PI3K inhibition impairs BRCA1/2 expression and sensitizes BRCA proficient triple negative breast cancer to PARP inhibition

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ABSTRACT
Poly-ADP-ribose-polymerase (PARP) inhibitors are active in tumors with defects in DNA homologous recombination (HR) due to BRCA1/2 mutations. The phosphatidylinositol 3-kinase (PI3K) signaling pathway preserves HR steady state. We hypothesized that in BRCA proficient Triple Negative Breast Cancer (TNBC), PI3K inhibition would result in HR impairment and subsequent sensitization to PARP inhibitors. We demonstrate in TNBC cells that PI3K inhibition leads to DNA damage, downregulation of BRCA1/2, gain in poly-ADP-ribosylation and subsequent sensitization to PARP inhibition. In TNBC patient-derived primary tumor xenografts, dual PI3K and PARP inhibition with BKM120 and olaparib reduced tumor growth in tumors displaying BRCA1/2 downregulation following PI3K inhibition. PI3K-mediated BRCA downregulation was accompanied by ERK phosphorylation. Overexpression of an active form of MEK1 resulted in ERK activation and downregulation of BRCA1, while the MEK inhibitor AZD6244 increased BRCA1/2 expression and reversed the effects of MEK1. We subsequently identified that the ETS1 transcription factor was involved in the ERK-dependent BRCA1/2 downregulation and that knock-down of ETS1 led to increased BRCA1/2 expression, limiting the sensitivity to combined BKM120 and olaparib in 3D culture.
SIGNIFICANCE

Treatment options are limited for patients with TNBC. PARP-inhibitors have clinical activity restricted to a small subgroup of patients with BRCA mutations. Here we demonstrate that PI3K blockade results in HR impairment and sensitization to PARP inhibition in TNBC without BRCA mutations, providing a rationale to combine PI3K and PARP inhibitors in this indication. Our findings could expand greatly the number of patients with breast cancer that would benefit from therapy with PARP inhibitors. Based on our findings a clinical trial with BKM120 and olaparib is being initiated in patients with TNBC.
INTRODUCTION

Therapeutic options for triple negative breast cancer (TNBC) are limited and based on the use of multiple lines of chemotherapy (1). An exception to this paradigm is found in the small subset of TNBC that have defects in homologous recombination (HR)-mediated DNA repair due to BRCA1 and BRCA2 mutations, where therapy with poly-ADP-ribose-polymerase (PARP) inhibitors results in high antitumor activity (2-5). PARP enzymatic activity is necessary for the repair of single-strand breaks (SSBs) through the base-excision repair (BER) pathway. Several double-strand break (DSB) repair mechanisms exist, including HR, which uses a sister chromatid template for recombination; and non-homologous end joining (NHEJ), which uses DNA ligation for repair but exhibits lower fidelity than HR (5). When PARP is inhibited, unrepaired SSBs can degenerate to DSBs that, in BRCA-deficient cells, can no longer be repaired by HR, resulting in continuous and lethal DNA damage (4-6). BRCA1 and BRCA2 proteins are essential components of HR that are recruited to damaged DNA for repair of the DSB. Therefore, the loss of either BRCA1/2 results in HR-deficiency and sensitization to PARP inhibitors. HR-deficiency can also occur through other mechanisms that may promote sensitivity to PARP inhibition: a) methylation and silencing of the Fanconi Anemia genes (7), involved in DNA repair and associated with BRCA1/2, b) loss of Cdk1 activity, which maintains BRCA1 protein stability (8), or c) loss of the Rad51 expression (7, 9), a necessary recombinase in the HR complex that associates with BRCA1/2. TNBC also display aberrant activation of the phosphatidylinositol 3-kinase (PI3K) pathway, which occurs due to a variety of mechanisms including loss of negative
pathway regulators such as Phosphatase and Tensin homolog (PTEN) or Inositol polyphosphate 4-phosphatase type II (INPP4B)(10, 11), activating mutations of the PIK3CA gene (12) or overexpression of the epidermal growth factor receptor (13). Direct pharmacologic inhibition of the PI3K/AKT/mTOR signaling is, therefore, an attractive clinical strategy for this disease. In addition to regulating cellular processes including metabolism, growth, and survival (14), PI3K also stabilizes and preserves DSB repair by interacting with the HR complex under normal conditions (15), and is necessary for DNA repair during ionizing radiation (16). Here we investigate the effects of PI3K inhibition in perturbing DNA HR in preclinical models of TNBC containing PI3K-activating alterations. We found that PI3K blockade promotes HR-deficiency by downregulating BRCA1/2 and sensitizes BRCA proficient tumors to PARP inhibition.
RESULTS

