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The BET inhibitor INCB054329 reduces homologous recombination efficiency and augments PARP inhibitor activity in ovarian cancer

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Abstract

Objective—Homologous recombination (HR)-proficient ovarian tumors have poorer clinical outcomes and show resistance to poly ADP ribose polymerase inhibitors (PARPi). A subset of HR-proficient ovarian tumors show amplification in bromodomain and extra-terminal (BET) genes such as *BRD4*. We aimed to test the hypothesis that BRD4 inhibition sensitizes ovarian cancer cells to PARPi by reducing HR efficiency and increasing DNA damage.

Methods—HR-proficient ovarian cancer cell lines (OVCAR-3, OVCAR-4, SKOV-3, UWB1.289 + BRCA1) were treated with BRD4-targeting siRNA, novel (INB054329, INCB057643) and established (JQ1) BET inhibitors (BETi) and PARPi (olaparib, rucaparib). Cell growth and viability were assessed by sulforhodamine B assays *in vitro*, and in SKOV-3 and ovarian cancer patient-derived xenografts *in vivo*. DNA damage and repair (pH2AX, RAD51 and BRCA1 foci formation, and DRGFP HR reporter activity), apoptosis markers (cleaved PARP, cleaved caspase-3, Bax) and proliferation markers (PCNA, Ki67) were assessed by immunofluorescence and western blot.

Results—In cultured cells, inhibition of BRD4 by siRNA or INCB054329 reduced expression and function of BRCA1 and RAD51, reduced HR reporter activity, and sensitized the cells to olaparib-induced growth inhibition, DNA damage induction and apoptosis. Synergy was observed

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between all BETi tested and PARPi. INCB054329 and olaparib also co-operatively inhibited xenograft tumor growth, accompanied by reduced BRCA1 expression and proliferation, and increased apoptosis and DNA damage.

Conclusions—These results provide strong rationale for using BETi to extend therapeutic efficacy of PARPi to HR-proficient ovarian tumors and could benefit a substantial number of women diagnosed with this devastating disease.

Keywords

ovarian cancer; BET inhibitor; PARP inhibitor; homologous recombination

INTRODUCTION

Ovarian cancer is the deadliest gynecologic malignancy and the fifth leading cause of cancer death among women in the US [1]. High-grade serous ovarian cancer (HGSOC) is the most common and fatal subtype. Treatment options are limited for women with recurrent ovarian cancer, particularly those with chemoresistant disease. Poly ADP ribose polymerase inhibitors (PARPi) are promising new drugs that have shown clear advantage in *BRCA*-mutated ovarian cancer [2, 3] and in tumors with deficiencies in other homologous recombination (HR) DNA repair genes [4, 5]. By inhibiting single-strand break repair machinery, PARPi cause synthetic lethality in HR-deficient cells. Three PARPi (olaparib, rucaparib, and niraparib) were recently FDA-approved for treatment and maintenance in ovarian cancer. Despite their potential, PARPi are far less effective as single agents in the 50% of patients with high-grade serous tumors retaining HR proficiency [6, 7]. Developing strategies to expand the use of PARPi to HR-proficient tumors is a critical clinical challenge. One strategy is the development of rational PARPi combinations with other drugs.

The bromodomain and extraterminal domain (BET) proteins BRD2, BRD3 and BRD4 interact with acetylated lysine residues on histone tails via their bromodomain, and function as epigenetic readers of lysine acetylation to promote gene transcription. Of the BET proteins, BRD4 is most frequently altered in ovarian cancer, since the BRD4 gene is amplified in approximately 10% of high-grade serous ovarian tumors in the Cancer Genome Atlas database [6]. Targeting BRD4 for inhibition could be effective in this subset of *BRD4* amplified tumors that are associated with poor clinical outcomes [8, 9]. Small molecule BET inhibitors (BETi), such as JQ1, reversibly bind to the hydrophobic pockets in bromodomains, which disrupts the association of BET proteins acetylated histone tails and transcription factors such as histone deacetylases (HDACs) and the transcriptional elongation factor pTEFb [10]. This results in transcriptional repression of BET target genes, which are involved in promoting transcription of genes involved in the cell cycle, DNA repair, cell growth, cancer, and inflammation. BETi have emerged as an exciting new epigenetic therapeutic strategy for multiple types of cancer, including ovarian cancer [11–15]. JQ1 was the first in class [16], and novel BETi INCB054329 and INCB057643 are advancing to the clinic (Incyte Corporation, www.clinicaltrials.gov - ID numbers NCT02431260 and NCT02711137).

Our group has generated multiple lines of evidence demonstrating that epigenetic drugs such as histone deacetylase inhibitors (HDACi) improve responses to DNA damaging drugs in ovarian cancer cells [4, 17, 18]. We have shown that the HDACi vorinostat and panobinostat downregulates HR gene expression and repair efficiency in HR-proficient ovarian cancer cells and sensitizes chemoresistant cells to the cytotoxic effects of the PARPi olaparib and cisplatin [4, 18]. Because of the link between BRD4 and HDACs in promoting gene transcription [10] and recent observations that JQ1 improves olaparib response in ovarian cancer cells [15, 19], we tested novel clinically relevant BETi/PARPi drug combinations in HR proficient ovarian cancer cells *in vitro* and *in vivo*.

