitoclax (80 mg/kg), or the combination of olaparib (100 mg/kg) + navitoclax (80 mg/kg) for 28 days. Error bars are ± SEM. Asterisks indicate a significant separation between the combination (olap/nav) and all other treatment cohorts using the Student t test (P < 0.05). H, CHLA10 xenografts treated daily with olaparib (100 mg/kg), venetoclax (100 mg/kg), or the combination of olaparib (100 mg/kg) + venetoclax (100 mg/kg) for 30 days. Error bars are ± SEM.
Figure 2. Combination of olaparib and navitoclax does not augment toxicity. NSG mice were treated with olaparib (100 mg/kg), navitoclax (80 mg/kg), or the combination of olaparib (100 mg/kg) + navitoclax (80 mg/kg) compared to a no-treatment control (no Rx). After the indicated 3- or 7-day treatment period, blood was collected and sent to IDEXX BioResearch (idexxbioresearch.com) for a complete blood count. The recovery cohort was treated for 7 days with the combination of olaparib (100 mg/kg) + navitoclax (80 mg/kg) and allowed 24 hours without treatment before blood was collected. Three-day treatment with navitoclax not significant compared with 3-day treatment with the combination olaparib + navitoclax, nor was 7-day navitoclax compared with 7-day olaparib + navitoclax.
combination, which was ineffective (Fig. 1H), consistent with the in vitro results (Supplementary Fig. S5A). The olaparib and navitoclax combination did not induce substantial weight loss in the mice or any overt signs of toxicity, suggesting the combination is well tolerated (Supplementary Fig. S6B). To assess possible hematologic toxicity when using olaparib and navitoclax in combination, we performed a complete blood count on NSG mice in vivo. Red blood cell and reticulocyte counts were not significantly affected by the combination. We did observe a significant decrease in platelet count as well as neutrophil count following navitoclax treatment that has previously been reported with its use (31); however, importantly, olaparib did not augment platelet loss at either time point. Also of importance, there was no augmentation of neutropenia or other toxicity by the combination compared with any single-agent dosing at either time point (Fig. 2). As thrombocytopenia is the major dose-limiting effect of navitoclax (42), we also assayed populations of cells following a 24-hour recovery period after 7 days of treatment. Impressively, these mice nearly fully recovered pretreatment platelet levels (Fig. 2). These data demonstrate olaparib plus navitoclax may be both effective and tolerated as a novel combination therapy in EWFTs.

Most Ewing sarcoma cell lines do not undergo marked apoptosis following olaparib therapy

Following our findings in the CHLA9 and CHLA10 pair, we next expanded to a panel of Ewing sarcoma cell lines to determine the ability of olaparib to induce apoptosis. Akin to CHLA10, these cells had poor apoptotic responses to olaparib (Fig. 3A), in contrast to the CHLA9 cells. However, all Ewing sarcoma cells underwent G2–M accumulation, as previously reported (Supplementary Fig. S6C; ref. 43). Caspase 3 activity confirmed both the differential apoptosis between the CHLA9 cells and other EWFTs cell lines, as well as the apoptosis sensitization by navitoclax (Supplementary Fig. S7A). These data suggest our findings of mitigated apoptotic responses to olaparib uncovered in the CHLA10 cells extend to other EWFTs models.

We next determined whether these other resistant EWFTs models had higher levels of BCL-2 or BCL-XL, as our model would predict. Indeed, in comparison with the CHLA9 cells, these models had higher levels of BCL-2 and/or BCL-XL (Fig. 3B), associated with their poor apoptotic responses to olaparib (Fig. 3A). We have uncovered an important role for both BCL-2 and BCL-XL in olaparib response in EWFTs (Figs. 1 and 3), and it would strengthen the case of the importance of BCL-2 and BCL-XL in EWFTs survival if these cancers were sensitive to pharmaceutical targeting of these 2 proteins. We therefore examined in the updated GDSC screen (www.cancerRxgene.org) whether Ewing sarcoma cells were more sensitive to navitoclax (Fig. 3C) compared with all other solid tumor cell lines grouped together. In fact, Ewing sarcoma cell lines were substantially more sensitive (P = 8.69 × 10−5), with 8 of 21 cell lines demonstrating IC50s of 700 nmol/L and below (Fig. 3C).

