Co-treatment with BEZ235 Enhances Sensitivity of *BRCA1*-negative Breast Cancer Cells to Olaparib

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Abstract. *The poly(ADP-ribose) polymerase (PARP) inhibitor, olaparib has been reported as having preferential anti-proliferative effects on breast cancer 1 (BRCA1)-deficient breast and ovarian cancer cells and was recently approved by the US Food and Drug Administration (FDA) for advanced, BRCA1-mutated ovarian cancer. Herein, we show that BEZ235, a protein kinase inhibitor, enhanced the tumor cell-killing effect of olaparib in BRCA1-mutated breast cancer cells in vitro. BEZ235 reduced olaparib-induced phosphorylation of p53 binding protein 1 (53BP1) and 53BP1 foci formation, as well as phosphorylation of AKT (S473). Long-term colony-formation assay revealed more strong synergistic effects of this combination in SUM149PT and MDA-MB-468 breast cancer cell lines. BEZ235 treatment combined with olaparib may be a candidate for effective therapeutic treatment of BRCA1-mutated breast cancer.*

Breast cancer 1 (*BRCA1*) is a tumor-suppressor gene and its mutations are found in a high percentage of hereditary breast and ovarian cancer cases (1). In 1990, a research group led by Mary-Claire King assigned chromosome 17q21 (2), by linkage analysis, as containing the locale of a gene for inherited susceptibility to breast cancer in families with early-onset disease (2, 3). Subsequently, Mark Skolnick and his colleagues at Myriad Genetics cloned the *BRCA1* gene, composed of 22 coding exons distributed over ~100 kb of genomic DNA (4, 5). The full-length *BRCA1* cDNA encodes a large protein of 1,863 amino acids (4). The BRCA1 protein has diverse roles in various cellular processes such as cell-cycle control, DNA damage response (DDR), genomic integrity maintenance, protein ubiquitination, transcriptional regulation, signal transduction, and oxidative stress regulation through interacting with various proteins (6). Regardless of cumulative studies on the functions of the BRCA1 protein, the exact mechanisms of its tumor-suppressor function are not yet fully understood (7, 8).

Since BRCA1-defective cancer cells, either by loss of function or mutations of *BRCA1*, bear defects in DNA damage repair (9, 10), targeting poly(ADP-ribose) polymerase (PARP), an enzyme that is important to repair of single-strand breaks in DNA, by small-molecule inhibitors is a promising anticancer approach to treating BRCA1-defective cancer (11, 12). Indeed, a PARP inhibitor, olaparib, was recently approved by the US Food and Drug Administration (FDA) for advanced, *BRCA1*-mutated ovarian cancer (13). However, like many anticancer drugs, resistance of cancer cells to PARP inhibitors has been reported (14-20). As an attempt to overcome this resistance and enhance the potency of PARP inhibitors, we tested a small set of protein kinase inhibitors (PKIs) and identified BEZ235 as a potential agent to augment the efficacy of olaparib in BRCA1-defective breast cancer cells.

Materials and Methods

Cell culture and reagents. Both MDA-MB-436 and SUM149PT cell lines have BRCA1 allelic loss and each cell line shows mutation status of 5396 + 1G>A and 2288delT (21). MDA-MB-436 cell line was obtained from the Tissue Culture Shared Resource of Georgetown University Medical Center (Washington, DC, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) containing 10% heat-inactivated fetal bovine serum (HI-FBS; HyClone, Logan, UT or Omega Scientific, Inc., Tarzana, CA, USA) and 100 units/ml penicillin/streptomycin (Lonza). SUM149PT cell line was maintained according to the supplier's recommendation (Asterand,

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Detroit, MI, USA) with Ham's F-12 media (Corning Cellgro, Manassas, VA, USA) containing 5% HI-FBS (HyClone). The viability of cultured cells was monitored by the trypan blue dye exclusion method using the Luna™ Automated Cell Counter (Logos Biosystems, Gyeonggi-do, Korea). Enzyme inhibitors were purchased from following sources: olaparib and BEZ235 from LC Labs (Woburn, MA, USA); NSC 109555 and PD407824 from Tocris Bioscience (Minneapolis, MN, USA); and KU55933 and NU7441 from Selleck Chemicals (Houston, TX, USA). Stock solutions of compounds, except for BEZ235, were made in dimethyl sulfoxide (DMSO) and stored at −20˚C in small aliquots; BEZ235 was dissolved in dimethylformamide (DMF).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays. Cells (2,500~3,000 cells/well) in 96-well plates were treated with compounds in triplicate as indicated in the Figures. DMSO or DMF was used as vehicle control. Cell viability was determined at 72 h after treatment with compounds by MTT assay as described previously (22, 23). The half-maximal effective concentration (EC_{50}) values were calculated by CompuSyn software V1.0 (ComboSyn, Paramus, NJ, USA). The interaction between the two drugs was evaluated by using the combination index (CI) as described previously (24) using CompuSyn software.

Colony-formation assays. For multi-parallel comparison of drug combinations, colony-formation assays were performed in 96-well plates (25). Cells (100-500 cells/well) were plated in 96-well plates on the day before treatment. Cells were then treated with each compound or combination of each compound in triplicate for 72 h as indicated in the figure legend. After treatment with compounds, cells were replenished with fresh normal growth media and further cultivated for an additional 4 days. The surviving colonies were visualized and measured by MTT staining.