*PI3K suppression impairs homologous recombination*

TNBC is exquisitely sensitive to chemotherapy. This is due, at least in part, to intrinsic genomic instability of TNBC cells as a result of deficient DNA repair (17). Since PI3K signaling is known to maintain HR steady state (15), we asked whether inhibition of PI3K would further promote DNA damage in TNBC. To test this hypothesis, we first used siRNA to knock-down the expression of *PIK3CA*, the gene encoding for the alpha isoform of the catalytic p110 subunit of the PI3K enzyme complex, in MDA-MB-468 cells, a BRCA wild-type PTEN null TNBC cell line. *PIK3CA* knock-down resulted in accumulation of phosphorylated Histone 2AX (γH2AX), a protein that localizes to damaged DNA (18) recruiting DNA repair effectors to these sites (19) (Fig 1A and B). γH2AX accumulation, occurring mainly in the S and G2M phases of the cell cycle, was further enhanced by concomitant treatment with olaparib, a small molecule PARP inhibitor. In the same assay, siRNA for *BRCA1* served as positive control for γH2AX accumulation following PARP inhibition. Concomitantly, we observed that suppression of *PIK3CA* was accompanied by downregulation of BRCA1, indicating that PI3K suppression *per se* can inhibit the expression of proteins necessary for HR (Fig 1C, Supplementary Fig 1). Importantly, *BRCA1* downregulation by itself had little effect in increasing γH2AX nuclear foci formation. Subsequently, we investigated whether pharmacological inhibition of PI3K could recapitulate the effects on BRCA expression observed by *PIK3CA* knock-down (Fig 1D). Consistently, treatment with a pan PI3K inhibitor (NVP-BKM120, hereafter referred as BKM120), resulted in decreased expression of BRCA1/2 and
concomitant gain of poly ADP-ribosylated proteins (PAR), a product (and marker) of PARP activation (20). qRT-PCR showed that BRCA1/2 downregulation occurred, at least in part, at transcriptional levels and was not limited to MDA-MB-468 cells (Fig 1E, Supplementary Fig 2).

**HR deficiency induced by PI3K suppression sensitizes to PARP inhibition**

In the setting of decreased HR activity, PARP inhibition results in chromatid aberrations leading to cell lethality (5). In order to evaluate whether HR impairment following PI3K suppression conferred increased sensitivity to PARP inhibition, we tested the *in vitro* activity of two PARP inhibitors, olaparib (Fig 2, Supplementary Fig 3A) and ABT888 (Supplementary Fig 3B), in cells transfected with *PIK3CA* siRNA or treated with BKM120. Knock-down of *PIK3CA* decreased the IC-50 for olaparib in MDA-MB-468 (Fig 2A) and BT20 (Supplementary Fig 3B) TNBC cells. Knock-down of *PIK3CB* also led to sensitization to olaparib or ABT888, albeit to a lesser extent (data not shown). The combination of olaparib and BKM120 was superior to either single agent in inhibiting colony formation in soft agar in MDA-MB-468 cells (Fig 2B). The superiority of the combination was observed in additional TNBC BRCA-wild-type cell lines (MDA-MB-231: RAS mutated; HCC1143: PTEN null; HCC70: PTEN null) as well as with different PI3K inhibitors (Fig 2C).
**BKM120-mediated BRCA downregulation sensitizes to PARP inhibition in patient-derived primary tumor xenografts**

In order to study the effects of BKM120 in modulating BRCA expression and consequent sensitization to olaparib in models that better represent patient tumors, we decided to use three TNBC patient-derived tumor xenografts developed from three different breast cancer patients at our institution (Table 1). These models have been reported to resemble both the morphological and molecular characteristics of the original patient tumors from which they have been expanded (21, 22). Indeed, tissue architecture, hormone receptor levels, HER2 and PTEN expression of our xenografts were indistinguishable from their original tumors (Supplementary Fig 4). In concordance with the high frequency of PTEN loss reported in TNBC (12, 23), PTEN expression was barely detectable in any of the TNBC models, whereas an activating *PIK3CA* mutation (H1047R) was detected in one (TNBC1). Of note, both PTEN loss and *PIK3CA* mutations have been previously reported to co-exist in breast cancer (23).

Accumulation of nuclear γH2AX foci following PI3K inhibition was variable in the three TNBC models (Fig 3A). TNBC1 and TNBC3 showed a two fold increase in γH2AX staining whereas no significant changes occurred in TNBC2 following BKM120 treatment. The inability of TNBC2 to accumulate γH2AX foci PI3K inhibition may be due to its intrinsic resistance (and lack of significant cell death (24, 25)) to BKM120 used as single agent (data not shown). Downregulation of BRCA expression upon BKM120 treatment occurred in TNBC1 and TNBC2, and was accompanied by an increase in PAR levels (Fig 3C). We therefore hypothesized that...
combined PI3K and PARP inhibition was likely to be effective in these two xenograft models. BKM120 as single agent temporarily reduced tumor growth in TNBC1 and TNBC3 but was scarcely efficacious in TNBC2. As expected, olaparib monotherapy was ineffective in all three models. However, the combination of BKM120 with olaparib was superior to single agents in reducing TNBC1 and TNBC2 tumor growth (Fig 4). Consistently, lack of BRCA downregulation and PARP activation (TNBC3, Fig 3B and 3C) coincided with little or no benefit from dual PI3K and PARP suppression (Fig 4, TNBC3).