Interestingly, expression of EWS-FLI1 in HEK293T cells led to increased BCL2, PRKCB, EZH2, and STEAP1 expression with the expected decrease in BCL2 expression with the expected decrease in EZH2

Figure 3.

EWFTs are resistant to olaparib which correlates with increased BCL-2 and navitoclax sensitivity. A, FACS analysis of apoptosis after 24-hour treatment with 5 μmol/L olaparib. Error bars are ± SEM. Asterisks indicate a significant separation between the olaparib treatment groups of the CHLA9 and CHLA10 cells, using the Student t test (P < 0.01). B, Western blot analysis of the indicated antibodies in EWFT cell lines. C, IC50 of navitoclax plotted for solid tumor cell lines and 21 Ewing sarcoma cell lines from http://www.cancerRxgene.org/. A Mann–Whitney nonparametric test was performed (P = 8.69 × 10−5).
expression (Supplementary Fig. S7E), and increase in expression of the normally EWS-FLI1 downregulated LOX, while EWS-FLI1 upregulated targets STEAP1, NPY1R, and PRKCB expression were reduced following EWS-FLI1 downregulation, albeit not all significantly (45–47). Altogether, these data further demonstrate the importance of BCL-2 and BCL-XL in EWTs, which appear to play complimentary roles, constituting a critical survival signal for EWS-FLI1-driven EWTs.

Navitoclax sensitizes a panel of Ewing sarcoma cells to olaparib

We moved to validate navitoclax as a sensitizing agent to olaparib in EWTs, as was determined in the CHLA9 and CHLA10 pair (Fig. 1). In fact, we noted marked sensitization by navitoclax to olaparib-induced apoptosis in a panel of Ewing sarcoma cells as evidenced by cleaved PARP1 (Fig. 4A) and FACS measurement of Annexin-V–positive cells (Fig. 4B). Consistent with the CHLA pair, apoptosis sensitization was sufficient for navitoclax to markedly reduce total viable cells as determined by both crystal violet assays (Fig. 4C) and 72-hour cell-viability assays (Fig. 4D and E). These data again indicate that olaparib-induced apoptosis can be rescued by the addition of navitoclax in Ewing sarcoma.

Navitoclax sensitizes Ewing sarcoma cells to olaparib by augmentation of DNA damage and disruption of BIM complexes

We next examined the DNA-damaging activity of the olaparib/ navitoclax combination using γH2AX immunofluorescence staining as a marker for DNA damage. Interestingly, the DNA damage observed following olaparib exposure was substantially increased (P < 0.0001) with the addition of navitoclax, which by itself did not induce DNA damage (Fig. 5A; Supplementary Fig. S8A). These data are consistent with a direct role of BCL-2 and BCL-XL in the augmentation of DNA damage (48).

Navitoclax disrupts BIM:BCL-XL and BIM:BCL-2 complexes, to induce apoptosis (20, 49–51), and can sensitize kinase inhibitors in different cancers through modulation of the BCL-2 family (reviewed in ref. 7). We therefore asked whether reduction of BIM protected from the olaparib/navitoclax combination. As demonstrated in Fig. 5B, reduction of BIM by siRNA led to a concomitant loss in cleavage of PARP1. Immunoprecipitation of BIM complexes verified navitoclax-disrupted BIM:BCL-2 complexes (Fig. 5C; Supplementary Fig. S8B and S8C). These data indicate that olaparib/navitoclax induce apoptosis in EWTs through disruption of BIM complexes, whereas this complex disruption leads to BIM-mediated apoptosis (51, 52). Together, these data demonstrate multiple mechanisms in which navitoclax sensitizes Ewing sarcoma to olaparib.