The standard colony-formation assay (clonogenic assay) was performed in 6-well plates, as described previously (26). In brief, SUM149PT cells (500-1,000 cells/well) were sub-cultured into 6-well plates. The day after sub-culture, cells were treated with the indicated concentration of BEZ235 or olaparib in triplicate and incubated for 24 h. The cells were then further incubated with BEZ235 or DMSO (instead of olaparib as a vehicle) or DMF (vehicle control) for an additional 48 h. After treatment with compounds, cells were supplied with fresh normal growth media and incubated for an additional 7 days. Colonies were stained with 0.5% crystal violet solution in Phosphate buffered Saline (PBS) containing 25% methanol. Crystal violet-stained colonies were solubilized by 0.1 M NaH_2PO_4:ethanol (1:1) solution and the absorbance of each well was measured by ELx808 (BioTek, Winooski, VT, USA).

Western blots and antibodies. Western blot analyses were performed as described previously (22). Antibodies used in this study were against the following: phospho-ATM and Rad3 related (phospho-ATR) (S428), phospho-AKT (Ser473), AKT, phospho-checkpoint kinase 1 (phospho-CHK1) (S296), and phospho-checkpoint kinase 2 (phospho-CHK2) (T68) from Cell Signaling (Danvers, MA, USA); checkpoint kinase 2 (CHK2) from Santa Cruz (Santa Cruz, CA, USA); p53 binding protein 1 (53BP1) and phospho-53BP1 (S25) from Novus Biologicals (Littleton, CO, USA); and β-actin and horseradish peroxidase-conjugated secondary antibodies from Sigma (St. Louis, MO, USA).

Confocal microscopy of 53BP1 foci. SUM149PT cells (30,000 cells/well) were subcultured into an 8-well chamber slide (Nalge Nunc Int., Rochester, NY, USA). The day after subculture, the cells were treated with 30 μM olaparib, 10 μM BEZ235, or the combination of both drugs for 2 h. Then the cells were fixed by 3.7% formaldehyde in Dulbecco's PBS for 15 min at room temperature. The fixed cells were washed four times with Dulbecco's PBS and permeabilized by 0.5% Triton X-100 in Dulbecco's PBS for 5 min at room temperature. The cells were washed again four times with Dulbecco's PBS containing 0.5% Tween-20 and blocked with 1X blocking buffer (Sigma) at 4˚C overnight. 53BP1 foci were detected by 53BP1 antibody followed by Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). The nuclei of cells were visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. Confocal microscopy was performed with an LSM510 META confocal microscope (Carl Zeiss, Jena, Germany) at the Microscopy and Imaging Shared Resource of Georgetown University Medical Center.

Results

Inhibitors of DDR kinases did not affect the effect of olaparib in SUM149PT cells. Since PARP inhibitors affect DDR, we first analyzed the status of DDR kinases including ATR, CHK1, and CHK2 by western blot analysis (Figure 1A). After treatment with 30 μM of olaparib, the levels of phospho-ATR, phospho-CHK1, and phospho-CHK2 were changed over time. The level of phospho-ATR (S428) was increased at 24 h after treatment. The levels of phospho-CHK1 and CHK2 were also increased as early as 2 h after treatment with olaparib.

Since many previous results suggest that co-targeting DDR kinases may sensitize to the effect of PARP inhibitors (27-32), we further tested the effects of olaparib in the presence of DDR kinase inhibitors: a CHK2 inhibitor NSC 109555 (33), a CHK1 and WEE1 inhibitor PD407824 (34), an Ataxia telangiectasia mutated (ATM) inhibitor KU55933 (35), and a DNA-dependent protein kinase (DNA-PK) inhibitor NU7441 (36). SUM149PT cells were treated with olaparib in the presence of these inhibitors for 3 days and viable cells were measured by the MTT assay (Figure 1B). Under these conditions, none of the kinase inhibitors enhanced the effect of olaparib.

BEZ235 potentiated the effect of olaparib in BRCA1-defective breast cancer cells. To identify the potentiating effect of olaparib, we performed a series of MTT assay with PKIs in the presence of olaparib. As a result, we found that BEZ235 enhanced the effect of olaparib in SUM149PT cells. BEZ235 was originally identified as a dual inhibitor of phosphadylinositol-3-OH kinase (PI3K) and mammalian target of rapamycin (mTOR) (37). Recently, BEZ235 was further characterized as an inhibitor of ATM, ATR, and DNA-PK (38). Interestingly, another PI3K/mTOR inhibitor PI-103 (39) did not potentiate the