Here we show that specific BRD4 knockdown and BRD4 inhibition with the novel BETi INCB054329 reduced expression of HR components and co-operatively reduced cell growth and increased DNA damage and apoptosis induced by PARPi and cisplatin. Consistent with these observations, INCB054329 reduced HR proficiency, and co-operatively induced PARPi-induced DNA damage and apoptosis in cultured cells and in cell line and patient-derived xenografts (PDX) *in vivo*. Our studies provide strong rationale for extending the use of PARPi, in combination with BETi, to a significant proportion of women diagnosed with HR-proficient ovarian cancer.

MATERIALS AND METHODS

Cell culture and reagents

The epithelial ovarian cancer cell lines SKOV-3, OVCAR-3, UWB1.289+BRCA1 wild-type (BRCA1 WT) and UWB1.289 BRCA1 null (BRCA1 Null) cell lines (American Type Culture Collection, Manassas, VA), and OVCAR-4 (National Cancer Institute, Bethesda, MD) were maintained in culture as previously described [4, 17]. Cell lines were authenticated by the Vanderbilt VANTAGE Core using the GenePrint 10 kit (Promega, Madison, WI). All cell lines used tested negative for mycoplasma. The BETi INCB054329 and INCB057643 were provided by Incyte Corporation (Wilmington, DE) and AZD-2281 (olaparib) was provided by Astra Zeneca Pharmaceuticals (Wilmington, DE). The BETi JQ1 and the PARPi rucaparib were purchased from Selleck Chemicals (Houston, TX), while cisplatin was from Sigma Chemical Co. (#479306), St Louis, MO). BETi and PARPi were reconstituted in DMSO, while cisplatin was dissolved in PBS. 0.01% dimethyl sulfoxide (DMSO; Sigma) was used as vehicle for experiments combining BETi and PARPi in cultured cells. All treatments received equal amounts of DMSO or PBS/DMSO vehicle, as applicable. A mixture of PBS and 0.01% DMSO was the vehicle for experiments combining cisplatin and BETi. For transient BRD4 knockdown, SKOV-3 cells were transfected with ON-TARGETplus non-targeting (NT) or two distinct BRD4-targeting siRNA duplexes (siBRD4) (using RNAiMAX transfection reagent (Thermo Fisher Scientific, Inc., Waltham, MA).

Cell proliferation and cytotoxicity assays

Sulforhodamine B (SRB) assays were used to determine cell proliferation and cytotoxicity as previously described [18]. Absorbance was measured at 510nm using a Spectramax M5 spectrophotometer (Molecular Devices, Sunnyvale, CA) in the High-Throughput Screening

Laboratory at Vanderbilt. The interaction between fixed ratios of BETi and PARPi or cisplatin was measured with the Combination Index (CI) method. A CI level of < 0.9, CI = 0.9–1.1 and CI > 1.1 indicates synergy, additivity and antagonism respectively, between drug combinations. Clonogenic assays were performed and quantified as described [18].

Immunostaining

Following drug treatment, OVCAR-3 and SKOV-3 ovarian cancer cells were fixed, permeabilized and stained with mouse monoclonal anti-phospho H2AX (Ser139) (pH2AX) (EMD Millipore, Billerica, MA), rabbit polyclonal anti-RAD51 (Millipore), or rabbit monoclonal anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) as described [17]. Immunohistochemistry (IHC) for anti-Ki-67/mib-1 and cleaved caspase-3 in formalin-fixed, paraffin-embedded tumors, secondary antibodies for immunofluorescence (IF) and IHC, and image acquisition and analysis was as previously described [17].

High-content fluorescent microscopy

Following drug treatment, we performed high-content IF imaging of cells using the ImageXpress Micro XL imaging platform (Molecular Devices (Sunnyvale, CA). Images of cells stained with DAPI (Sigma) to mark cell nuclei and for pH2AX were acquired in nine fields of view per well. Analysis of pH2AX intensity normalized to DAPI staining, adherent cell number and the cell cycle indices %G₀/G₁, %S and %G₂/M were performed using MetaXpress software.

Plasmid-based DNA repair assays

For measuring drug effects on HR, the HR reporter plasmid pDRGFP and endonuclease encoding pCBASce1 (I-Sce1) (both gifts from Maria Jasin; Addgene plasmids #26475 and #26477, respectively) [20, 21] were used. For measuring NHEJ efficiency, the pEJ5-GFP NHEJ reporter plasmid (gift from Jeremy Stark; Addgene plasmids #4426) [22] and I-Sce1 were used. OVCAR-3 and SKOV-3 cells grown on coverslips were transfected with the plasmids using Lipofectamine 2000 according to manufacturer's instructions (Thermo Fisher), drug treated for 24 hours and and visualized for GFP expression using fluorescence microscopy as previously described [17].

