RALBP1 Depletion Downregulates DNA Repair and Potentiates PARP Inhibitor Activity in BRCA Wild Type and Mutated Breast Cancer Cell Lines

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INTRODUCTION

Rlip (RLIP76 protein encoded by *RALBP1*) is a Ral-regulated, membrane-associated ATPase enzyme that facilitates Phase 2 detoxification, plays a role in clathrin-dependent endocytosis (CDE), and is vital for cancer cell formation and survival. DNA repair is essential for both normal and cancer cell survival, as it protects cancer cells from DNA damage caused by chemotherapy or radiation. Poly (ADP-ribose) polymerases (PARPs) play a crucial role in the repair of DNA damage. By targeting these repair mechanisms in cancer cells, it is possible to improve the effectiveness of cancer treatments. A notable example is dysfunctional homology-directed repair (HDR), which results from inactivating mutations in the BRCA1 and BRCA2 genes, contributing to radiation resistance in human cancer cells. Therefore, identifying new targets within double-strand break (DSB) repair pathways is crucial. A core concept in using PARP inhibitors (PARPi) for treating patients with BRCA1/2 mutations is the concept of synthetic lethality. Our research found that Rlip depletion or inhibition, combined with PARPi, works synergistically in both BRCA1/2-mutated and BRCA1/2-wild-type breast cancer cells.

OBJECTIVES

Hypothesis. We hypothesized that cancer cells with homologous recombination deficiency (HRD) or defects after Rlip depletion will be hypersensitive to PARP inhibitors and synergize with PARPi to selectively kill BRCA1-mutated triple negative, (TNBC) and overwhelm tumor cell defenses by enhancing DNA damage, oxidative stress, resulting in killing the cells based on the synthetic lethality principle and reducing chemotherapy-related toxicity.

Objectives: The present study tests a new therapeutic paradigm: Rlip depletion, combined with low-dose of PARP (poly ADP ribose) inhibitor Olaparib, to enhance cancer cell cytotoxicity, DNA damage and cell death.

MATERIALS AND METHODS

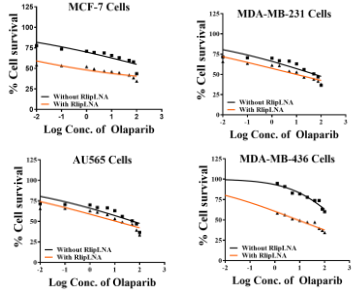
PARP inhibitor, AZD2281 (Olaparib) was tested on the proliferation and survival of different cancer cell lines, including MDA-MB-231 (TNBC), MCF-7 (ER+), AU565 (HER2+), and MDA-MB-436 (BRCA1 mutant TNBC), with and without Rlip depletion using an antisense locked nucleic acid (LNA), 10μg/ml for 24h. For all experiments, 10 μM Olaparib was used for 24-48h in complete growth medium. Cell survival and death was measured by MTT assay. Cell cycle analysis was performed by flow cytometry. Expression levels of γH2AX and Rad51 proteins were assessed via Western blotting and immunofluorescence staining. The comet assay was used to measure DNA damage and fragmentation.

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RESULTS

Effect of PARP (poly ADP ribose) inhibition on cell death with and without Rlip depletion



EC50 (μM)	PARP inhibitor (Olaparib 10μM) alone	Olaparib+ RlipLNA
MCF-7	278.20	0.0242
MDA-MB-231	62.11	2.01
AU565	62.11	5.58
MDA-MB-436	318.60	6.93

Figure 1. Inhibition of Rlip increased Olaparib-induced cell death in human breast cancer cells. A. MDA-MB-231, MCF-7, AU565, and MDA-MB-436 cells were Rlip depleted by Rlip-LNA (10 μg/ml) or control scrambled LNA transfection for 24h before Olaparib treatment (10 μM). The effect of varying concentrations of Olaparib on cell proliferation was determined for all cell lines by MTT assay after 48h of treatment. **B.** Combination index values for PARP inhibitors and Rlip depletion were calculated by the Hill equation, using Prism 6.0 (GraphPad Software Inc., San Diego, CA) and with Chou-Talalay's method with CompuSyn software. The effects were highly synergistic (CI<1) with combination in MCF-7 cells. MDA-MB-231 cells showed additive effects, whereas the combination of PARPi and Rlip depletion was antagonistic in AU565 and MDA-MB-436 (CI>1), but the doses of both inhibitors were significantly reduced ($p<0.001$) after Rlip depletion. Analysis of variance (ANOVA) with Tukey's post-hoc analysis was used for group comparison.

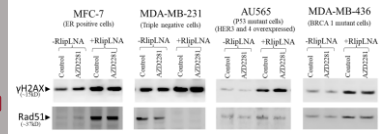


Figure 2. Rlip depletion with Olaparib increases γH2AX protein expression in all breast cancer cells tested. Whereas Rad51 did not show any significant increase in any cell types other than MCF-7 cells. Cells were depleted for Rlip with RlipLNA than treated with 10μM of Olaparib for 24h. Total cell lysates were used to run the Western blots. Representative blots (n=3) are shown.

