स्तन कैंसर चिकित्सा हेतु महौषध

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टैलाज़ोपैरिब और रेस्वेराट्रोल के संयोजन से ऑटोफ़ैगी का विनियमन व्यापक डीएनए क्षति और सहक्रियात्मक कोशिका प्रेरण

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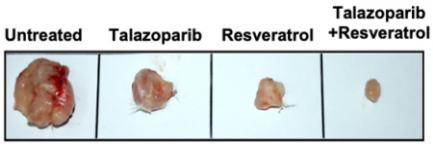

अकेले टैलाज़ोपेरिब, अकेले रेसवेराट्रोल या उनके संयोजन के साथ उपचार के बाद MCF-7 जेनोग्राफ्ट वाले एससीआईडी चूहों से निकाले गए ट्यूमर की प्रतिनिधि छवियाँ

सारांश

समरूप पुनर्संयोजन (एच. आर.) कुशल कैंसरों में पॉली (ADP-Ribose) पोलीमरेज़ अवरोधकों (PARPi) की सीमित प्रभावशीलता के कारण उनकी उपयोगिता का विस्तार करने के लिए PARPis के साथ नये औषधि संयोजनों की आवश्यकता होती है। BRCA1/2 की स्थिति की परवाह किए बिना स्तन कैंसर में PARP1 का अत्यधिक निष्पीडन कीमो-सुग्राहीकारक के साथ PARPi के प्रभावी लक्ष्यीकरण के लिए एक चिकित्सीय अवसर प्रस्तुत करती है। ऑटोफैगी स्तन कैंसर में नवीन PARPi प्रतिरोध प्रदान करता है। हमने इस अध्ययन में, स्तन कैंसर में टैलाज़ोपेरिब (BMN373, PARPi) के प्रभावों को बढ़ाने में एक प्राकृतिक अणु कीमो-सुग्राहीकारक, रेस्वेराट्रोल के व्यवहार की जांच की। रेस्वेराट्रोल ने इन-विट्रो में एचआर-कुशल स्तन कैंसर कोशिकाओं में टैलाज़ोपेरिब-प्रेरित कोशिका मृत्यु (क्षय) को प्रभावी ढंग से संवेदनशील बनाया। रेस्वेराट्रोल विषम टैलाज़ोपेरिब प्रेरित प्रो-सर्वाइवल ऑटोफैजिक फ्लक्स, लाइसोसोमल-मेम्ब्रेन-पारगम्यता (एल. एम. पी.) के माध्यम से ऑटोफैगोसोम-लाइसोसोम संलयन को कम करके, टैलाज़ोपेरिब-प्रेरित डी. एन. ए. क्षति को बढ़ाता है। विशेष रूप से, टैलाज़ोपेरिब प्लस रेस्वेराट्रोल संयोजन ने पूर्व-नैदानिक एससीआईडी-माइस मॉडल जीवे में ट्यूमर की मात्रा को प्रभावी ढंग से कम किया। यह काम रेस्वेराट्रोल को नैदानिक सेटिंग्स में एचआर-कुशल स्तन कैंसर के लिए टैलाज़ोपेरिब के साथ एक संभावित कीमो-सुग्राहीकारक के रूप में प्रस्तुत करता है।

Adjuvants for Breast Cancer Therapy

4

Dysregulation of Autophagy by the Combination of Talazoparib Plus Resveratrol Induces Extensive DNA Damage & Synergistic Cancer Cell Death

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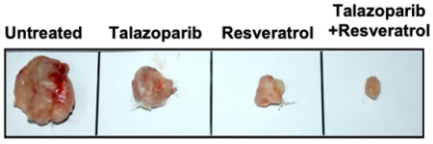

Representative images of tumours excised from SCID mice bearing MCF-7 xenografts post treatment with talazoparib alone, resveratrol alone or their combination