Western Blotting

For harvested tumors or cell lines, whole cell protein isolation, hydrochloric acid extraction of histones, western blotting and signal detection were performed as previously described [23, 24]. Antibodies used were rabbit polyclonal anti-BRD4 (Millipore), mouse monoclonal anti-BRCA1 (Millipore), rabbit polyclonal anti-RAD51 (Millipore), rabbit polyclonal anti-cyclin E (Abcam, Cambridge, MA), rabbit polyclonal anti-PARP (Cell Signaling Technology), rabbit polyclonal anti-Bax (Millipore), mouse monoclonal anti-PCNA (Santa Cruz Biotechnology, Inc., Dallas, TX), mouse monoclonal anti-p21 (Thermo Fisher), mouse monoclonal anti-phospho H2AX (Ser139) (Millipore), phospho(T2609)-DNA-PKcs (Thermo Scientific), DNA-PKcs (Santa Cruz Biotechnology, Dallas, TX). Loading control was β -actin (Sigma).

Animals

Experiments performed were approved by the Vanderbilt University Animal Use and Care Committee and animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care. Six to eight-week-old female athymic Nude-*Foxn1^{nu}*, (Nude; Envigo, Indianapolis, IN) and NOD.CB17-Prkdcscid Il2rgtm1Wjl/SzJ (NSG; Jackson Laboratory, Bar Harbor, ME) were purchased for drug treatment experiments. 5×10^6 SKOV-3 tumor cells in a 200 μ L of mixture of PBS and Matrigel (1:1 v/v) (BD Biosciences, San Jose, CA) were injected into the right flank of Nude mice. After the tumors reached approximately 200mm³, we treated the mice in cohorts as follows (n=10): vehicle control (0.5% methylcellulose [Sigma] and N,N-dimethylacetamide [Sigma] by oral gavage), INCB054329 (25mg/kg twice daily by oral gavage), olaparib (100mg/kg PO daily by oral gavage) and the INCB054329/olaparib combination via intraperitoneal injection for 3 weeks. Animals were examined biweekly for the effects of tumor burden and tumor growth, and tumor measurements were performed weekly. Tumor volume was calculated weekly from caliper measurements of the smallest (SD) and largest diameter (LD) using the formula: volume = $[LD \times SD^2] \times \pi/6$ [24]. 24 h after the final dose of drug, the mice were euthanized according to protocol and necropsy performed. Tumors were excised and either snap-frozen for western blot analyses or formalin-fixed for immunohistochemistry (IHC).

For drug treatment experiments in ovarian cancer patient-derived (PDX) tumors, we used a previously characterized model [25]. P4 generation PDX tumors were injected intraperitoneally (IP) into NSG mice and tumors left to engraft for 2 weeks prior to beginning drug treatment (Fig 6A). Mice were treated with vehicle, INCB054329, olaparib and the INCB054329/olaparib combination using the doses described above for 4 weeks. At collection, the volume of abdominal ascites and the weight of harvested abdominal tumors was measured as indices of tumor burden. Tumors were processed for western blot analyses or IHC as analysis as above.

Chromatin Immunoprecipitation (ChIP)

For ChIP experiments, 2×10^7 OVCAR-3 cells were used. Cells were cross-linked with 1% formaldehyde (Sigma-Aldrich), harvested into cell lysis buffer, and sonicated with 2×5 -s pulses. The sonicated chromatin was immunoprecipitated with 5 μ g of rabbit polyclonal ChIP-grade anti-BRD4 antibody (Millipore), 5 μ g of a rabbit polyclonal acetylated histone H3 antibody (Millipore), or 5 μ g of normal rabbit IgG (Santa Cruz Biotechnology). DNA-protein complexes were isolated with protein A/G Plus agarose beads (Santa Cruz Biotechnology) for 4 h at 4°C, washed, eluted in 0.1% SDS, 0.1M NaHCO₃ elution buffer, and cross-links were reversed overnight at 65°C in 0.3 M NaCl. Input samples were also incubated in this way. DNA was purified using phenol:chloroform extraction and ethanol precipitation. A validated primer set was used to amplify DNA adjacent to the transcription start site (TSS) of the *BRCA1* promoter based on a previous report (ChIP-BRCA1) [26]. An additional primer set was designed to amplify a non-related region of DNA (4kb upstream from the TSS of the *p21* promoter; ChIP-Neg) [24]. Quantitative real-time RT-PCR was performed, with DNA content in Input and immunoprecipitation samples measured relative

to a standard curve of OVCAR-3 cell genomic DNA. All experimental values were expressed relative to relevant Input DNA content. Primer sequences were as follows:

ChIP-BRCA1: Forward 5'-CTGACAGATGGGTATTCTTTGACG-3'; Reverse 5'-GCATATTCCAGTTCCTATCACGAG-3')

ChIP-neg: Forward 5'-AGTCTTGCCTGCCTTCAGAG-3'; Reverse 5'-ACGAAGGGCTTGTTTATAGG-3'.