Taken together, these results suggest that 1) the degree of HR impairment and consequent sensitization to PARP inhibition induced by PI3K blockade may vary among different TNBC populations and 2) the sensitivity to dual PARP and PI3K treatment may be dependent on the degree of BRCA1/2 downregulation and consequent increased PARP activity (PAR) following PI3K inhibition.

*ERK inhibition prevents BKM120-mediated decrease in BRCA expression and increase in PARP activity*

Next we searched for possible mechanisms that could explain the variability in BRCA1/2 expression (and consequent susceptibility to PARP inhibition) following PI3K suppression. Interestingly, in both TNBC1 and TNBC2 we observed a concomitant increase of ERK phosphorylation upon BKM120 treatment (Fig 3C). This finding was not surprising as ERK activation following PI3K blockade has been previously described by us and others in breast cancer (26-28). In order to explore whether BRCA1/2 expression may be regulated by the ERK pathway, we undertook
several approaches. First, we exogenously expressed an active form of MEK1 (increasing ERK phosphorylation) in MDA-MB-468 cells and asked whether elevated ERK activity per se led to downregulation of BRCA1. In Fig 5A and 5B we show that this in fact was the case. Moreover, pharmacological MEK inhibition using the small molecule MEK inhibitor AZD6244 was sufficient to increase the expression of BRCA1 and BRCA2 and reverse the effects of MEK1 overexpression. These observations prompted us to perform a Jaspar promoter analysis (29) searching for ERK-related transcription factors binding to either BRCA1 or BRCA2 promoters. This analysis identified ETS1 as the transcription factor with the highest number of BRCA1/2 binding motifs (Supplementary Table 1). As a matter of fact, ETS transcription factors have been described to be phosphorylated (and activated) by the ERK pathway (30) and to repress the BRCA1 promoter (31). To test whether ETS1 transcription factor was involved in the ERK-dependent BRCA1/2 downregulation upon PI3K blockade, we measured the levels of phosphorylated ETS1 (pETS1_T38) in MDA-MB-468 cells treated with BKM120, AZD6244 and the combination of both (Fig 5C). Together with the expected increase in ERK activation, BKM120 treatment also increased the levels of pETS1_T38 (Fig 5C, Supplementary Fig 5). In Figure 5D we confirm that PI3K suppression and ERK activation lead to transcriptional BRCA1/2 downregulation. In figure 5E and 5F we show that these results hold true also in vivo. In fact, TNBC1 treated with BKM120 displayed ERK activation with increased pETS1 levels that led to downregulation of BRCA1/2. This phenomenon was prevented by MEK suppression by AZD6244.
In order to further confirm the role of ETS1 in regulating ERK-dependent BRCA1/2 expression, we specifically knocked-down ETS1 by shRNA or siRNA (Fig 6A, 6C, Supplementary Fig 6) and showed that this manipulation was sufficient to increase BRCA1/2 expression and concomitantly inhibit PARP activity in two TNBC cell-lines (Fig 6A, B, C, D). Furthermore, ETS1 downregulation in MDA-MB-231 cells limited the sensitivity to dual PI3K and PARP inhibition (without altering colony formation of cells treated with single agents, Fig 6E).

Taken together, these data indicate that HR impairment following PI3K blockade may be dependent of ERK activation that in turn increases ETS1 phosphorylation (and activity) to repress BRCA1/2 expression.
DISCUSSION

HR-deficiency associated with BRCA1/2 mutation results in dependence on PARP-mediated DNA repair and profound sensitivity to PARP inhibitors (2-4, 7). In contrast, the clinical activity of PARP inhibitors in non-BRCA mutant tumors to date has been disappointing (32), resulting in limited applicability of these agents in the clinic. It has been proposed that somatic loss of BRCA function by promoter methylation and consequent transcriptional silencing may also induce HR-deficiency, which would result in an additional fraction of patients who may potentially benefit from PARP inhibitors (33).