ABSTRACT

The limited efficacy of Poly (ADP-Ribose) polymerase inhibitors (PARP*i*) in homologous recombination (HR) proficient cancers necessitates novel drug combinations with PARP*i*s to expand their utility. Over-expression of *PARP1* in breast cancers irrespective of BRCA1/2 status presents a therapeutic opportunity for effective targetting of PARP*i* with chemo-sensitizers. Autophagy confers *de novo* PARP*i* resistance in breast cancers. In this study, we investigated the role of resveratrol, a natural molecule chemosensitizer in enhancing the effects of talazoparib (BMN673, PARPi) in breast cancers. Resveratrol effectively sensitized talazoparib-induced cell death in HR-proficient breast cancer cells *in vitro*. Resveratrol impaired talazoparib-induced pro-survival autophagic flux, by attenuating autophagosome-lysosome fusion *via* lysosomal-membrane-permeabilization (LMP), enhancing talazoparib-induced DNA damage. Notably, talazoparib *plus* resveratrol combination effectively reduced tumour volume *in vivo* in pre-clinical SCID-mice model. This work presents resveratrol as a potential chemosensitizer with talazoparib for HR-proficient breast cancers in clinical settings.

KEYWORDS: *Autophagy, Resveratrol, PARP inhibitor, Resistance, Talazoparib*

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Introduction

Poly(ADP-Ribose) polymerase (PARP) inhibitors, used predominantly in the treatment of breast, ovarian, prostate and pancreatic cancers with homologous recombination deficiency, work on the principle of synthetic lethality [1]. Notably, this precision therapy has been marred by various intrinsic and acquired resistance mechanisms [2]. Moreover, PARP*i* therapy is by and large inefficient in HR-proficient cancers. Therefore, novel combinatorial approaches involving PARP*i* are warranted to target HR-proficient cancers and PARP*i*-resistant HR-deficient cancers which have recrudescent HR. Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural stilbene found in many food items such as grapes and berries. Resveratrol possesses potent anti-cancer activity and its effects, in the cellular context, are manifested through modulation of multiple pathways including autophagy [3]. Additionally, resveratrol sensitizes the effects of different drugs to better their anti-cancer potential [3]. We have previously reported that talazoparib (Tal), a potent, 3rd generation PARPi induces robust autophagic flux in cells which plays a role in *de novo* resistance to talazoparib in HR proficient breast cancer cells [4]. In the current study, we aimed to understand i) if resveratrol treatment could sensitize the effects of talazoparib in the induction of breast cancer cell death, ii) whether autophagy modulation was involved in this sensitization process.

Materials and Methods

PARP1 expression in breast cancer tissue *vis-à-vis* normal tissue was performed on the TCGA (The Cancer genomic Atlas) breast cancer dataset using Gene Expression Profiling Interactive Analysis (GEPIA) tool. For *in vitro* mechanistic analyses, MCF-7 cells breast adenocarcinoma cell line was used as the model system. Cells were routinely cultured in in Dulbecco's Modified Eagle's medium with 10 % fetal bovine serum and 1 % penicillin-streptomycin solution in a CO_2 incubator (95 % relative humidity; 5 % CO_2; 37 ^{0}C). Cell viability was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Synergy analysis of the cell viability data was carried out using Combenefit software. Cumulative cell death was ascertained by clonogenic assay, and apoptosis was assessed using propidium iodide staining-based sub-G1 analysis by flow cytometry. Autophagy reporter cell lines- GFP-LC3 MCF-7 and tf-LC3 (tandem fluorescence, mRFP-EGFP-LC3)- were generated by lipofectamine-based transfection of plasmids in wild-type MCF-7 cells. These cells were further used for autophagy assessment by confocal microscopy. The level of autophagic proteins and DNA damage markers was assessed by immunoblotting. Analysis of lysosomal integrity and functional homologous recombination using flow cytometry was performed by acridine orange staining and DR-GFP reporter assay, respectively. MCF-7 xenograft SCID mice were utilized for the assessment of anti-cancer efficacy of the combination of agents. Statistical analyses (t-test, ANOVA as applicable) were performed using GraphPad Prism 5.0 software. Values indicated in graphs are Mean ± SD or SEM. Significance in *P* values are as **p*<0.05, ***p*<0.01, ****p*<0.001 compared to respective untreated/vehicle group. Inter-group comparisons are indicated wherever applicable in the graphs.

Results and Discussion

PARP1 is overexpressed in breast cancer patients

PARP1 is a vital protein contributing to a highly diverse set of essential processes in cells including DNA repair, DNA replication, transcription etc [5]. Analysis of the breast cancer patient sample dataset available in TCGA using GEPIA tool (http://gepia.cancer-pku.cn/) showed that PARP1 levels were significantly upregulated in breast cancer irrespective of BRCA1/2 status (Fig. 1A). This indicated a therapeutic opportunity in targeting PARP1 in breast cancers irrespective of HR status.