Statistics

Unless otherwise indicated, values shown were the mean + SE of 3 independent experiments with * $p < 0.05$ relative to control using the Student's t test.

RESULTS

Inhibition of BRD4 downregulates BRCA1 and reduces homologous recombination (HR) efficiency in ovarian cancer cells

A major subtype of HR-proficient HGSOc tumors are those harboring *CCNE1* amplifications [6]. Another newly discovered subtype of HR-proficient tumors with similarly poor outcomes have amplifications in *BRD4* [8, 9]. Published copy number analysis data from The Cancer Genome Atlas (TCGA) show frequent amplifications in *CCNE1* (106/557 tumors, 19.0%) and *BRD4* (57/557, 10.2%), and relatively fewer amplifications in other BET protein-encoding genes, *BRD2* (7/557, 1.3%) and *BRD3* (1/557, 0.8%). *BRD4* amplification overlaps with cyclin E (*CCNE1*) amplification in 26/57 (45.6%) of high-grade serous ovarian tumors (Fig. 1A). This finding is consistent with frequent amplification of chromosome 19 in ovarian cancer because chromosome 19 contains both *CCNE1* (19q12) and *BRD4* (19p13) genes [8]. *BRD2* is located on 6p21, a region that is also associated with amplifications in ovarian cancer [27]; whereas, *BRD3* is located on 9q34, which is rarely amplified [28].

We have previously shown that epigenetic HDAC inhibitors reduce expression of HR genes (e.g. *BRCA1* and *RAD51*) and the cell cycle gene *CCNE1* in HR-proficient ovarian cancer cells, resulting in increased DNA damage and apoptosis, and sensitization to PARPi such as olaparib [4, 18]. Given the frequency of *BRD4* amplifications and overlap with *CCNE1* amplifications in ovarian tumors, we examined the effects of inhibiting BRD4 on expression levels of BRCA1 and cyclin E. First, we knocked down BRD4 using two independent siRNAs in SKOV-3 cells and demonstrated a decrease in BRCA1 expression in knockdown and olaparib-treated cells (Fig 1B&C). Moreover, we observed significant increases in pH2AX, an established marker of double-strand DNA damage and cleaved PARP, a marker of apoptosis. We performed complementary chromatin immunoprecipitation assays to verify that BRD4 knockdown specifically reduced occupancy of BRD4 and was associated with reduced levels of acetylated histone H3 at the *BRCA1* locus (Fig 1D&E). These results suggest that BRD4 inhibition plays a role in mediating downregulation of BRCA1 and cyclin E and thus, enhances DNA damage-induced apoptosis induced by PARPi in HR-proficient SKOV-3 ovarian cancer cells.

The effects of specific BRD4 knockdown on BRCA1 and cyclin E expression were recapitulated by the BETi INCB054329. In OVCAR-3 and SKOV-3 cells treated with INCB054329, protein expression levels of BRCA1 and cyclin E were significantly reduced (Fig 2A–C), although there were no effects on BRD4 expression. While treatment with olaparib alone had minimal effects on expression of BRCA1 and cyclin E, we demonstrated co-operative inhibitory effects between INCB054329 and olaparib on BRCA1, RAD51 another HR mediator, and cyclin E expression (Fig 2A–C). These results support targeting BRD4 as an approach to downregulate HR genes BRCA1 and RAD51, as well as cyclin E expression, and as a result, sensitize HR-proficient ovarian cancer cells to PARPi.

To determine whether BETi also reduced the function of BRCA1, we measured drug effects on formation of BRCA1 foci following DNA damage. We performed independent IF assays measuring HR efficiency by RAD51 foci formation to validate our results [4]. In OVCAR-3 and SKOV-3 cells pre-treated for 6h with the known inducer of double-strand DNA breaks, cisplatin (0.5 μ M), there was an approximately 40–50% reduction in the frequency of INCB054329-treated cells expressing BRCA1 foci RAD51 compared to vehicle control (Fig 2D&E). In parallel, there was a reduction in the percentage of cells expressing RAD51 foci by INCB054329 alone and combined with olaparib (Fig 2F&G). HR events were also measured in an independent assay by the production of GFP in cells transiently co-transfected with the HR reporter DRGFP and I-Sce1 endonuclease plasmids, which was assessed by IF analysis [4, 18]. As shown in Fig 2H&I, INCB054329 reduced the percentage of GFP-positive by approximately 50% when administered as a single drug or when combined with olaparib. Consistent with the RAD51 and BRCA1 foci data, olaparib alone had no significant effects on HR efficiency. We observed similar results for JQ1 in combination with olaparib on BRCA1 and cyclin E expression and in the HR efficiency assays (Supplemental Fig S1).