Mouse models of Ewing sarcoma are sensitive to olaparib plus navitoclax

To robustly test this novel combination of olaparib and navitoclax, we expanded our analyses to 3 models of Ewing sarcoma, the SK-ES-1 xenograft (ATCC HTB-86), and 2 PDXs. Mice were treated daily with olaparib (100 mg/kg), navitoclax (80 mg/kg), or the combination of olaparib (100 mg/kg) + navitoclax (80 mg/kg). In all 3 models, there was limited activity of either agent when dosed as a mono-therapy. However, the combination of olaparib and navitoclax markedly inhibited tumor growth in the SK-ES-1 model and PDX SA10233 and, impressively, almost completely shrank tumors in the other PDX model, SA13542 (Fig. 6A and B). Again, the combination did not markedly affect mouse weights or induce any overt signs of toxicity (Supplementary Fig. S6B).

Assessment of the tumor lysates from the SA13542 PDX confirmed marked apoptosis induction with the combination, but not single agents (Fig. 6C), and that both PDX models expressed BCL-XL and with the SA10233 model expressing high levels of BCL-2 (Supplementary Fig. S8D). These data, along with the CHLA10 chemorefractory mouse model (Fig. 1C), demonstrate compelling activity of the combination of olaparib and navitoclax in vivo.

Discussion

Through an unbiased high-throughput drug screen, olaparib was discovered to have marked in vitro activity in Ewing sarcoma (11). Despite several other reports demonstrating hypersensitivity of Ewing sarcoma to PARP inhibition (10, 12–14, 23, 53), subsequent clinical evaluation in a heavily pretreated cohort of patients with Ewing sarcoma with single-agent olaparib showed only modest efficacy (15). Here, we demonstrated an important role for deficient apoptosis following olaparib therapy in Ewing sarcoma, with the antiapoptotic proteins BCL-2 and BCL-XL playing key roles. We believe these experimental findings at least in part explain the disappointing clinical data.

PARP inhibitors prevent single-stranded (ss) DNA break repairs. This mechanism underlies PARP1 activity in BRCA-deficient cancers, which are inherently deficient in double-stranded (ds) DNA break repair (54). In Ewing sarcoma, PARPi sensitivity has been proposed to occur for several reasons: First, PARPi expression is higher in Ewing sarcoma (55), probably as a direct result of EWS-FLI1 (10, 55), and higher PARP1 expression is a cause of enhanced PARPi sensitivity (56), most likely through the mechanism of PARP trapping at ssDNA breaks (57, 59). Second, Ewing sarcoma, like BRCA- deficient cancers, appears to have a deficient dsDNA repair system (12). Third, FLI1 drives high SLFN11 expression (60), a gene tightly linked to DNA-damaging agent efficacy (24, 61). Fourth, EWS-FLI1 expression is sufficient to increase dsDNA breaks (10). Fifth, EWS-FLI1 causes R-loop accumulation, increases replication stress, and interferes with BRCA1 function (62).

Although there are several factors that may have contributed to olaparib's lack of efficacy in patients with chemotherapy-resistant Ewing sarcoma, it is likely that a biological resistance mechanism served to rescue tumor cells from direct PARP inhibition. We propose that there is an inherent deficiency in many Ewing sarcomas to undergo apoptosis following olaparib treatment resulting from a protective effect of BCL-2 and BCL-XL (Figs. 1 and 3). Furthermore, exposure and resistance to chemotherapy appear to contribute to this state of apoptotic resistance to olaparib, as evidenced by our results in the CHLA10 cell line (Figs. 1E and 3A) and observations in patients' tumor specimens (Fig. 1C). It is likely that DNA-damaging agents used in induction chemotherapy lead to additional pressure on the Ewing sarcoma tumor and, as a result, the emergence of cells particularly reliant on BCL-2/BCL-XL for survival. Overall, further studies will be necessary to elucidate the precise relationship between these prosurvival BCL-2 members and Ewing sarcoma tumorigenesis.