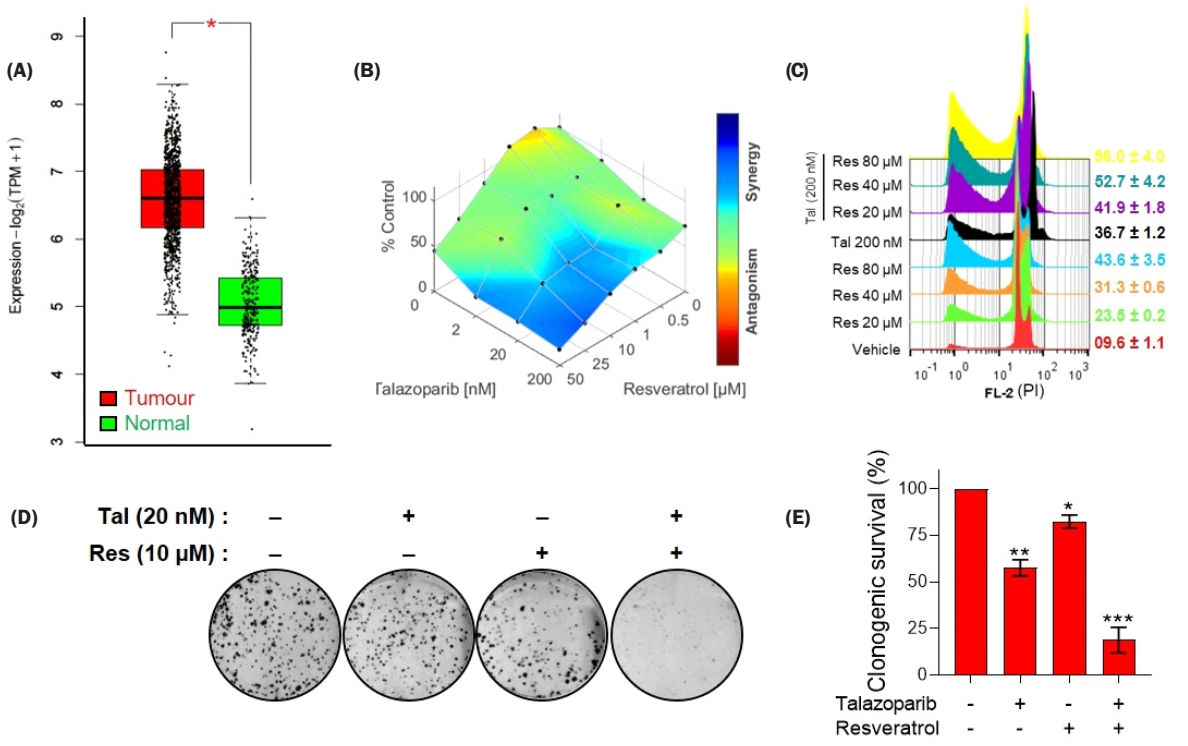

Fig.1: **Resveratrol sensitizes talazoparib induced cell death in MCF-7 cells.** *(A) PARP1 expression in breast cancer and normal patients. (B) Combenefit visualization plot for the MCF-7 cell viability assessment using MTT assay. (C) Assessment of apoptosis in MCF-7 cells by sub-G1 assay by flow cytometry. (D, E) Colony formation assay for the assessment of cumulative breast cancer cell death. (Adapted from Pai Bellare G and Patro BS, 2022[7]).*

Resveratrol sensitizes talazoparib induced cell death in breast cancer cells

Further, we assessed the effect of resveratrol (Res) in sensitizing the effects of talazoparib (Tal) in HR proficient MCF-7 breast cancer cells. MTT-based cell viability assay indicated a concentration dependent effect of individual treatments of talazoparib and resveratrol in MCF-7 cells (Fig. 1B). Interestingly, a synergistic increase was observed in the induction of cell death in MCF-7 cells in the combination of talazoparib *plus* resveratrol at multiple concentrations as indicated in Combenefit analysis. Apoptosis induction at 72 h was found to be greater in the combination of talazoparib and resveratrol compared to individual treatments (Fig. 1C). We also observed that resveratrol effectively sensitized the effects of talazoparib in MCF-7 colony formation assay compared to individual treatments (Fig. 1D, E). Together, the combination of talazoparib and resveratrol synergistically induced breast cancer cell death *in vitro*.