BETi increase non-homologous end-joining (NHEJ) efficiency in ovarian cancer cells

Drug effects on NHEJ events were measured by the production of GFP in cells transiently co-transfected with the NHEJ reporter EJ5GFP and I-Sce1 endonuclease plasmids [22]. As shown in Fig 2J, INCB054329 increased the percentage of GFP-positive cells by 40–50% in OVCAR-3 and SKOV-3 cells. Olaparib alone did not have a significant effect on NHEJ, although there was a trend towards stimulation in these cells ($p=0.057$ and $p=0.072$, respectively). An increase in NHEJ was sustained when the cells were treated with the combination of INCB054329 and olaparib (Fig 2J). Drug effects were further confirmed by measuring expression of the established NHEJ protein marker, phosphorylated(T2609)-DNA-PKcs (Fig 2K).

BETi synergize with DNA damaging drugs in HR-proficient ovarian cancer cells

Having established that BETi reduce HR efficiency, we predicted that BETi sensitize HR-proficient ovarian cancer cells to the cytotoxic effects of PARPi. As shown in Fig 3A&C, INCB054329 reduced cell viability at increasing concentrations and synergistically enhanced the effects of olaparib in an expanded panel of ovarian cancer cell lines. Synergistic effects were also observed between INCB054329 and the PARPi rucaparib in HR-proficient ovarian cancer cells (Fig 3B&C). These synergistic effects were more

pronounced in HR-proficient ovarian cancer cells than in HR-deficient, BRCA null cells, which are sensitive to PARPi. Clonogenic assays confirmed BETi sensitization to the PARPi olaparib in SKOV-3 cells (Fig 3D&E). Finally, SRB assays revealed synergy between olaparib and two other BETi, JQ1 and INCB057643, a newer BETi in clinical development (Supplemental Fig S2A–D).

To directly assess drug effects on proliferation, we performed cell cycle analysis of DAPI-stained cells by high-content fluorescence staining using the MetaXpress platform. As shown in Fig 4A, INCB054329 and olaparib cooperatively reduced the number of adherent DAPI-labeled cells, and the proportion of cells in S phase in OVCAR-3 and SKOV-3 cells. The drug combination induced a G₀/G₁ growth arrest in SKOV-3 cells, but increased the percentage of OVCAR-3 cells in G₂/M phase (Fig 4A). These results were consistent with co-operative induction of expression of the cell cycle inhibitor, p21, by the drug combination (Fig 4B–D).

Having demonstrated synergistic reduction in cell growth and viability with BETi and PARPi combinations, we determined if the drug combinations resulted in further increases in DNA damage. DNA damage was assessed by measuring expression of the established marker of double-strand DNA breaks, pH2AX, a sensitive surrogate marker of cytotoxicity in ovarian cancer cells [17, 29]. First, we measured pH2AX expression by western blot. These results showed enhanced pH2AX expression in cells treated with the combination of INCB054329 and olaparib compared to either drug alone (Fig 4B–D). Our observations were confirmed by immunofluorescence analysis, which showed enhanced pH2AX expression in cells treated with INCB054329 alone and in combination with olaparib (Fig 4E&F). Comparable effects on pH2AX expression were observed between JQ1 and olaparib (Supplemental Fig S3A–D). There was a greater number of cells with high pH2AX expression (greater than 20 foci and pan-nuclear staining where individual foci are not countable) with combination drug treatment. These results were confirmed by western blot analysis (Supplemental Fig S3E&F).

To assess whether the BETi and PARPi drug combination resulted in increased cell death, we measured drug effects on expression of the established apoptotic marker, cleaved PARP. Consistent with the pH2AX data, there was significantly increased expression of cleaved PARP following combination treatment with INCB054329 and olaparib compared to each drug alone in OVCAR-3 and SKOV-3 cells (Fig 4C&D).

Finally, to show that BETi sensitizes ovarian cancer cells to other DNA damaging agents, we evaluated BETi in combination with the clinically utilized drug, cisplatin. As shown in Supplemental Fig 2E&H, INCB054329 or JQ1 synergized with cisplatin in SRB assays in OVCAR-3 and SKOV-3 cells. Furthermore, the combination of JQ1 and cisplatin induced higher levels of pH2AX and the apoptosis marker cleaved caspase-3 than either drug alone in IF assays (Supplemental Fig S4A–D).

Combination treatment with INCB054329 and olaparib demonstrates robust inhibitory effects in vivo

Nude mice injected subcutaneously with SKOV-3 cells were treated with either vehicle, INCB054329 (25 mg/kg PO twice daily), olaparib (100 mg/kg PO) or the INCB054329 and olaparib combination for 3 weeks. Treatment with olaparib, but not INCB054329, led to significant tumor growth inhibition as a single agent compared to the vehicle control (Fig 5A–C). Tumors in the INCB054329 and olaparib combination group were significantly smaller compared to vehicle and either drug alone within 2 weeks of treatment. By the end of the 19 day treatment period, the INCB054329 and olaparib combination led to a greater than 80% reduction in tumor volume and weight at sacrifice, compared to control mice (Fig 5A–C). These differences were unlikely to be a result of generalized toxicity of drug treatment, since mouse weights were stable over the treatment period for all treatment groups (Fig 5A). We confirmed down-regulation of BRCA1 protein expression in tumors treated with INCB054329 alone or combined with olaparib (Fig 5D&E). Consistent with the relative effects on tumor size, the combination treatment co-operatively reduced expression of the pharmacodynamic marker of proliferation (PCNA). In addition, the drug combination increased expression levels of the cyclin-dependent kinase inhibitor p21, and markers of apoptosis (cleaved PARP and Bax), and DNA damage (pH2AX) to a significantly greater extent than single drug treatment alone (Fig 5D&E). Inhibitory drug effects on tumor cell growth and induction of apoptosis were independently confirmed by immunohistochemical analysis (Fig 5F–H).