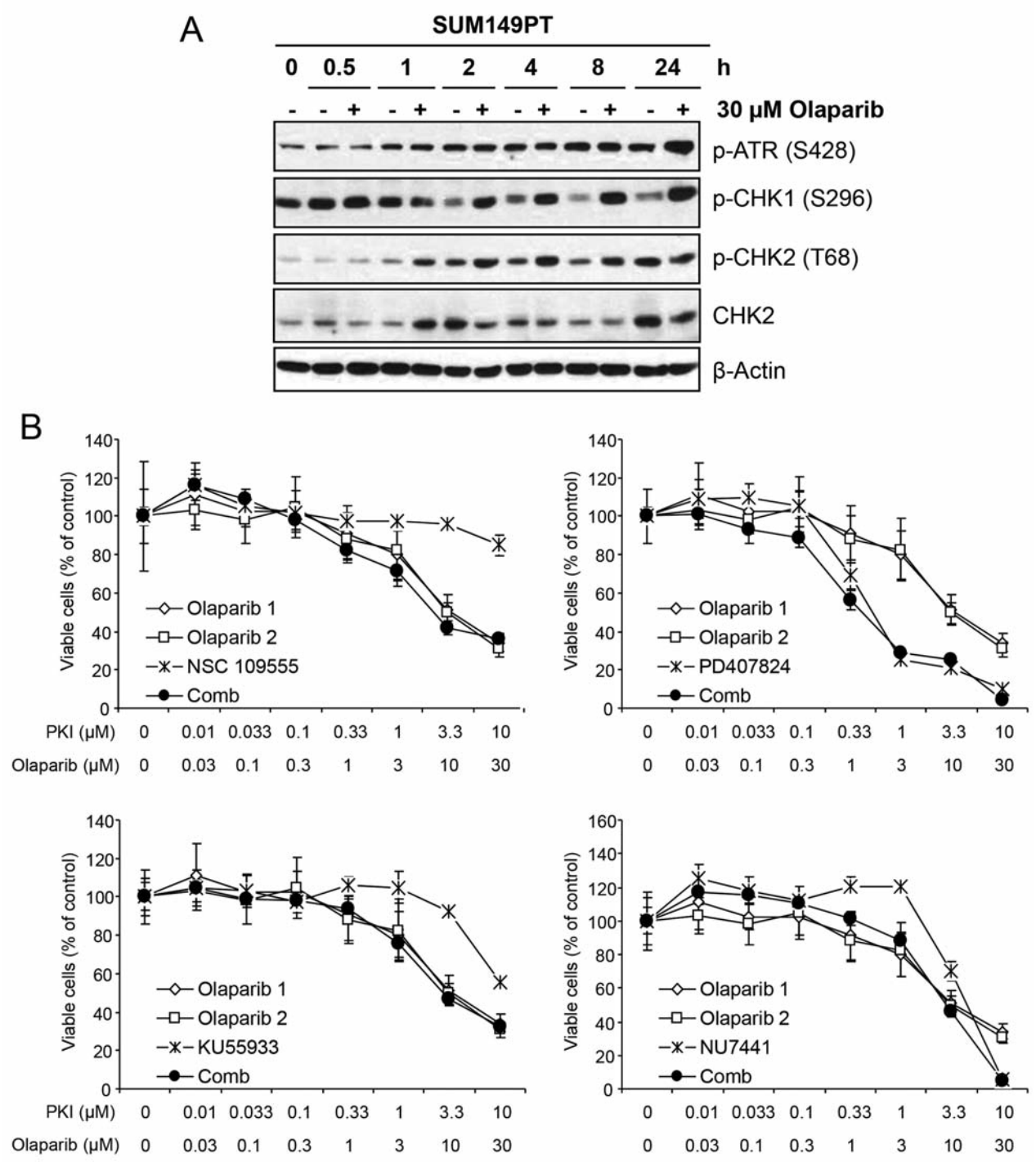


Figure 1. *DNA damage response (DDR) kinase inhibitors did not potentiate the effect of olaparib in SUM149PT cells. A: Western blot analysis of SUM149PT cells after treatment with olaparib, a poly(ADP-ribose) polymerase (PARP) inhibitor. The cells were treated with either DMSO (vehicle control, 0 μM) or 30 μM olaparib for the indicated time intervals and the lysates from these cells were analyzed by antibodies against phospho-ATM and Rad3 related (p-ATR (S428)), phospho-checkpoint kinase 1 (p-CHK1 (S296)), phospho-checkpoint kinase2 (p-CHK2 (T68)) and checkpoint kinase 2 (CHK2). β-Actin was used as a loading control. B: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay of SUM149PT cells. Cells were treated with either olaparib, DDR kinase inhibitors (NSC 109555, a CHK2 inhibitor; PD407824, a CHK1 and WEE1 inhibitor; KU55933, an ataxia telangiectasia mutated (ATM) inhibitor; NU7441, a DNA-dependent protein kinase (DNA-PK) inhibitor) or combination of them (Comb) for 72 h and the viability of cells were measured by the MTT assay. Representative data from experiments performed in triplicate are shown as the mean±SD. Olaparib 1 and olaparib 2 represent independent MTT assay results of triplicated experiments using same olaparib compound. Comb represents viability plot of combined treatment with olaparib and DDR kinase inhibitor.*