The strategy to sensitize Ewing sarcoma to PARP inhibition via BCL-2/BCL-XL coinhibition is different from other explored...
Figure 4.
Combination of olaparib and navitoclax is effective in multiple Ewing sarcoma cell lines. A, Western blot analysis of apoptosis indicated by cleaved PARP1 after 24-hour treatment with no-treatment control (no Rx), 5 μmol/L olaparib, 1 μmol/L navitoclax, or 5 μmol/L olaparib + 1 μmol/L navitoclax. B, FACS analysis of apoptosis after 24-hour treatment with 5 μmol/L olaparib, 1 μmol/L navitoclax, or 5 μmol/L olaparib + 1 μmol/L navitoclax. The percentage of apoptosis induced by drugs is normalized to the no-treatment control. Error bars are ±SEM. C, Crystal violet staining after 5-day treatment with 5 μmol/L olaparib, 1 μmol/L navitoclax, or 5 μmol/L olaparib + 1 μmol/L navitoclax. D, Dose–response curves in Ewing sarcoma cell lines after 72-hour treatment with increasing concentrations of olaparib. Viability was determined using CellTiter-Glo. Data are graphed as percent viable cells from no-treatment control (no Rx), performed in quadruplicate. Error bars are ±SEM. E, Ewing sarcoma cell lines after 72-hour treatment with 1 μmol/L navitoclax or 1 μmol/L navitoclax in the presence of increasing concentrations of olaparib, and viability was determined using CellTiter-Glo. Data are graphed as percent viable cells from no-treatment control (no Rx), performed in quadruplicate. Error bars are ±SEM.
strategies to sensitize Ewing sarcoma to PARP inhibition; these include the addition of DNA-damaging agents that intensifies the amount of active DNA damage in the cell, like irinotecan and temozolomide (13). Temozolomide has also been demonstrated to enhance PARP1 trapping (59) and, interestingly, the combination of temozolomide and PARP inhibition cooperatively downregulates MCL-1, sensitizing to mitochondrial-mediated death (13). Although temozolomide–PARPi combinations are poorly tolerated in preclinical Ewing sarcoma mouse models (12), irinotecan delivered to an orthotopic Ewing sarcoma mouse model in dosing schedules consistent with the pediatric population demonstrated marked activity (12). Consistent with these results, the combination of the PARP veliparib and irinotecan was well tolerated in a recent phase I trial, including reaching a dose sufficient for PARP inhibition in adult cancers (63). Of note, BCL-X<sub>i</sub> blocks the ability of irinotecan to induce apoptosis and BCL-X<sub>i</sub> inhibition results in a switch from irinotecan-induced senescence to apoptosis (64). Therefore, it is possible that PARPi/irinotecan combinations in other Ewing sarcoma models will face the same issues we have found PARP inhibition monotherapy to face, namely a refractory apoptosis response. The PARPi/irinotecan combination is currently being evaluated in pediatric patients with solid tumors (NCT02392793).

The BCL-2 family of proteins monitors the integrity of the mitochondria and integrates the signals of many pathways at the mitochondria (65). Importantly, Jawaheri and colleagues (66) elegantly demonstrated that EWS-FLI1 overexpression in mesenchymal stem cells, the presumed cell of origin for Ewing sarcoma, was sufficient for blocking differentiation but led to high rates of apoptosis; retroviruses containing BCL-2, BCL-X<sub>i</sub>, or MCL-1 expression plasmids was able to rescue apoptosis and, importantly, led to sarcoma formation, which was not accomplished in the parallel, control-transduced cells. These data together with the data in this article present a compelling case where antiapoptotic activity of BCL-2 family members, particularly BCL-2 and BCL-X<sub>i</sub>, plays an intricate role in Ewing sarcoma tumorigenesis and affects Ewing sarcoma therapy.

It has been well known for several decades that BCL-2 has a protective role against DNA damage–induced apoptosis (48, 67). In addition, BCL-X<sub>i</sub> expression has been reported to correlate inversely with the sensitivity of cancer cell lines to multiple antitumor agents, including those acting via a DNA-damaging mechanism (23). This becomes particularly relevant in the light that Ewing sarcoma have deficient DNA damage responses (12). Adding to the intrigue, Brohl and colleagues recently reported 13% of patients with Ewing sarcoma have germline loss-of-function mutations in DNA repair genes (68). It is therefore tempting to speculate that, in order for EWS-FLI1–translocated Ewing sarcoma to develop and thrive, there must be an acquired reliance on the antiapoptotic proteins BCL-2 and BCL-X<sub>i</sub> to maintain survival. Consistent with this notion, our analyses of HTS data revealed navitoclax has substantial single-agent activity (IC<sub>50</sub> less than 700 nmol/L) across approximately 40% of Ewing sarcoma cell lines (Fig. 3C).

