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Co-targeting poly(ADP-ribose) polymerase (PARP) and histone deacetylase (HDAC) in triple-negative breast cancer: Higher synergism in BRCA mutated cells

Hélène Marijon[a,b,*], Dhong Hyun Lee[a], LingWen Ding[c], Haibo Sun[a], Sigal Gery[a], Aimery de Gramont[b], H. Phillip Koeffler[a,c]

[a] *Division of Hematology and Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA, United States*
[b] *Oncology Department, Franco-British Institute, Paris, France*
[c] *National Cancer Institute and Cancer Science Institute, National University of Singapore, Singapore*

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ABSTRACT

Purpose: Despite similarities with *BRCA*-mutated breast cancers, triple-negative breast cancers (TNBC) remain resistant to poly(ADP-ribose) polymerase (PARP) inhibitors as single agents. Histone deacetylase inhibitors (HDACi) can decrease expression of proteins involved in DNA repair. We thus hypothesized that a HDACi (suberoylanilide hydroxamic acid (SAHA) or belinostat) could sensitize TNBC to the PARP inhibitor olaparib.
Methods: Human TNBC cells were co-treated with olaparib and either SAHA or belinostat, and their effects on survival, proliferation, cell cycle, apoptosis and DNA repair pathways were evaluated. Subcutaneous xenografts were used to determine the effect of the combination treatment *in vivo*.
Results: HDACi and olaparib synergistically inhibited proliferation of a panel of 8 TNBC cell lines *in vitro* and in nude mice harboring TNBC xenografts *in vivo*. We noted a weaker synergism in *PTEN*-deficient TNBC cells and a stronger synergism in *BRCA1*-mutated TNBC cells. In the *BRCA1*-mutated cell line HCC-1937, we observed a drastic decrease in the expression of proteins involved in homologous recombination (HR), leading to a large imbalance of the ratio P-H2AX/RAD51. In *BRCA1* wild type (wt) cell lines, effect of the combination treatment relied on DNA damage-induced cell cycle arrest followed by induction of apoptosis.
Conclusion: In summary, these results provide a preclinical rationale to combine a HDACi with a PARP inhibitor to reduce HR efficiency in TNBC and sensitize these aggressive tumors to PARP inhibition.

1. Introduction

Poly(ADP-ribose)polymerase (PARP) is a superfamily of 17 proteins that can add mono- or poly-ADP ribose residues to their targets. Thus, PARP proteins post-transcriptionally regulate several proteins involved in DNA repair, telomere maintenance, and also ERK, NFκB and Wnt signaling [1]. PARP1 is the best known PARP protein. It plays a critical role in the base excision repair pathway, thus allowing cells to repair single strand breaks. In 2005, Farmer et al. demonstrated that inhibition of PARP caused synthetic lethality in *BRCA1*$^{-/-}$ and *BRCA2*$^{-/-}$ cells, but not in *BRCA*$^{+/-}$ cells [2]. This led to the clinical development of PARP inhibitors in patients with somatic *BRCA* mutations. In a phase 2 study, treatment with the PARP inhibitor olaparib (400 mg, twice daily) achieved a 41% objective response in patients with *BRCA* mutated metastatic breast cancers [3].

Triple-negative breast cancers (TNBC) are tumors lacking expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and human epidermal growth factor receptor 2 (Her2) as measured by immunohistochemistry. TNBC accounts for 15% of all breast cancers. Although TNBC show a better response to chemotherapy compared with non-TNBC in the neoadjuvant setting, these patients, paradoxically, are more likely to have recurrent disease and greater mortality versus metastatic non-TNBC patients [4]. Most *BRCA* mutated breast cancers are triple-negative. In addition, two thirds of TNBC have gene expression profiles similar to those of BRCA deficient tumors, and one-third of TNBC have *BRCA1* promoter methylation [5]. However, despite these similarities between TNBC and *BRCA* mutated breast cancers, PARP inhibitors as monotherapy failed to improve the outcome of TNBC patients [6].

Two approaches have recently moved forward. First consists of developing models to determine biomarkers that could predict sensitivity to PARP inhibitors. Second is to impair pharmacologically DNA

* Corresponding Author: Oncology Department, Franco-British Institute, 4 rue Kleber 92300 Levallois-Perret, France.
E-mail address: helene.marijon@ihfb.org (H. Marijon).

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repair functions in order to sensitize tumors to PARP inhibitors. For example, PI3K inhibitors can sensitize *BRCA* wild type TNBC to PARP inhibitors through ETS1-mediated downregulation of *BRCA1* [7]. Inhibitors of histone deacetylase (HDAC) can decrease the expression of proteins involved in homologous recombination (HR) in prostate cancers [8] and can induce DNA damage which can be repaired in normal but not transformed cells [9]. Suberoylanilide hydroxamic acid (SAHA, vorinostat) and belinostat are the first HDAC inhibitors (HDACi) approved by the US Food and Drug Administration for the treatment of cutaneous T cell lymphoma [10].

In our study, we demonstrate that in a panel of TNBC cell lines, olaparib and HDACi synergistically inhibit cell growth *in vitro,* as well as *in vivo* by induction of DNA damage. Analysis from the results obtained in our panel of 8 TNBC cells shows that synergism is higher in *BRCA* mutated cells and weaker in *PTEN* mutated cells. These results provide additional rationale for the development of this combination in TNBCs.