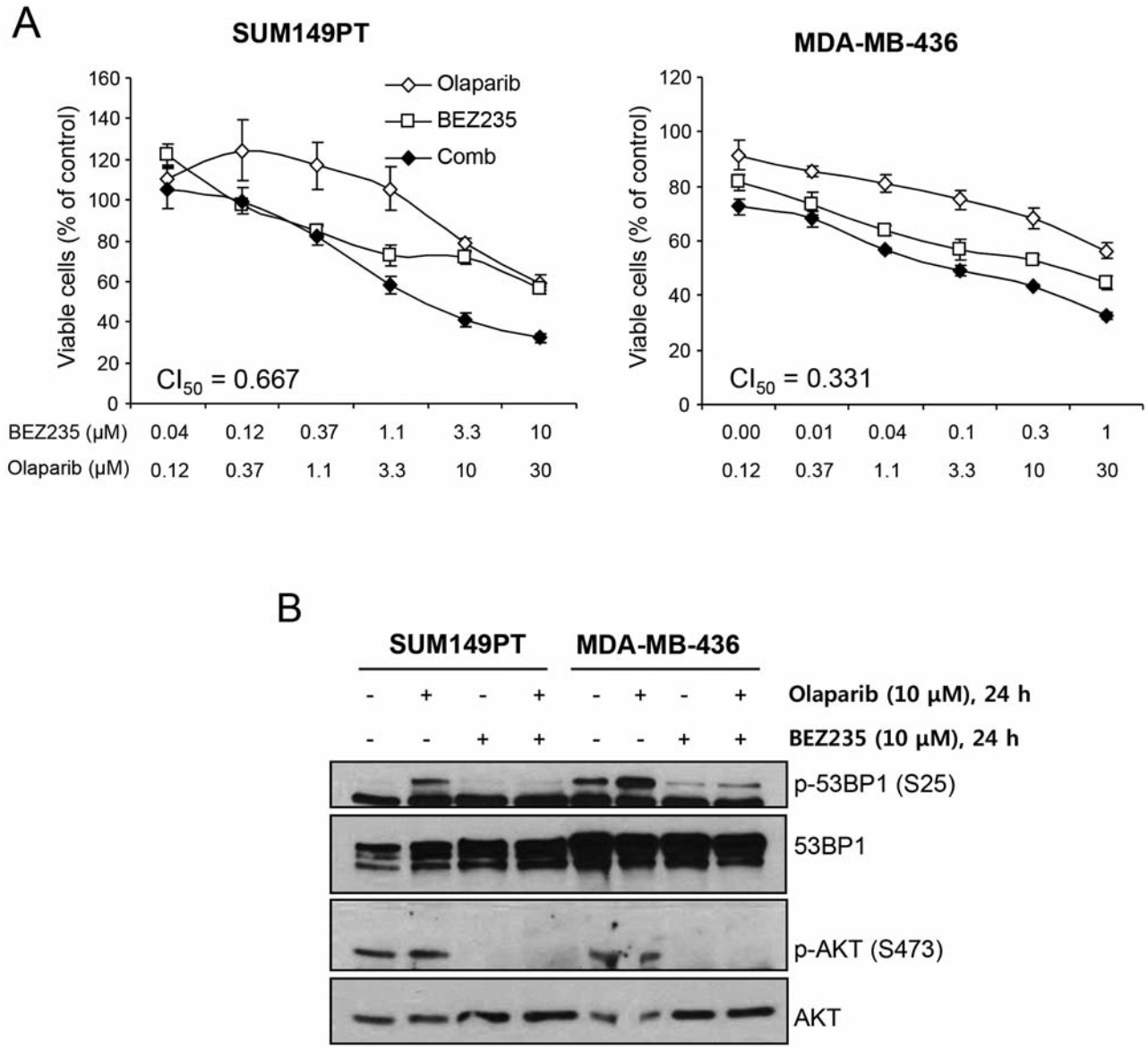
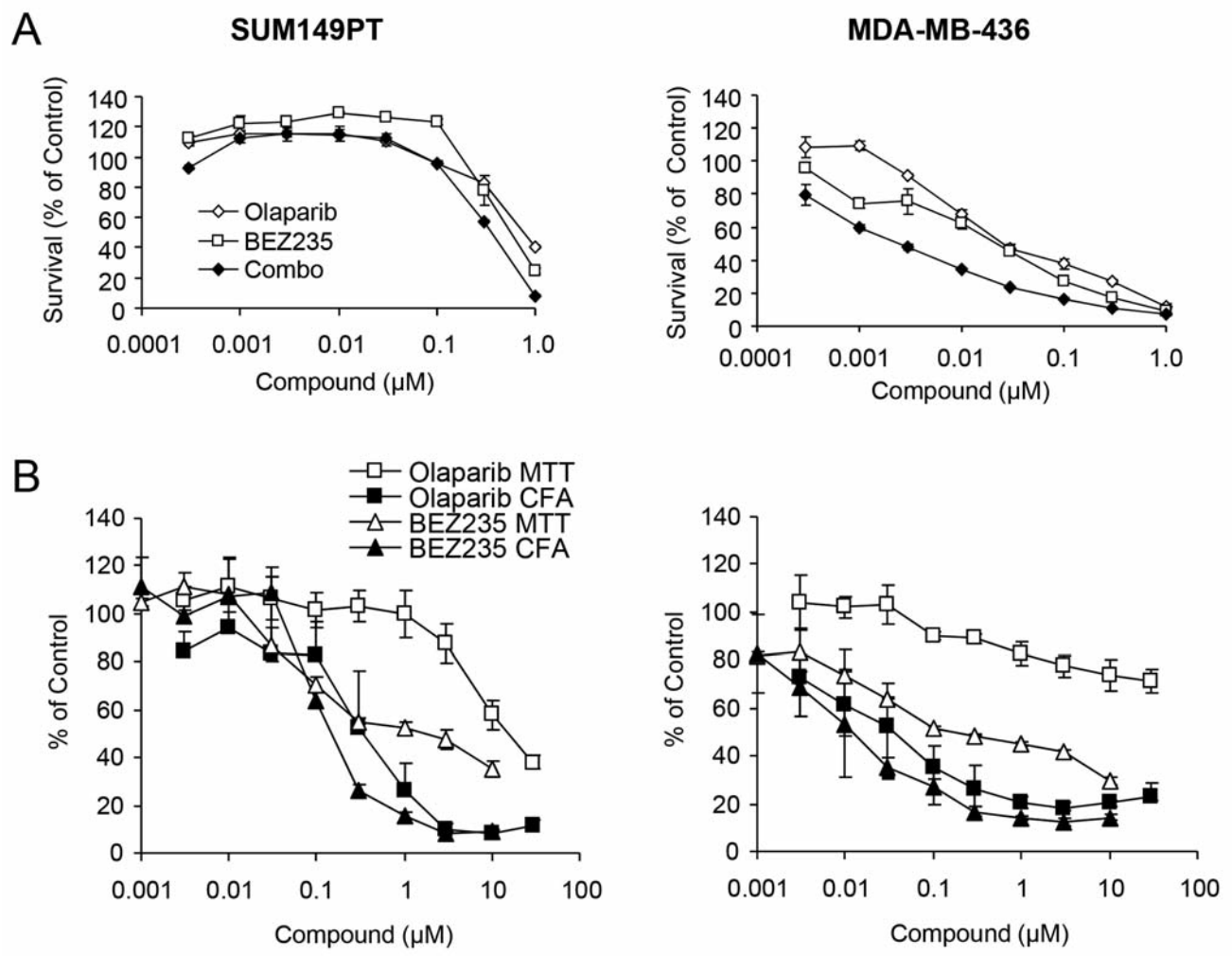