This notion is further supported by our findings in the CHLA cells derived from a patient prior to and following chemotherapy treatment. The postchemotherapy CHLA10 cells, derived at progressive disease, had higher BCL-2 expression relative to the matched chemotherapy-naïve CHLA9 cells (Fig. 1B) and, unlike CHLA9 cells, failed to undergo cell death following olaparib therapy (Figs. 1E and 3A). It is important to note that we did not account for other changes that occurred during the acquisition of chemotherapy resistance in this model, which could...
contribute to the resistance of these cells to olaparib. For instance, Mendoza-Naranjo and colleagues demonstrated the CHLA10 cells have enhanced flux through the PI3K pathway compared with the CHLA9 cells, which is a result of increased ErbB4 expression (69) and which may be contributing to olaparib resistance. Notwithstanding, the fact that navitoclax was sufficient to resensitize the cells to olaparib reflects the importance of BCL-2 and BCL-XL. Interestingly, in the chemotherapy-naïve CHLA9 cells, where olaparib was very effective (Fig. 1A), navitoclax fully sensitized these cells to a low dose of olaparib (Supplementary Fig. S5D), which did not have marked single-agent anticancer activity. These data reveal an important interplay between PARP inhibition and BCL-2/XL inhibition, which likely contributes to the impressive activity of dual PARP and BCL-2/XL inhibition in Ewing sarcoma (Figs. 1, 3, and 4) and, again, supports the notion that BCL-2 and BCL-XL are important to counteract the intrinsic deficiencies in Ewing sarcoma DNA damage repair, which are exacerbated by PARP inhibition. Indeed, BCL-2/BCL-XL inhibition causes accumulation of DNA damage following PARP inhibition (Fig. 5A; Supplementary Fig. S8A). Therefore, the robust activity of PARP inhibition and navitoclax is most likely due to both BCL-2 and BCL-XL inhibition, making these cells more vulnerable to DNA damage-induced apoptosis, but also increasing the DNA damage itself. The result is a substantial increase in apoptosis (Figs. 4A–B), mediated by BIM (Fig. 5B and C), which translates to impressive in vivo activity.

Overall, we demonstrate Ewing sarcoma tumors do not undergo a marked apoptotic response following olaparib therapy; however, cotargeting BCL-2 and BCL-XL dramatically sensitizes these tumors to olaparib in several mouse models of Ewing sarcoma, including chemotherapy-resistant Ewing sarcoma and 2 PDX models of Ewing sarcoma. As we found neither drug augmented hematologic toxicity of the other.

Figure 6.
Olaparib and navitoclax combination is effective in mouse models of Ewing sarcoma. A, SK-ES-1 xenografts treated daily for 26 days (top) and patient-derived xenografts (bottom) treated daily for 31 days (PDX SA13542) or 27 days (PDX SA10233) with olaparib (100 mg/kg), navitoclax (80 mg/kg), or the combination of olaparib (100 mg/kg) + navitoclax (80 mg/kg). Error bars are ±SEM. Asterisks indicate a significant separation between the combination (olap/nav) and all other treatment cohorts using the Student t test (P < 0.05). B, Fold change in tumor volume, please note data are from the experiment shown in Fig. 6A (PDX SA13542) after 31 days of treatment, and the x axis denotes individual xenografts. C, Western blot analysis of cleaved PARP1 from PDX tumor lysates after 3 daily treatments of no-treatment control (no Rx), olaparib (100 mg/kg), navitoclax (80 mg/kg), or the combination of olaparib (100 mg/kg) + navitoclax (80 mg/kg).
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.A.R. Heisey, T.L. Lochmann, K.V. Floros, C.M. Coon, K.M. Powell, S. Jacob, M.L. Calbert, M.S. Ghotra, G.T. Stein, S.C. Smith


References

Navitoclax Sensitizes Ewing Sarcoma to Olaparib


The Ewing Family of Tumors Relies on BCL-2 and BCL-X\textsubscript{L} to Escape PARP Inhibitor Toxicity


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