Autophagy is dysregulated in cells treated with the combination of talazoparib and resveratrol

Talazoparib induces pro-survival autophagy leading to *de novo* resistance to talazoparib in HR proficient MCF-7 breast cancer cells [4]. Thus, further, we ventured to assess the status of autophagy under the talazoparib, resveratrol and the combination treatment conditions. Employing EGFP-LC3 reporter transfected MCF-7 cells [6], it was observed that the number of autophagosomes as indicated by the green punctae were increased under talazoparib only, resveratrol and talazoparib *plus* resveratrol treated conditions compared to the control at 48 h, indicating a robust autophagosome formation (Fig. 2A, B).

Further, we employed tf-LC3 reporter MCF-7 cells to understand the status of the autophagic flux in cells [6]. Talazoparib alone treatment showed increased red punctae (RFP^+ and GFP^-, autophagolysosomes) indicative of an effective

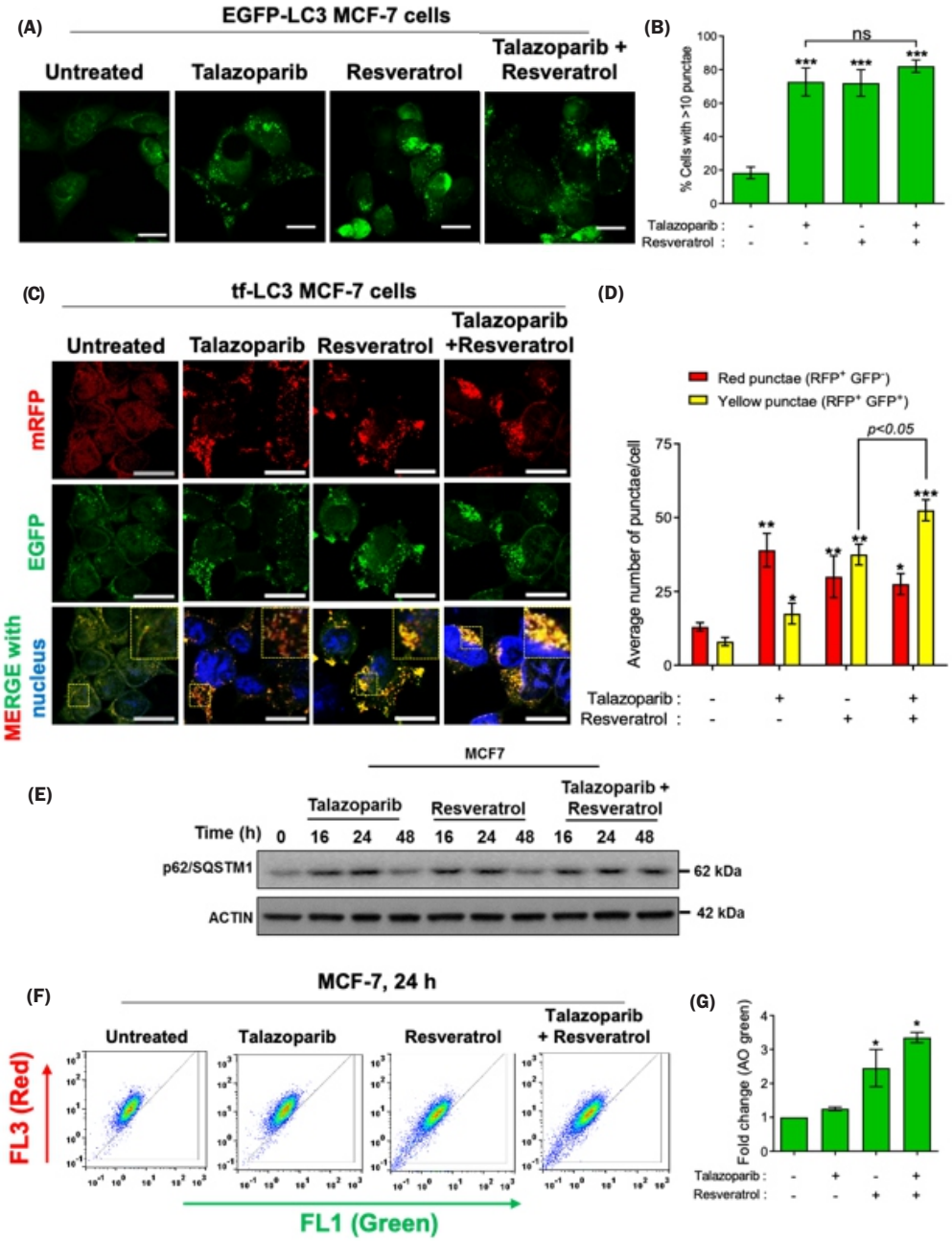

Fig.2: **Resveratrol dysregulates talazoparib induced pro-survival autophagy.** *(A, B) Determination of autophagy induction at 48 h using EGFP-LC3 stably expressing MCF-7 cells by confocal microscopy with quantification. (C, D) Assessment of autophagic flux in MCF-7 cells stably expressing tf-LC3 by confocal microscopy. (E) Immunoblot analysis showing the level of p62 and loading control β-Actin post respective treatment for 24 h. (F, G) Analysis of LMP using acridine orange (10 μM) staining assay by flow cytometry with quantification. Scale bars: 20 μm. (Adapted from Pai Bellare G and Patro BS, 2022 [7]).*

autophagic flux through fusion of autophagosomes with lysosomes at 48 h (Fig. 2C, D). Intriguingly, resveratrol treatment enhanced yellow punctae (RFP^+ and GFP^+; unfused autophagosomes), which was significantly enhanced in response to combination treatment (Fig. 2C, D), indicating the inhibition of fusion of autophagosomes with lysosomes (i.e., defective autophagy flux) in the presence of resveratrol. Similarly, p62 protein (SQSTM1, an autophagic adapter protein), whose levels decrease upon autophagic activation in a time-dependent manner, was found to be stabilized in resveratrol and combination treatment in contrast to talazoparib only treatment (Fig. 2E). Induction of lysosomal membrane permeabilization (LMP) by resveratrol leads to loss of lysosomal integrity. Acridine