Drug effects were also determined in a BRCA wild type HGSOc patient-derived xenograft (PDX) model, derived as previously described [25]. Following intra-peritoneal injection of the P4 generation PDX tumor into NSG mice, and a 2 week engraftment period, mice were treated for 4 weeks with the drug regimen described above (Fig 6A). As shown in Fig 6B&C, the INCB054329 and olaparib combination co-operatively reduced tumor burden, as measured by ascites and weight of harvested disseminated peritoneal tumors. Consistent with the SKOV-3 xenograft model, western blot analysis of harvested tumors showed co-operative inhibition of BRCA1 expression and the proliferation marker PCNA, along with induction of apoptosis and DNA damage (Fig 6C&D). These results suggest BETi mediated reduction in HR efficiency, as indicated by a reduction in BRCA1 expression, was associated with enhanced DNA damage and PARPi cytotoxicity in a clinically relevant HR-proficient PDX model of HGSOc.

DISCUSSION

Extending the efficacy of PARPi to chemoresistant HR-proficient ovarian tumors is an important clinical challenge. We have previously shown that epigenetic HDAC inhibitors and the first-in-class BETi JQ1 reduce HR and sensitize these cells to PARPi [4, 18, 19]. In this study, we focused on inhibition of BRD4 with clinically relevant BETi. We showed that INCB054329 reduced HR efficiency and sensitized ovarian cancer cells to the DNA damaging, cytotoxic effects of PARPi and cisplatin. These effects were not associated with a reduction in BRD4 expression in our study, which is consistent with other studies in ovarian cancer cell lines suggesting that BETi block BRD4 interaction with transcriptional co-

activators [19, 30]. The combinatorial BETi and PARPi effects were observed in a panel of cultured HR proficient ovarian cancer cells, which included cyclin E-amplified OVCAR-3 and cyclin E-overexpressing OVCAR-4 cells, and in xenograft models. Although BETi showed a consistent pattern of sensitization to PARPi in HR proficient ovarian cancer cells, there were differences in effects on the cell cycle. The combination of INCB054329 and olaparib induced a G₀/G₁ arrest in SKOV-3 cells, but a G₂/M arrest in OVCAR-3 cells. Since cyclin E associates with its partner kinase CDK2 to promote G₁/S progression [31], it is possible that higher basal levels of cyclin E in OVCAR-3 cells allows progression into S phase even following BETi/PARPi treatment in contrast to SKOV-3 cells.

There are several possible mechanisms underlying the synergy between BETi and PARPi in ovarian cancer cells. We have shown that BETi downregulated BRCA1 expression, which was accompanied by specific reduction of BRD4 occupancy at the *BRCA1* promoter. This observation is consistent with a recent report [15]. Examination of publically available databases showed a general downregulation of HR components by JQ1 treatment in other ovarian cancer cells [30], although there is variability in the specific HR genes downregulated between cell lines. We also showed that there was a co-operative reduction in expression of BRCA1, RAD51 and cyclin E between BETi and olaparib, particularly in SKOV-3 cells. The precise mechanism(s) for this co-operative downregulation remains to be defined. It is possible that the drugs act directly to reduce transcriptional elongation in these genes, as both BET proteins and PARP-1 are known to induce transcriptional elongation [10, 32]. An alternative explanation is that expression of these genes could be reduced secondarily to cell cycle arrest induced by the drug combination, since BRCA1 expression is known to be highest in S phase, decreases through G₂, and is lowest in G₀/G₁ [33, 34]. Recent studies have identified additional mechanisms of action of BETi combined with DNA damaging agents such as PARPi and CHK1 inhibitors, including mitotic catastrophe [19] and upregulation of the heterochromatin protein CBX5, leading to reduced chromatin access of DNA damage response mediators [35].

BETi treatment reduced HR efficiency and was accompanied by NHEJ upregulation. It remains unclear whether upregulated NHEJ is a resistance mechanism to BETi, or it contributes to PARPi sensitization. Evidence supporting the latter concept is emerging. It has recently been proposed that NHEJ deficiency is a mechanism of resistance to PARPi in ovarian cancer cells, since NHEJ is an error-prone repair pathway of double-strand DNA breaks [36]. Moreover, Aurora A kinase inhibitors increase NHEJ and sensitize ovarian cancer cells to PARPi [37].