Figure 2. *BEZ235, a dual inhibitor of phosphadylinositol-3-OH kinase (PI3K) and mammalian target of rapamycin (mTOR) potentiated olaparib effect in BRCA1-defective breast cancer cells. A: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay of SUM149PT and MDA-MB-436 cells. SUM194PT and MDA-MB-436 cells were treated with BEZ235 or olaparib, a PARP inhibitor or combination of them (Comb) as indicated, for 72 h and the viable cells were measured by MTT assay. Data from two independent experiments performed in triplicate are shown as the mean±SD. CI_{50} is combination index (CI) at half-maximal effective concentration (EC_{50}) obtained from Compusyn software (ComboSyn Inc), as described in the Materials and Methods. $CI_{50}>1$, antagonism; $CI_{50}<1$, synergy. B: Western blot analysis of SUM149PT and MDA-MB-436 cells treated with olaparib and BEZ235 combination. The cells were treated with compounds as indicated for 24 h and the cell lysates were analyzed by antibodies against p53 binding protein 1 (53BP1), phospho-53BP1 (p-53BP1 (S25)), phospho-AKT (p-AKT(S473)) and AKT as indicated. Since the level of AKT was not changed under these conditions (data not shown), AKT was used as a loading control.*

effect of olaparib in SUM149PT cells (data not shown). As shown in Figure 2A, the olaparib–BEZ235 combination synergistically reduced the viable SUM149PT and MDA-MB-436 cells in MTT assays with the combination index at half-maximal effective concentration (CI_{50}) of 0.667 and 0.331, respectively.

We further performed western blot analysis of cells treated with the olaparib/BEZ235 combination (Figure 2B). Treatment with olaparib did not affect the level of phospho-AKT (S473) which is activated in BRCA1-defective SUM149PT and MDA-MB-436 cells (40). As expected, BEZ235 reduced the level of phospho-AKT both as a single-



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Cell line	Compound	EC_{50} (μM)		Fold decrease
		MTT, 72 h	CFA, 72 h	
SUM149PT	Olaparib	15.8	0.31	51.1
	BEZ235	1.54	0.28	5.6
MDA-MB-436	Olaparib	N/D	0.028	N/D
	BEZ235	0.39	0.016	25.2