orange staining based flow cytometry analysis indicated a significant increase in LMP in the combination of talazoparib *plus* resveratrol compared to the talazoparib or resveratrol alone treatments at 24 h (Fig. 2F, G). This may have led to the observed inhibition or delay of the fusion of autophagosome with the lysosomes (i.e. late-stage autophagy inhibition) [8,9]. Taken together, talazoparib induced pro-survival autophagy in MCF-7 cells was significantly hampered by resveratrol treatment which may have led to the increased cell death observed in the combination treatment conditions.

Co-treatment of talazoparib and resveratrol induces extensive DNA damage and suppresses homologous recombination

Autophagy supports effective repair of DNA damage via faithful homologous recombination pathway [10]. Late stage autophagy inhibition by resveratrol under resveratrol alone and combination treatment could potentially lead to extensive DNA damage. In agreement with this, DNA damage induced by talazoparib, assessed by γ-H2AX(S139) level, was greatly enhanced by resveratrol in a concentration dependent manner at 24 h (Fig. 3A). Interestingly, resveratrol induced γ-H2AX(S139) level was further enhanced in the presence of talazoparib. Further, DR-GFP reporter based functional assay for HR showed that HR was marginally increased in talazoparib treated conditions and decreased under resveratrol treatment. Notably, HR was significantly downregulated in the combination of talazoparib and resveratrol in concordance with the inhibition of autophagy in response to resveratrol treatment (Fig. 3B, C).

Talazoparib plus resveratrol reduces tumour burden in pre-clinical models of breast cancer

The efficacy of the resveratrol mediated sensitization of PARP inhibitor effects were further evalauated in MCF-7 xenograft SCID mice model (Fig. 4A). The individual treatments of talazoparib (1 mg/kg body weight) and resveratrol (50 mg/kg body weight) reduced the tumour volume. However, the reduction in tumour volume was significantly greater upon treatment with 15 doses of the combination of talazoparib *plus* resveratrol in comparison to the individual treatments (Fig. 4A-C) with no observable side-effects. Together, the data suggested high efficacy of the combination treatment of talazoparib *plus* resveratrol over individual treatments in pre-clinical model.