Effect of PARP inhibition on DNA damage with and without Rlip depletion

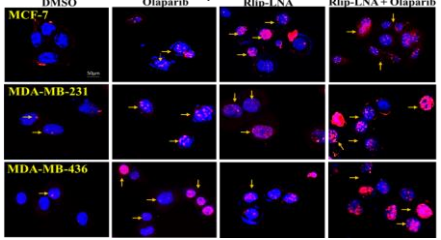


Figure 3. Olaparib induces γH2Ax foci in the nucleus after Rlip depletion/inhibition. After Rlip depletion, equal number of cells were plated in 4 well chamber slides in complete growth medium. After cells adhered were treated with 10μM of Olaparib for 24h. In DMSO and Olaparib alone groups cells were plated without Rlip depletion. As observed, with RlipLNA cells showed increased number of γH2AX foci in the nucleus, which was more than either Olaparib or RlipLNA alone groups, Representative images are presented in figure. All images are at 40X. Blue nucleus with DAPI, arrows indicating the γH2Ax foci.

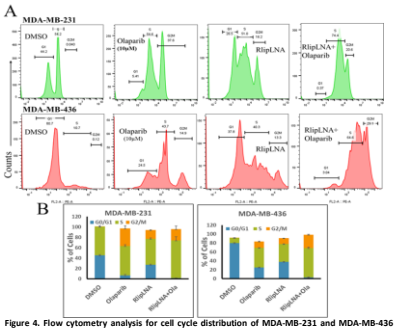


Figure 4. Flow cytometry analysis for cell cycle distribution of MDA-MB-231 and MDA-MB-436 cells after Rlip depletion. A. Rlip depletion combined with Olaparib treatment, the percentage of cells entering into the S phase is significantly ($p<0.05$, and <0.01) higher than Olaparib alone in both cell types, whereas the MDA-MB-436 cells showed significantly ($p<0.01$) higher % of cells in G2M phase (29.7±0.84 vs 14.35±0.77 respectively) as calculated by . Representative data (n=3) is shown. Each phase was calculated using the cell FlowJo software. **B.** Quantitative analysis of the percentages of cells in G1, S, and G2/M are presented as mean=SD as indicated.

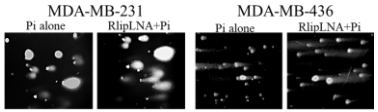


Figure 5. Alkaline comet assay demonstrated more DNA strands break in MDA-MB-231 and MDA-MB-436 cells after Rlip depletion. Olaparib treatments after Rlip depletion exhibited significantly higher number of cells undergoing DNA strand breaks. Representative (n=2) images are shown.

KEY OUTCOMES

- Rlip depletion significantly increased the reduction in cell survival caused by PARPi treatments across all tested cell lines. Combination Index (CI) values were calculated using the Hill equation and Chou-Talalay's method with CompuSyn software. The effects were highly synergistic (CI< 1) with Olaparib in MCF-7 cells.
- Olaparib showed additive effects in MDA-MB-231 cells, whereas the combination of PARPi and Rlip depletion was antagonistic in AU565 and MDA-MB-436 (CI>1), but the EC50 doses of both inhibitors were significantly reduced ($p< 0.001$) after Rlip depletion.
- The number of γ-H2AX foci, characteristic of DNA damage, increased significantly in nuclei ($p<0.01$) after Rlip depletion compared to inhibitor treatment alone, as observed by Immunocytometric staining.
- γH2AX protein reactivity in nuclei was accompanied by γH2AX protein expression in total cell lysates as shown by Western blot analysis following Rlip depletion. Though, in MCF-7 and MDA-MB-231 cells, DMSO or Olaparib alone showed notable amount of γH2AX, indicating there was some cytoplasmic accumulation of γH2AX, might be associated with some pro-apoptotic factors.
- Cell cycle analysis with Rlip depletion and Olaparib treatments showed either an increase in the S phase or G2/M (MDA-MB-436) phase arrest, suggesting cells attempt to undergo mitosis with unrepaired DNA damage, leading to elevated DNA double-strand break signaling, cell cycle arrest, and apoptosis.
- Alkaline comet assay confirmed more DNA damage with Rlip depletion compared to treatments with the inhibitor alone.

CONCLUSIONS

Our findings suggest that drugs that inhibit or deplete Rlip could help sensitize breast cancer cells, regardless of their BRCA status or whether they are wild-type ER+ or triple-negative breast cancers, to PARP inhibitors. The immunogenicity of HRD-associated malignancies and the immunomodulatory effects of PARP inhibition could be combined with Rlip inhibitors to treat HRD cancers. These findings clarify the underlying mechanisms and are expected to lead to the development of first-in-class therapies.

ACKNOWLEDGMENTS

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