Our study suggests that PI3K inhibition could be exploited to induce HR-deficiency and sensitization to PARP inhibition in BRCA-proficient TNBCs. In *in vitro* models, we show that suppression of the PI3K pathway was accompanied by BRCA1/2 downregulation and increase in PARP activity (increased PAR levels) indicating that cells undergoing PI3K suppression become more dependent on this DNA repair mechanism. As a matter of fact, dual inhibition of both PARP and PI3K activity resulted in greater suppression of cell proliferation, as well as of anchorage-independent and -dependent colony formation. However, *in vivo* we observed that BRCA1/2 downregulation following PI3K inhibition was variable among our tested models. In those patient-derived xenografts showing BKM120-dependent BRCA mass downregulation, the addition of olaparib significantly suppressed tumor growth. No such effects were observed when BRCA1/2 levels remained unchanged by PI3K inhibition. This would suggest that BRCA expression rather than DNA damage following PI3K inhibition predict response to PARP inhibition. This observation is in
accordance with recent data indicating that response to chemotherapy in TNBC
occurs exquisitely in tumors with impaired BRCA nuclear expression/localization
independently of the magnitude of DNA damage (34).

Intriguingly, in the tumors responding to combined therapy, BRCA1/2
downmodulation was accompanied by concomitant activation of the ERK pathway
following PI3K inhibition. Moreover, the use of a MEK inhibitor prevented ERK
phosphorylation and limited BRCA1/2 downregulation induced by BKM120
treatment. These results indicate that BRCA1/2 gene transcription may be
regulated, at least in part, by the ERK pathway. Furthermore, our data suggest that
ETS1 is among the transcription factors mediating this ERK-dependent BRCA
downregulation. Interestingly, it has been recently proposed that the antitumor
effects of olaparib may be limited to ETS positive tumors (35). Our study support
this conclusion and argue that ETS1-dependent ERK activation is sufficient to
sensitize TNBC to dual PI3K and PARP blockade.

It remains to be determined the magnitude of BRCA1/2 downregulation following
PI3K blockade that is needed for the cancer cells to acquire sensitivity to PARP
inhibitors. Single allelic mutation of BRCA1 is sufficient for the induction of HR-
deficiency (36), suggesting that changes (and not only complete loss) in BRCA1 or
BRCA2 expression could act synergistically with PARP inhibition in abolishing
tumor growth.

In summary, we demonstrate that PI3K inhibition in TNBC results in BRCA
downregulation, activation of PARP, and ultimately sensitization to PARP inhibition.
Importantly, BRCA1/2 downregulation following PI3K inhibition seems to be a
conditio sine qua non to achieve strong antitumor activity when a PARP inhibitor is combined in BRCA-wt TNBC. Our findings provide the rationale to investigate the clinical efficacy of dual PARP and PI3K inhibition in triple negative breast cancer, an approach that could expand the fraction of patients who may benefit from PARP inhibitors.
MATERIALS AND METHODS

Cell culture and inhibitors

MDA-MB-468, MDA-MB-231, HCC70, HCC1143 and BT20 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were tested and authenticated by ATCC (DNA fingerprinting, karyotyping, and morphology study) and additionally by western blot and MassArray mutational analysis in our laboratory. MDA-MB-468 cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS). MDA-MB-231 cells were maintained in ATCC-formulated Eagle’s Minimum Essential Medium with 10% FBS. BT20 cells were maintained in RPMI-1640, 10%FBS, supplemented with basic minimal essential amino acids (Sigma; St. Lous, MO, USA), MEM non-essential amino acids (Sigma; St. Lous, MO, USA), and porcine insulin. HCC70 and HCC1143 cells were cultured in RPMI-1640 supplemented with 10% FBS. All cell culture was performed at 37 °C in 5% CO2. Low melting temperature seaplaque agarose was purchased from Lonza (Rockland, ME, USA). Matrigel was purchased from Invitrogen (Carlsbad, CA). GDC-0941, MK2206, AZD6244 and AZD2281 (olaparib) were purchased from Selleck chemicals (Selleck, Houston, TX, USA). ABT-888 was purchased from Active Biochem (Maplewood, NJ). Plasmids for control and MEK1 were kindly provided by Cory Johannessen at the BROAD Institute (Bostom, MA). siRNA against human BRCA1, PIK3CA, PIK3CB and non-targeting control #3 are ON-TARGET- plus Smartpool siRNAs from Dharmacon Thermal Scientific. Lipofectamine™ RNAiMAX Transfection Reagent was purchased from Life Technologies (Grand Island, NY). siRNA for control and ETS-1 were generated using Invitrogen siRNA
sequence generator (Carlsbad, CA), and purchased from SIGMA (St. Lous, MO, USA). pTRIPZ ETS-1 shRNA lentiviral (5 clones - RHS4740-NM_005238) Doxycycline regulated vector (shRNA-ETS-1) was purchased from OpenBiosystems (Huntsville, AL).