2. Materials and methods

2.1. Breast cancer cell lines

Eight human TNBC cell lines (HCC-1937, MDA-MB-231, MDA-MB-157, MDA-MB-453, MDA-MB-436, MDA-MB-468, Hs578T, BT-549) were used in our study. Each was obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell lines were authenticated by short tandem repeat profiling. Among these cell lines, two were *BRCA1* mutated (HCC-1937 and MDA-MB-436). TNBC cell lines were maintained in DMEM medium (Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, Logen, UT) in a humidified incubator at 37 °C supplied with 5% CO_2. Only cells in exponential growth phase were used in the study.

2.2. Chemical compounds

Olaparib was purchased from LC Laboratories (Woburn, MA). Pure olaparib was solubilized with DMSO and stored at 60 mM at −20 °C. SAHA was purchased from Cayman Chemical (Ann Arbor, MI), diluted in DMSO and stored at 1 mM at −20 °C. Belinostat was purchased from Selleck Chemicals (Houston, TX), diluted in DMSO and stored at 100 mM at −20 °C.

2.3. Measurement of cell growth and survival

For dose-response studies, cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 3,000–10,000 cells (depending on the cell line) were plated in 96-well plates and incubated overnight. On the following day, 100 μL of new media containing increasing concentrations of olaparib and/or SAHA were added. After 72 h incubation, 5 mg/mL MTT solution (Sigma-Aldrich, St Louis, MO) was added and incubated for 4 h. Absorbance was measured at 540 nm using an ELISA reader. For growth assays, experiments were performed similarly, plating cells at a lower density (800–2000 cells), and incubating them with the drugs up to 7 days.

Clonogenic ability of TNBC cells cultured with olaparib and/or SAHA was monitored by soft agar colony formation for 14 days, as previously described [11].

2.4. Analysis of drug interaction

Synergism between olaparib and HDAC inhibitors was determined quantitatively by isobologram and combination index (CI) analysis adapted from the median-principle methods of Chou and Talalay [12]. CompuSyn 1.0 software was used for CI analysis (ComboSyn, Paramus, NJ).

2.5. Growth of MDA-MB-157 xenografts in athymic nude mice with in vivo treatment

All animal experiments strictly followed the guidelines of Cedars-Sinai Medical Center and the National Institute of Health (NIH). Female nu/nu athymic nude mice (5–6 weeks old) from Harlan laboratories (Indianapolis, IN) were maintained in a pathogen-free condition with sterilized chow and water. MDA-MB-157 cells (5×10^6) were suspended in 100 μL of PBS and mixed with 100 μL of Matrigel solution (BD Biosciences, San Jose, CA); the mixture was injected subcutaneously on the upper flanks of nude mice. Six mice were randomly assigned to each experimental group: (i) PBS with 10% DMSO (diluent-specific control), (ii) olaparib (12.5 mg/kg body weight), (iii) SAHA (25 mg/kg body weight) and (iv) olaparib with SAHA. Drugs were administered intra peritoneally 5 days a week. Body weights and tumor sizes were monitored three times a week. Tumor volumes were determined using the following formula: A (length) x B^2 (width) x 0.5236. The experiment was stopped at Day 64, and all mice were sacrificed. At autopsy, tumors were excised, weighed and fixed in 10% PBS-buffered formalin and maintained in 70% ethanol. Fixed tumors were embedded in paraplast (Oxford Labware, St. Louis, MO), sliced and stained with hematoxylin and eosin (H&E) for histopathological examination.

2.6. Measurement of cell cycle and apoptosis

For cell cycle analysis, MDA-MB-157, MDA-MB-231 and HCC-1937 were exposed to either drug-free medium or olaparib (10 μM) and/or SAHA (2 μM). Cells were harvested and fixed with 70% ethanol at regular interval. Fixed cells were stained with propidium iodide (PI) for flow cytometry analysis using BD FACScan (BD Biosciences, San Jose, CA). For apoptosis assay, cells were exposed to either olaparib (30 μM) and/or SAHA (2 μM) for 72 h, harvested and stained with PI and fluorescein isothiocyanate (FITC) using Annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol.

2.7. Western blotting

MDA-MB-157, MDA-MB-231 and HCC-1937 were exposed to either drug-free medium or olaparib and/or SAHA. After different durations of exposure, cells were lysed with RIPA buffer (Millipore-Upstate, Temecula, CA) supplemented with protease inhibitor cocktail. For analysis of phosphorylated protein, a phosphatase inhibitor cocktail (PhosSTOP, Roche) was added to the lysis buffer. 40 μg proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with primary antibodies from either Cell Signaling Technology (P-NFkB, P-p38, P-ERK, P-AKT1, P-4EBP1, P-STAT3, P-Chk2 and P-H2AX) or Santa Cruz Biotechnology (STAT3, Chk1, Chk2 and RAD51). β-actin was used as a loading control. ImageJ was used to analyze band intensity [13].

2.8. Statistical analysis

In vitro experiments were repeated at least three times to ensure reproducibility. Two-tailed Student's t-test was used to compare differences between two groups. One-way ANOVA test was used to compare differences among three or more groups. P values less than or equal to 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism6 software.

3. Results

3.1. Synergistic effect of olaparib and HDAC inhibitors on viability of MDA-MB-231 and MDA-MB-157 cell lines in vitro and in vivo

MDA-MB-231 and MDA-MB-157 cell lines were exposed to various concentrations of either olaparib and/or SAHA for 72 h, and cell

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