Figure 3. *The effect of olaparib was more profound in the colony-formation assay (CFA) than in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A: 96-well CFA of SUM149PT and MDA-MB-436 cells treated with olaparib, a poly(ADP-ribose) polymerase (PARP) inhibitor in combination with BEZ235, a dual inhibitor of phosphadylinositol-3-OH kinase (PI3K) and mammalian target of rapamycin (mTOR). The cells were treated with olaparib, BEZ235 or combination of them (Comb) as indicated, for 3 days in 96-well plates. The concentration of compounds treated were as follows: 1 μM, 0.3 μM, 0.1 μM, 0.03 μM, 0.01 μM, 0.003 μM, 0.001 μM and 0.0003 μM. After incubation, the media containing compounds were removed from the cells and fresh normal growth media were supplemented to the cells. The cells were further incubated for 4 more days and the viable cells were measured by MTT assay. Data from two independent experiments performed in triplicate are shown as the mean±SD. B: Comparison of MTT assay and 96-well CFA. For the MTT assay, cells in 96-well plates were treated with olaparib or BEZ235 for 3 days and MTT assay was performed as described in the Material and Methods. For 96-well CFA, cells in 96-well plates were treated with same compounds for 3 days. The cells were further treated as described in the Materials and Methods and the viable cells were measured by MTT staining. The concentration of compounds treated are as follows: 30 μM, 10 μM, 3 μM, 1 μM, 0.3 μM, 0.1 μM, 0.03 μM, 0.01 μM, 0.003 μM and 0.001 μM. Representative data from experiments performed in triplicate are shown as the mean±SD. C: Comparison of the half-maximal effective concentration (EC_{50}) values obtained by MTT assay and 96-well CFA. The EC_{50} values were obtained by CompuSyn software (ComboSyn Inc) from the data shown in B.*