Flow cytometry for γH2AX
Twenty four hours after transfection with 50nMsiRNA and RNAi MAX transfection reagent according to manufacturer’s protocol, cells were treated with PARP inhibitor or vehicle control for 48 hours. Cells were then trypsinized into single cell suspension and fixed with 1% ice-cold paraformaldehyde solution for 15 minutes, followed by incubation with 70% ethanol at −20°C overnight. Cells were permeabilized with BSA-T-PBS (1% BSA and 0.2% Triton X-100 in PBS) and incubated with γH2AX antibody (BD Pharmingen™, Alexa Fluor® 488 Mouse anti-H2AX, pS139) at 4 °C overnight. Cells were then washed, re-suspended in propidium iodide (PI) staining solution and run on FACSCalibur System (BD Biosciences, San Jose, California). Data were analyzed with Flowjo (Tree Star, Inc., Ashland, OR).

Cell viability assay
Eighteen hours after transfection with the indicated siRNA, cells were trypsinized and plated in 96-well plates in triplicates. Six hours later, various concentrations of olaparib or ABT-888 were added. After growing for 7 days, cell viability was assessed by Cell Titer-Glo (Promega, Madison, WI).
Clonogenic Assay

Eighteen hours after transfection with the indicated siRNAs, cells were trypsinized and plated in 6-well plates at clonal density in triplicates. Six hours later, various concentrations of olaparib or ABT-888 were added. Medium were refreshed every 4 days. After growing 14 to 18 days, cell colonies were stained with methylene blue and counted by Labworks imaging software (UVP, Upland, CA).

Anchorage-dependent and-independent growth in 3-D culture

A 1% seaplaque agarose media mixture was solidified in 24 well plates. A cell-mixture of 0.4% agarose in media and 5000 cells were plated on top of the 1% agarose layer. After 24 hours, cells were treated with inhibitors as indicated and media containing inhibitors was changed once per week. Images were taken at a 10X objective.

3D-anchorage culture of breast cancer cells was performed in 100% matrigel. Briefly, polyhema-coated 96-well plates were coated with 25 μl of matrigel, centrifuged and allowed to gel at 37°C. 5000 cells were plated on top in media containing 2% matrigel. After 3D structures were formed (3days), cells were treated as indicated for 7 days. Phase contrast microscopy was used to image 3D structures.

Generation of ETS-1 shRNA MDA-MB-231

Lentiviral pTRIPZ was generated according to manufacturer’s protocol, and target MDA-MB-231 cells were infected with a pool of 5 ETS-1 shRNA viral constructs.
Cells were puromycin selected for 2 weeks, then treated with or without Doxycycline (20ng.ml) for 4 days.

**Western blots**

Tumor samples were homogenized in ice-cold lysis buffer (TRIS-HCl pH 7.8 20 mM, NaCl 137mM, EDTA pH 8.0 2mM, NP40 1%, glycerol 10%, supplemented with NaF 10 mM, Leupeptin 10μg/mL, Na₂VO₄ 200 μmol/L, PMSF 5mM and Aprotinin (Sigma-Aldrich, MO). Cell lines were also collected in ice-cold lysis buffer. Lysates were cleared by centrifugation at 13000 rpm for 10 min at 4°C and supernatants were removed and assayed for protein concentration using the Pierce BCA Protein Assay Kit (Thermo Scientific, IL). Thirty micrograms of total lysates were resolved by SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes. Membranes were hybridized with the following primary antibodies: phospho-Akt (pAkt-Ser473), phospho-Akt (pAkt-Thr308), phospho-S6 (pS6-Ser240/244), phospho-ERK (pERK-Thr202/Tyr204), tERK (CST, Danvers, MA), BRCA1, BRCA2 and PAR (Abcam, Cambridge, UK), phospho-ETS1 (pETS1-Thr38) (Invitrogen, CA) in 1% nonfat dry milk. Mouse and rabbit horseradish peroxidase–conjugated (HRP-) secondary antibodies (Amersham Biosciences) and chicken-HRP (Abcam) were used at 1:2000 in TBS-T/1% nonfat dry milk. Protein-antibody complexes were detected by chemiluminescence with the Immobilon Western HRP Substrate (Millipore, MA) and images were captured with a FUJIFILM LAS-3000 camera system.
Quantitative PCR

qRT-PCR was performed using Taqman probes from Applied Biosystems, according to the manufacturer’s recommendations. Reactions were carried out in an ABI 7000 sequence detector (Perkin Elmer) and results were expressed as fold change calculated by the ΔCt method relative to the control sample. The ribosomal subunit 18S was used as internal normalization controls.

RNA expression microarray analysis

Tumor RNA was extracted by RNeasy mini kit according to manufacturer’s instructions, and 100ng were hybridized onto Affymetrix HuGene microarrays (Santa Clara, CA). Data was analyzed as previously described (37).