We observed strikingly similar effects of the INCB054329/olaparib combination between cultured cells and in xenograft models in vivo. We chose the SKOV-3 cell line for our initial in vivo drug treatments because they readily form reproducible subcutaneous tumors in nude mice [4, 29], and we wished to determine drug effects on pharmacodynamic markers in defined tumors. Having successfully optimized a treatment regimen, we then performed experiments using an established IP ovarian cancer PDX model [25], which mimics the peritoneal disease spread in humans. A potential limitation of combining INCB054329 and PARPi treatment is toxicity, as toxic side-effects of both BETi and olaparib in the clinic are well-recognized [38, 39]. Although we showed that mouse weights remained stable in all

drug treatment groups, suggesting minimal overall toxicity in the mice, the safety profile of the BETi/PARPi drug combination in patients will need to be carefully characterized in Phase 1/II clinical trials.

In conclusion, the response of HR-proficient ovarian cancer cells to PARPi is enhanced by BETi. Our preclinical results indicate that the efficacy of PARPi in cyclin E high, HR-proficient ovarian cancer is improved by downregulating key HR components and cyclin E expression with BETi drugs. As BETi advance in the clinic, these results provide strong rationale for using BETi to extend the therapeutic efficacy of PARPi and other DNA damaging drugs to HR proficient, chemoresistant ovarian cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- BETi synergize with PARPi in HR-proficient ovarian cancer cells
- BETi downregulate HR genes and reduce HR efficiency
- BETi promote PARPi-induced DNA damage and apoptosis in vitro and in vivo

Figure 1. BRD4 knockdown reduces BRCA1 expression and sensitizes cells to olaparib
A) TCGA high-grade serous ovarian tumors with *BRD2*, *BRD3* and/or *BRD4* amplification overlap with a significant subset of *CCNE1* (cyclin E) amplified tumors. Copy number analysis data extracted from www.cbioportal.org. SKOV-3 cells were transiently transfected with distinct non-targeting (NT) or siRNAs targeting BRD4 (siBRD4). After 24 h transfection, the cells were treated with vehicle (0.01% DMSO) or olaparib (10μM) for an additional 24 hours. **B)** Western blot analysis of expression of BRD4, BRCA1, cleaved PARP and pH2AX. Actin and H2AX were used as loading controls. **C)** Densitometry of blots from A). Results are expressed relative to corresponding actin or histone H3 levels. Values are mean+SEM of 3 independent experiments; *p<0.01 compared to NT; ^ap<0.01 relative to olaparib-NT alone, Student's t-test. **D)** Chromatin immunoprecipitation strategy for analysis of BRD4 binding to the *BRCA1* locus. Primers were designed against a region proximal to the TSS (ChIP-BRCA1) and a negative control region (ChIP-Neg). **E)** ChIP-QPCR analysis of anti-BRD4 and anti-acetylated histone H3 (AcH3) in OVCAR-3 cells treated with NT or siBRD4 for 48 hours, or vehicle or INCB054329 (1μM) for 6h.

Figure 2. INCB054329 alone and combined with olaparib reduces HR and increases error-prone NHEJ in HR-proficient ovarian cancer cells

A) Western blot analysis of expression of BRD4, BRCA1, RAD51 and cyclin E in OVCAR-3 or SKOV-3 cells treated with vehicle (0.01% DMSO), INCB054329 (1 μ M) and olaparib (10 μ M) or the combination (24h). Densitometry analysis of blots for **B)** OVCAR-3 and **C)** SKOV-3 cells. Results are expressed relative to corresponding actin expression. OVCAR-3 or SKOV-3 cells pre-treated with 0.5 μ M cisplatin for 6h were then treated as above for a further 24 h. **D)** Foci for BRCA1 (green) were stained by IF. DAPI stained nuclei are in blue. Representative images are for SKOV-3 cells. **E)** The percentage of cells positive for BRCA1. **F)** Foci for RAD51 (green) and p2AX (red) were stained by IF. Representative images are for SKOV-3 cells. **G)** The percentage of cells positive for RAD51. Greater than 5 foci per nucleus was considered positive staining for BRCA1 and RAD51. HR repair efficiency of DNA double-strand breaks (DSBs) was also measured using the DRGFP assay. **H–I)** IF staining for GFP (green) in OVCAR-3 or SKOV3 cells co-transfected with the pDRGFP and I-Sce1 plasmids (both 1 μ g), and then treated as above for 24h. Representative images are for SKOV-3 cells. Results are expressed as the percentage of GFP-expressing cells. NHEJ repair efficiency was measured using the EJ5GFP assay. **J)** The percentage of GFP-expressing cells in OVCAR-3 or SKOV3 cells co-transfected with the EJ5GFP and I-Sce1 plasmids (both 1 μ g), and then treated as above for 24h. **K)** Drug effects on protein expression of the NHEJ marker, phospho(T2609)-DNA-PKcs and total DNA-PKcs, measured by western blot. For IF assays, at least 100 cells were counted ($\times 40$) for each assay and treatment. Values are mean+SEM of 3 independent experiments; * $p < 0.01$ compared to control; ^a $p < 0.01$ relative to olaparib alone, ^b $p < 0.01$ relative to INCB054329 alone, Student's t-test. Scale bars represent 20 μ m in D) and F), and 40 μ m in I).