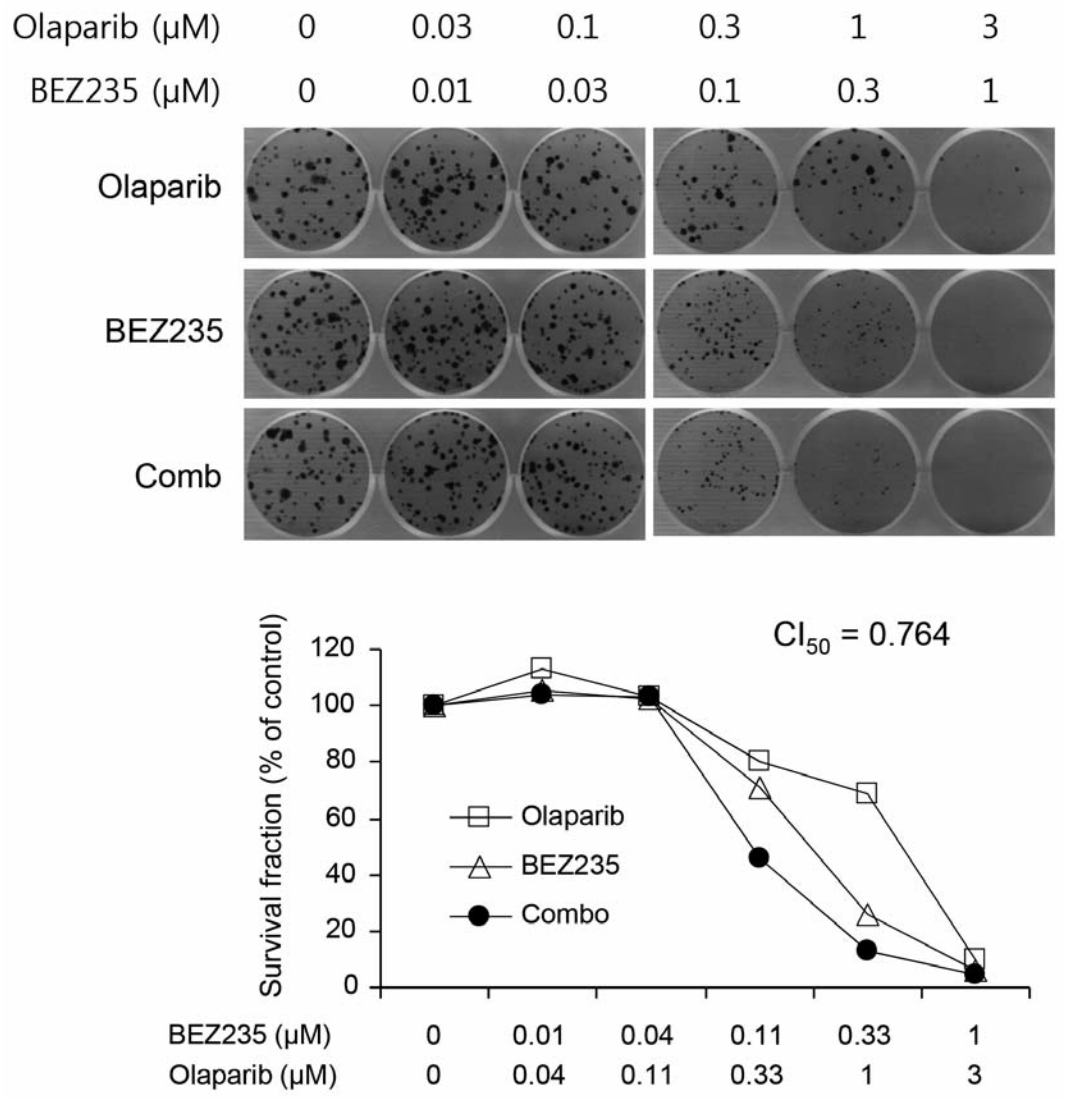

Figure 4. *Olaparib a poly(ADP-ribose) polymerase (PARP) inhibitor in combination with BEZ235 a dual inhibitor of phosphadylinositol-3-OH kinase (PI3K) and mammalian target of rapamycin (mTOR) reduced the survival of SUM149PT cells. The colony-formation assay of SUM149PT cells was performed in 6-well plates. The cells were treated with serially diluted olaparib and BEZ235 with a fixed molar ratio of 3:1 (the highest concentration of olaparib 3 μM, BEZ235 1 μM; the lowest concentration of olaparib 0.03 μM, BEZ235 0.01 μM). The detailed drug treatment scheme and cultivation duration for colony formation after removal of drugs are described in the Materials and Methods section. Representative data from experiments performed in triplicate are shown. CI_{50} is combination index (CI) at half-maximal effective concentration (EC_{50}) obtained from Compusyn software (ComboSyn Inc) as described in the Material and Methods section. CI_{50} >1, antagonism; CI_{50} <1, synergy.*

agent and in the presence of olaparib. Interestingly, treatment with olaparib induced the phosphorylation of 53BP1 (S25). 53BP1 is a marker for DNA double-strand breaks (DSBs) and accumulates at sites of DSBs (41). Although the 53BP1 protein is phosphorylated at multiple sites during DDR, it is not yet clear whether these phosphorylations are directly coupled to DNA binding (42). As shown in Figure 2B, BEZ235 reduced olaparib-induced phosphorylation of 53BP1 in both cell lines.

Olaparib–BEZ235 combination synergistically reduced the long-term survival of BRCA1-defective breast cancer cells. To determine the effect of olaparib and BEZ235 combination on the long-term survival of BRCA1-defective breast cancer cells, we performed 96-well colony-formation assays. The cells were sub-cultured into 96-well plates and treated with olaparib and BEZ235 at 1:1 molar ratio for 3 days. Then the media containing compounds were removed from the cells and fresh normal media without compounds were

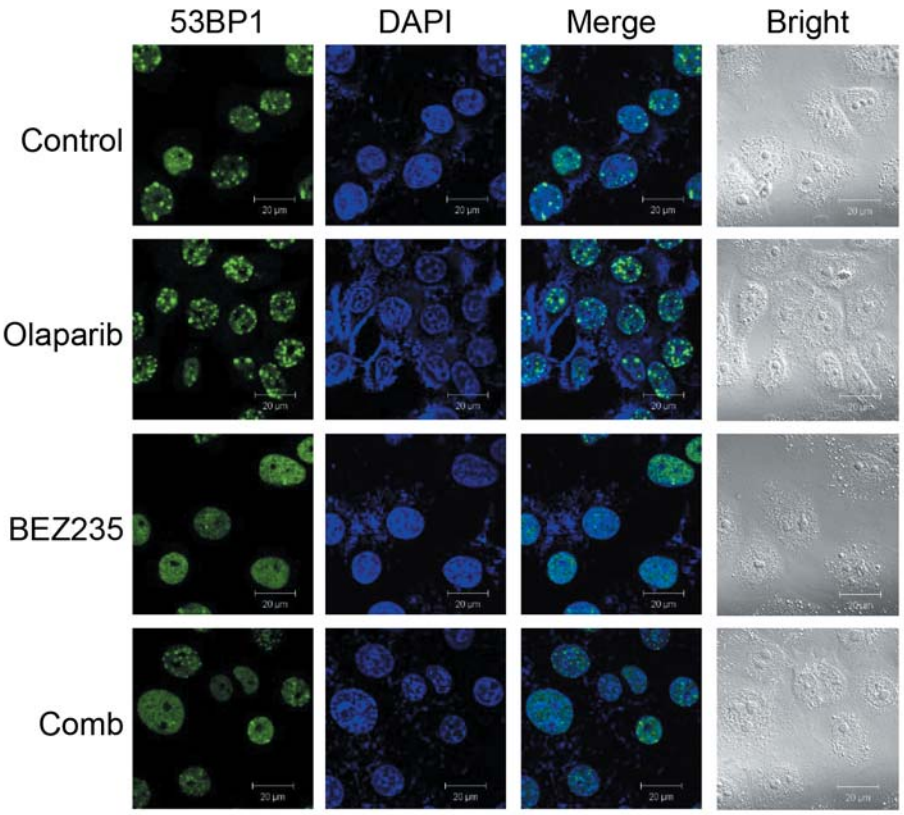

Figure 5. *BEZ235, a dual inhibitor of PI3K and mTOR reduces the olaparib-induced formation of p53 binding protein (53BP1) foci in SUM149PT cells. SUM194PT cells treated with 30 μM olaparib, 10 μM BEZ235 or combination of them (Comb) for 2 h. Immunofluorescence staining was performed as described in the Materials and Methods section and the images of 53BP1 foci, chromatin (DAPI) and bright-field (Bright) were obtained by confocal microscopy.*

supplemented to the cells. And then the cells were further incubated to form colonies and the viable cells of these colonies were determined by MTT staining. Under these conditions, the olaparib–BEZ235 combination had a synergistic effect only on MDA-MB-436 cells (Figure 3A). Unexpectedly, both olaparib and BEZ235 exhibited much more potency in the colony-formation assay than in the MTT assay (Figure 3B and C).