Establishment of patient-derived tumor grafts in nude mice

Patient consent for tumor use in animals was obtained under a protocol approved by the Vall d’Hebron Hospital Clinical Investigation Ethical Committee and Animal Use Committee. Tumors were subcutaneously implanted in 6 week old female HsdCpb:NMRI-Foxn1nu mice (Harlan Laboratories, Italy). Animals were supplemented with 1μM estradiol (Sigma) in the drinking water. After tumor graft growth, tumor tissue was re-implanted into recipient mice, which were randomized upon implant growth.
In vivo treatment study

Patient-derived tumor grafts: animals were randomized in 6-8 mice per group when tumor volumes reached 100-200 mm³. Animals were treated by oral gavage with NVP-BKM120 6xQD (dissolved in NMP-PEG) or AZD6244 (dissolved in Methylcellulose/Tween). Olaparib was re-suspended for intraperitoneal administration in 10% Captisol. Tumor grafts were measured with calipers and tumor volumes were determined using the formula: \((\text{length} \times \text{width}^2) \times (\pi/6)\). At the end of the experiment animals were sacrificed by CO₂ inhalation. Tumor volumes are plotted as mean ± SE.

Immunohistochemistry and immunofluorescence

Tumor specimens and tumor grafts were fixed immediately after biopsy or excision in a 10% buffered formalin solution for a maximum of 24h at room temperature before being dehydrated and paraffin embedded under vacuum conditions. For tumor grafts, tissue micro-arrays (TMA) were constructed including triplicate cores from each graft. TMA slides underwent deparaffinization and antigen retrieval using PT Link system (DAKO) following manufacturers' instructions. Antibodies against the following targets were from Dako (Glostrup, Denmark): ER (1:50), PR (prediluted), HER2 (prediluted) and PTEN (1:100) from CST (Danvers, MA). Immunohistochemical staining was performed as follows: 4µm sections from formalin-fixed and paraffin embedded material were deparaffinized and hydrated. Antigen retrieval was performed using a T/T Mega microwave system following manufacturer's instructions and DAKO reagents. After peroxide blocking, slides
were incubated with primary antibody, secondary antibody and finally developed
with freshly prepared 0.05% 3,3-diaminobenzidine and counterstained with
hematoxylin. Positive and negative controls were run along with the tested slides
per each marker.

Immunofluorescence analysis of γH2AX in MDA-MB-468 cells was performed on cells
seeded onto coated cover slips and then transfected 24 hours later with control
siRNA, BRCA1 siRNA or PIK3CA siRNA. Either vehicle control or 1 μM olaparib was
added to the medium 24 hours after transfection. Two days after drug treatment,
cells were fixed with 3% formaldehyde, permeabilized with 0.1% Triton X-100, and
blocked with 10% FBS-containing PBS medium for 1 hour. Subsequently, cells were
incubated with γH2AX antibody (BD Pharmingen™, Alexa Fluor® 488 Mouse anti-
H2AX, pS139; 1:1000), washed, counter-stained by Hoechst 33342 to visualize the
nuclei, and mounted for immunofluorescence analysis. 10 fields per sample were
quantified with a threshold of 10 foci or more being considered positive.

Immunofluorescence analysis of γH2AX (Millipore, 1:100) in tumor samples was
performed on formalin-fixed paraffin-embedded tissue-microarrays. After
deparaffinization, antigen retrieval was performed by incubation in 10 mM citrate
buffer, pH 6.0 (DAKO, Carpinteria, CA), in a heated (97°C) water bath for 40 minutes.
Nonspecific binding was blocked by immersing the sections in a TBS/5% BSA
solution for 10 minutes. Sections were incubated with a mouse monoclonal antibody
at a dilution of 1:100 for 60 minutes. Anti-mouse Alexa Fluor 568 goat anti-mouse
IgG (Molecular Probes, Eugene, OR) diluted 1:700 for 30 minutes. Sections were
counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Vysis,
Downers Grove, IL). All incubations were performed at room temperature. Images were acquired using a confocal microscope and quantified by a Pathologist blinded to the identity of the samples.

Statistical analysis

One-way ANOVA with Bonferroni post-test was done using GraphPad Prism (GraphPad Software, La Jolla, USA). Error bars represent the SE. All experiments were repeated at least three times.
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REFERENCES


34. Powell S. Homologous recombination defects found in all sub-types of sporadic breast cancer AOCR 2012 Annual Meeting. 2012;abstract MW11.


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Table 1

<table>
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* Low estrogen receptor expression by IHC
TABLE LEGENDS

Table 1. Characterization of TNBC patient-derived tumor grafts. Expression of PTEN and PIK3CA or BRCA1/2 mutational statuses of three tumor grafts derived from either primary or metastatic lesions of different TNBC patients. A minimum of 3 independent samples were used for PTEN quantification by immunohistochemistry. Molecular subtype analysis was performed using PAM50 molecular analysis of Affymetrix HuGene microarrays.