Figure 3. INCB054329 and PARP inhibitors synergize in ovarian cancer cells

SRB assays showing effects of increasing concentrations of **A)** INCB054329 and olaparib and **B)** INCB054329 and rucaparib (72h treatment) in HR-proficient ovarian cancer cell lines, SKOV-3, OVCAR-3, OVCAR-4, and BRCA1 WT cells, and HR-deficient BRCA1 NULL cells. **C)** Combination Index (CI) for ED(Effective Dose)50, ED75 and ED90 was calculated by isobologram analysis. CI < 1 is synergistic. All CI's were $p < 0.05$ compared to a CI of 1 with the exception of BRCA1 NULL cells, Student's t-test. **D)** Effects of treatment of SKOV-3 cells with vehicle (0.01% DMSO), INCB054329 (1 μ M) and olaparib (10 μ M) or the combination (24h) in clonogenic assays. Cells were cultured for 7 days following drug withdrawal. **E)** Clonogenicity was measured by cumulative staining intensity in triplicate wells. Values are mean+SEM of 3 independent experiments. * $p < 0.01$ compared to vehicle; ^a $p < 0.01$ relative to olaparib alone; ^b $p < 0.01$ relative to INCB054329 alone; ^c $p < 0.01$ relative to rucaparib alone, all Student's t-test.

Figure 4. Co-operative effects of INCB054329 and olaparib on cell growth inhibition, and induction of DNA damage and apoptosis

A) High-content IF analysis of pH2AX intensity normalized to DAPI staining, adherent cell number and the cell cycle indices %G₀/G₁, %S and %G₂/M in OVCAR-3 or SKOV-3 cells treated with vehicle (0.01% DMSO), INCB054329 (1μM) and olaparib (10μM) or the combination (24h). **B)** Western blot analysis of expression of p21, cleaved PARP and pH2AX in OVCAR-3 or SKOV-3 cells treated as above. Densitometry analysis of blots for **C)** OVCAR-3 and **D)** SKOV-3 cells. Results are expressed relative to corresponding actin or histone H3 levels. **E–F)** SKOV-3 cells stained for pH2AX are in red and DAPI-stained nuclei are in blue. Images were acquired and analyzed using the MetaXpress imaging platform. Values are mean±SEM of 3 independent experiments; *p<0.01 compared to control; ^ap<0.01 relative to olaparib alone, ^bp<0.01 relative to INB054329 alone, Student's t test. Scale bars represent 10μm.

Figure 5. Combination INCB054329/olaparib treatment markedly reduces growth of HR-proficient ovarian tumor xenografts

Nude mice with subcutaneous SKOV-3 tumors were treated with vehicle, INCB054329 (25 mg/kg PO bid), olaparib (100 mg/kg PO) or the INCB054329/olaparib combination for 3 weeks (n=10). Time course measurements of **A**) tumor volume and mouse weights at weekly intervals during treatments. **B**) Box and whiskers plot (minimum to maximum, line is the mean) of tumor weight at sacrifice. Tumors are shown in **C**). **D**) Western blot analysis of cleaved PARP, BRCA1, Bax, PCNA, p21 and p2AX protein expression in harvested tumors. Actin was the loading control. **E**) Densitometry analysis of expression relative to corresponding actin levels. IHC analysis of **F**) proliferation marker Ki67 and **G**) apoptosis marker cleaved caspase-3 in formalin-fixed, paraffin-embedded tumors. **H**) Representative images of H&E, Ki67 and cleaved caspase-3 IHC. Values are mean+SD; * p<0.05 single drug effect relative to vehicle; ^ap<0.01 combination drug effect relative to olaparib; ^bp<0.01 combination drug effect relative to INCB054329, Student's t test.

Figure 6. Combination INCB054329/olaparib treatment markedly reduces growth of BRCA non-mutated ovarian PDX

NSG mice with intra-peritoneal PDX tumors were treated with vehicle, INCB054329 (25 mg/kg PO bid), olaparib (100 mg/kg PO) or the INCB054329/olaparib combination for 4 weeks (n=5). **A)** Schematic showing the drug treatment schedule. The double arrows show the start of drug treatment. Box and whiskers plot (minimum to maximum, line is the mean) of **B)** ascites volume and **C)** weight of harvested abdominal tumors at sacrifice. **D)** Western blot analysis of cleaved PARP, BRCA1, PCNA and pH2AX protein expression in harvested tumors. Actin was the loading control. **D)** Densitometry analysis of expression relative to corresponding actin levels. Values are mean+SD; *p<0.05 single drug effect relative to vehicle; ^ap<0.01 combination drug effect relative to olaparib; ^bp<0.01 combination drug effect relative to INCB054329, Student's t test.