To determine the effect of the olaparib–BEZ235 combination on the long-term survival of SUM149PT cells, standard colony-formation assays were performed in 6-well plates. As shown in Figure 4, the combination synergistically reduced the survival of SUM149PT cells.

BEZ235 reduced the formation of olaparib-induced 53BP1 foci. Since 53BP1 is accumulated at DSB sites (41), we performed confocal microscopy to detect the foci of 53BP1 in SUM149PT cells. Cells were treated with compounds for 2 h and the 53BP1 foci were visualized by immunofluorescence. As shown in Figure 5, SUM149PT cells had a basal level of 53BP1 foci even in the vehicle (DMSO)-treated condition. Treatment with olaparib increased the number of 53BP1 foci in SUM149PT cells. Interestingly, BEZ235 reduced the number of both basal and olaparib-induced 53BP1 foci in SUM149PT cells.

Discussion

In the present study, we identified the synergistic anti-proliferative effect of BEZ235 on olaparib treatment in BRCA1-mutated breast cancer cell lines. We first screened the synergistic lethal effect between olaparib and DDR kinase inhibitors targeting CHK1 and WEE1, CHK2, ATM or DNA-PKs. The tested DDR kinase inhibitors hardly exerted any synergistic effect on olaparib treatment in SUM149PT, a *BRCA1*-mutated triple-negative breast cancer cell line. Inhibition of PARP activates ATM and homologous recombination-mediated repair of DNA damage (43). ATM-deficient lymphoma cell lines have been reported to be more sensitive to PARP inhibitors than proficient cell lines (27)

and depletion of ATM in MCF-7 and ZR-75-1 cell lines resulted in sensitivity to PARP inhibition (32). On the contrary, our current result using the ATM inhibitor KU55933 in combination with olaparib was not effective in SUM149PT. However, we observed 53BP1 phosphorylation and CHK2 phosphorylation, reflecting activation of ATM by olaparib treatment in SUM149PT cells.

We tested kinase inhibitors other than DDR kinases combined with olaparib and found that BEZ235, a PI3K/mTOR inhibitor, potentiated the effect of olaparib in BRCA1-defective breast cancer cells. These results partly concur with a previous report that the combined treatment of PI3K inhibitor (NVP-BKM120) and olaparib delayed tumor doubling time in a *BRCA1*-mutated mouse model and xenotransplants of human BRCA1-related breast cancer cells (44). PTEN-deficient endometrial carcinoma was also reported to be more sensitive to PARP inhibitors (45), as were various other types of cancer (46). However, we did not observe any synergic lethal effect between PI-103 (another PI3K inhibitor) and olaparib in SUM149PT cells. Recently, PI-103 was effective in radiosensitization when combined with olaparib and further addition of PI-103 did not enhance radiation-induced cell death in breast cancer cell lines (47). Possibly an additional insult to DNA damage seems to be required for appropriate synergistic lethal effect of PI-103 and olaparib in this cell line.

BEZ235 treatment reduced both olaparib-induced formation of 53BP1 foci and phosphorylation of 53BP1 in SUM149PT cells. It is unclear whether the inhibitory effect of BEZ235 on the PI3K/mTOR pathway is sufficient for synergistic lethal or growth-inhibitory effect in combination with olaparib, since BEZ235 was also reported to inhibit ATM, DNA-PK and ATR (38, 48). Simultaneous inhibition of these DDR kinases may possibly impair the single-strand and DSB DNA damage checkpoint pathways for restoring damaged DNA induced by olaparib and override the checkpoint control, ensuring unregulated cell proliferation and ultimately making replication defects irreversible or leading to lethality by the suppression of the PI3K/mTOR pathway. The *N*-terminal phosphorylation of 53BP1 is dependent on ATM activation and phosphorylated 53BP1 co-localizes with phosphorylated histone variant H2AX at S139 (γH2AX) (42, 49, 50). Upon DNA damage, 53BP1 recruits effector molecules including replication timing regulatory factor 1 (RIF1) for non-homologous end-joining in G_1 phase (51). At G_1-S phase transition, phosphorylated 53BP1 at DSB sites is removed by BRCA1, hence the loss of BRCA1 induces the accumulation of 53BP1 at DSB sites (51-53). In our current study, we found that olaparib treatment induced phosphorylation of 53BP1 and its accumulation at nuclear foci, while treatment with BEZ235 abrogated these effects in SUM149PT cells. Further investigation is needed to identify the role and mechanism related to the obliteration of functional 53BP1 and the possible mechanism of lethality by treatment with BEZ235. Interestingly in BRCA1-mutated mouse mammary tumors, the loss of 53BP1 is one mechanism of PARP resistance (14), suggesting the requirement of intact 53BP1 for lethality.

In the present study, we identified BEZ235 as a potentiator of the synthetic lethal and growth-inhibitory effect of olaparib in BRCA1-defective breast cancer cell lines, with suppression of both phosphorylation of 53BP1 and its accumulation at nuclear foci. The combination of olaparib and BEZ235 may be a candidate of effective synthetic lethality-inducing therapy against BRCA1-defective cancer.

Conflicts of Interest

The Authors confirm that there exist no conflicts of interest.

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