FIGURE LEGENDS

Figure 1. BRCA downregulation and γH2AX staining following PI3K inhibition in vitro. A, FACS analysis showing staining of γH2AX during the three phases of cell-cycle (G1, S, G2) in MDA-MB-468 cells transfected with control, BRCA1 or PIK3CA siRNAs and treated with either DMSO or 1μM olaparib. B, Immunofluorescence of γH2AX in MDA-MB-468 cells transfected with control, BRCA1 or PIK3CA siRNAs and treated with either DMSO or 1μM olaparib. Percentages of positive nuclei (10 foci or more/nucleus being considered positive) from 10 fields per sample are indicated. C, Western blot of cell lysates (7 days post transfection) from MDA-MB-468 cells transfected with control or PIK3CA siRNAs using the indicated antibodies. Tubulin was used as loading control. D, Western blot of lysates from MDA-MB-468 treated with BKM120 (750nM) for 7 days using the indicated antibodies. Total ERK (tERK) was used as a loading control. E, qRT-PCR measuring both BRCA1 and BRCA2 mRNA levels in MDA-MB-468 treated with BKM120. Measurements were normalized to
18S mRNA levels and expressed as fold change compared to controls (Log₂ scale). Data are shown as mean ± S.E. of 3 independent replicates for each condition.

**Figure 2.** Combined PI3K and PARP suppression in vitro. **A,** Viability (assayed by Cell Titer-Glo) of MDA-MB-468 cells transfected with control, *BRCA1* or *PIK3CA* siRNAs and treated with either DMSO or olaparib for 7 days. IC50 were calculated using GraphPad Prism program. **B,** MDA-MB-468 cells plated in anchorage-independent conditions treated with BKM-120 (750nM), olaparib (4µM), or the combination for 45 days, with weekly media changes. Cell colonies were stained with crystal violet. **C,** MDA-MB-231, HCC1143, and HCC70 cells plated in anchorage-dependent conditions treated with BKM-120 (750nM), olaparib (4µM), GDC-0941 (500nM) or the combination for 7 days. Magnification 10X.

**Figure 3.** γH2AX staining and BRCA downregulation following PI3K inhibition in vivo. **A,** Representative immunofluorescence staining for γH2AX (red) comparing placebo versus BKM120 treated (27.5mg/kg) TNBC xenografts. Nuclei are stained with DAPI (blue). Quantifications of γH2AX staining are from 6 different tumors for each condition. *p<0.001. Magnification 20X. **B,** qRT-PCR measuring both BRCA1 and BRCA2 mRNA levels of patient-derived TNBC1 and TNBC2 treated with BKM120. Measurements were normalized to 18S mRNA levels and expressed as fold change compared to controls (Log₂ scale). Data are shown as mean ± S.E. of 3 independent replicates for each condition. *p<0.001. **C,** Western blot of patient-derived xenografts of two independent tumors from different mice treated for 21
days with vehicle or BKM120 (27.5 mg/kg) using the indicated antibodies. Total ERK (tERK) is used as loading control.

**Figure 4.** Combined PI3K and PARP suppression *in vivo.* Tumor growth of TNBC1, TNBC2 and TNBC3 xenografts treated with vehicle control, BKM120 (27.5mg/kg), olaparib (50mg/kg), or the combination of both agents. Relative tumor volumes are displayed as mean ± S.E of a minimum of 6 tumors per arm. *p<0.001 combination versus BKM120 arm.

**Figure 5.** ERK-dependent BRCA downregulation. **A,** Western blot of protein lysates from MDA-MB-468 cells constitutively overexpressing control or MEK1 plasmids and treated with the MEK inhibitor AZD6244 (500nM) for 4 days using the indicated antibodies. Total ERK (tERK) is used as loading control. **B,** qRT-PCR measuring both BRCA1 and BRCA2 mRNA levels in MDA-MB-468-MEK1 cells treated with AZD6244. Measurements were normalized to 18S mRNA levels and expressed as fold change compared to controls (Log2 scale). Data are shown as mean ± S.E. of 3 independent replicates for each condition. **C,** Western blot of protein lysates from MDA-MB-468 cells treated with BKM120 (750nM), AZD6244 (500nM) or the combination of both for 4 days using the indicated antibodies. Total ERK (tERK) is used as loading control. **D,** qRT-PCR measuring both BRCA1 and BRCA2 mRNA levels in MDA-MB-468 cells treated with BKM120 and AZD6244. Measurements were normalized to 18S mRNA levels and expressed as fold change compared to controls (Log2 scale). Data are shown as mean ± S.E. of 3 independent replicates for each condition. **E,**
Western blot against the indicated proteins in two independent TNBC1 tumors treated for 4 days with BKM120 (50mg/kg), AZD6244 (10mg/kg) or the combination of both. Total ERK is used as loading control. F, qRT-PCR measuring both BRCA1 and BRCA2 mRNA levels in tumor grafts. Measurements were normalized to 18S mRNA levels and expressed as fold change compared to controls (Log$_2$ scale). Data are shown as mean ± S.E. of 3 tumors for each condition.

**Figure 6.** ETS1 knock down and resistance to the combination of BKM120 and olaparib. A, Western blot of protein lysates from MDA-MB-231 shRNA-ETS1 infected cells treated with Doxycycline (20ng/ml) for 4 days using the indicated antibodies. Total ERK (tERK) is used as loading control. B, qRT-PCR measuring both BRCA1 and BRCA2 mRNA levels in MDA-MB-231-shETS1 cells treated with Doxycycline. Measurements were normalized to 18S mRNA levels and expressed as fold change compared to controls (Log$_2$ scale). Data are shown as mean ± S.E. of 3 independent replicates for each condition. C, Western blot of protein lysates from BT20 shRNA-ETS1 infected cells treated with Doxycycline (20ng/ml) for 4 days using the indicated antibodies. Total ERK (tERK) is used as loading control. D, qRT-PCR measuring both BRCA1 and BRCA2 mRNA levels in BT20-shETS1 cells treated with Doxycycline. E, MDA-MB-231-shETS1 cells plated in anchorage-dependent conditions were treated with/without Doxycycline (20ng/ml) and BKM-120 (750nM), olaparib (4μM), GDC-0941 (500nM) or the combination for 14 days. Magnification 10X.
Figure 1

A) MDA-MB-468

<table>
<thead>
<tr>
<th>Olaparib (µM)</th>
<th>siControl</th>
<th>siBRCA1</th>
<th>siPIK3CA</th>
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<tbody>
<tr>
<td>0</td>
<td>G1: 0</td>
<td>S: 0</td>
<td>G2: 0</td>
</tr>
<tr>
<td>1</td>
<td>G1: 100</td>
<td>S: 100</td>
<td>G2: 75</td>
</tr>
</tbody>
</table>

B) MDA-MB-468 γH2AX

- siControl: 13.1%
- siBRCA1: 22.2%
- siPIK3CA: 30.3%

- DMSO: 31.6%
- Olaparib: 93.5%
- Olaparib: 82.4%

C) MDA-MB-468

- BRCA1
- PI3Ka
- Tubulin

D) MDA-MB-468

- BRCA1
- BRCA2
- PAR
- tERK

E) MDA-MB-468

- Log2 BRCA1 mRNA levels
- Log2 BRCA2 mRNA levels
- Log2 PAR mRNA levels
- Log2 tERK mRNA levels
Figure 2

A) MDA-MB-468

- **Surviving Fraction**
  - **μM Olaparib**
  - **siControl**
  - **siBRCA1**
  - **siPI3KCA**

**IC50**
- **siControl**: 1.461
- **siBRCA1**: 0.06
- **siPI3KCA**: 0.216

B) MDA-MB-468

- **BMX-Olap Olaparib BMX120 Control**

C) MDA-MB-231

- **Control**
- **BKM120**
- **Olaparib**
- **BKM+Olap**
- **GDC0941**
- **GDC+Olap**

HCC1143

HCC70
Figure 3

A) 

B) 

C) 

<table>
<thead>
<tr>
<th></th>
<th>TNBC1</th>
<th>TNBC2</th>
<th>TNBC3</th>
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<tr>
<td>Control</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BKM120</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 4

Responsive to BKM120 and Olaparib combination

Non-responsive to BKM120 and Olaparib combination

TNBC1

TNBC2

TNBC3

Relative tumor volume

Days

Control
BKM120
Olaparib
BKM+Olap
Figure 5

A) MDA-MB-468

Control  MEK1
AZD6244:  -  +  -  +

pERK

GAPDH

tERK

MEK 1/2

B) MDA-MB-468

Control  MEK1

C) MDA-MB-468

Control  BKM120  AZD6244  BKM120+AZD

pETS1

pERK

tERK

D) MDA-MB-468

Control  BKM120  AZD6244  BKM120+AZD

pETS1

E) TNBC1

Control  BKM120  AZD6244  BKM120+AZD

pAkt

pERK

tERK

PAR

pETS1

F) TNBC1
Figure 6

A) MDA-MB-231
- shETS1: - +
- pETS1 T38
- BRCA 1
- BRCA 2
- PAR
- tERK

B) MDA-MB-231
- shETS1: +
- pETS1 T38
- BRCA 1
- BRCA 2
- PAR
- tERK

C) BT20
- shETS1: - +
- pETS1 T38
- BRCA 1
- BRCA 2
- PAR
- tERK

D) BT20
- shETS1: +
- pETS1 T38
- BRCA 1
- BRCA 2
- PAR
- tERK

E) MDA-MB-231
- Control
- BKM120
- Olaparib
- BKM+Olap
- GDC0941
- GDC+Olap
- shETS1: - +
PI3K inhibition impairs BRCA1/2 expression and sensitizes BRCA proficient triple negative breast cancer to PARP inhibition


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