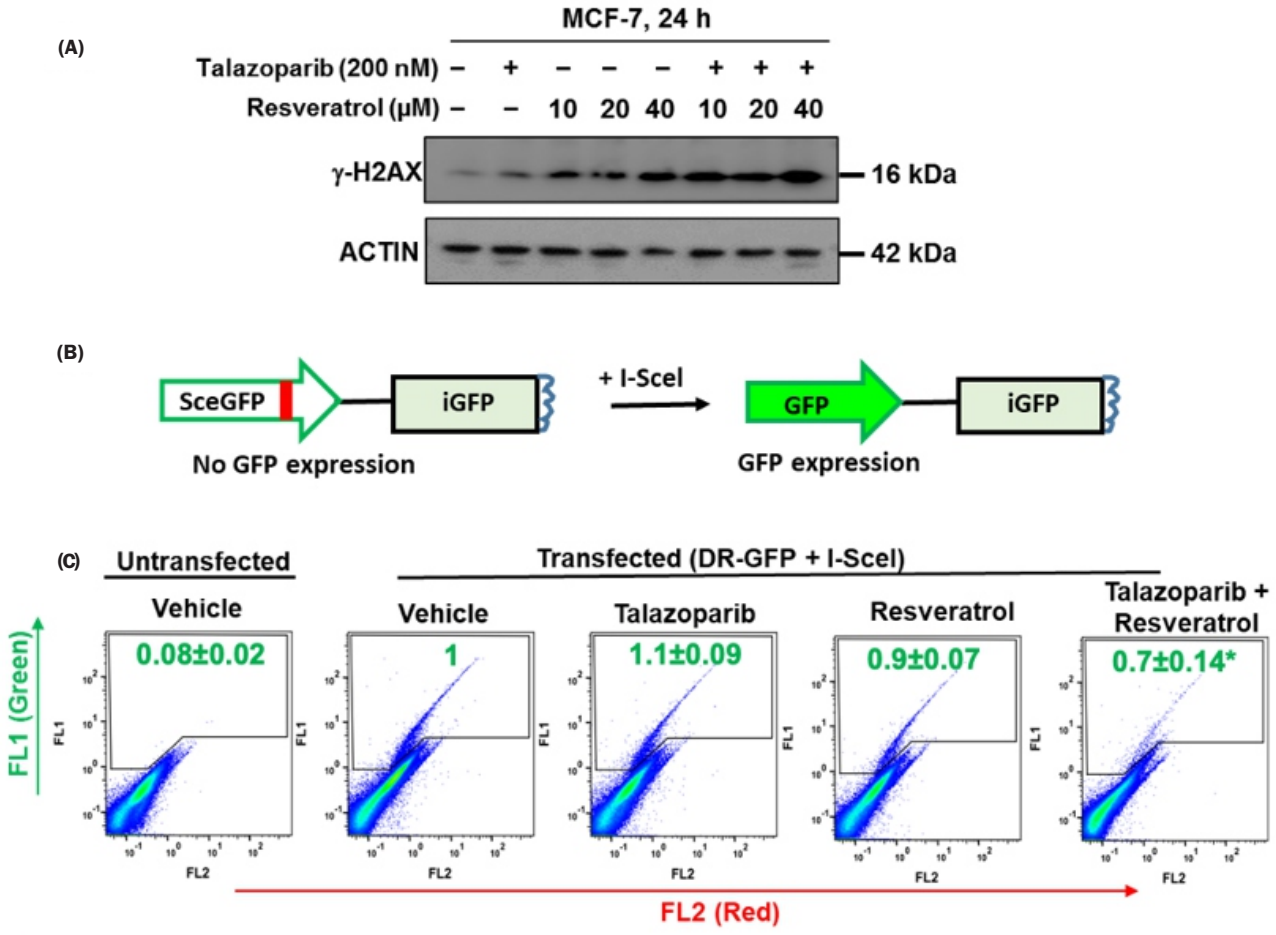

Fig.3: **Combination of talazoparib and resveratrol induces extensive DNA damage and suppresses homologous recombination.** *(A) Immunoblot analysis showing the level of DNA damage marker γ-H2AX with loading control β-Actin post indicated treatments for 24 h. (B) Scheme for the DR-GFP HR reporter assay. (C) Assessment of functional HR in the cells using DR-GFP HR reporter assay by flow cytometry. (Adapted from Pai Bellare G and Patro BS, 2022 [7]).*

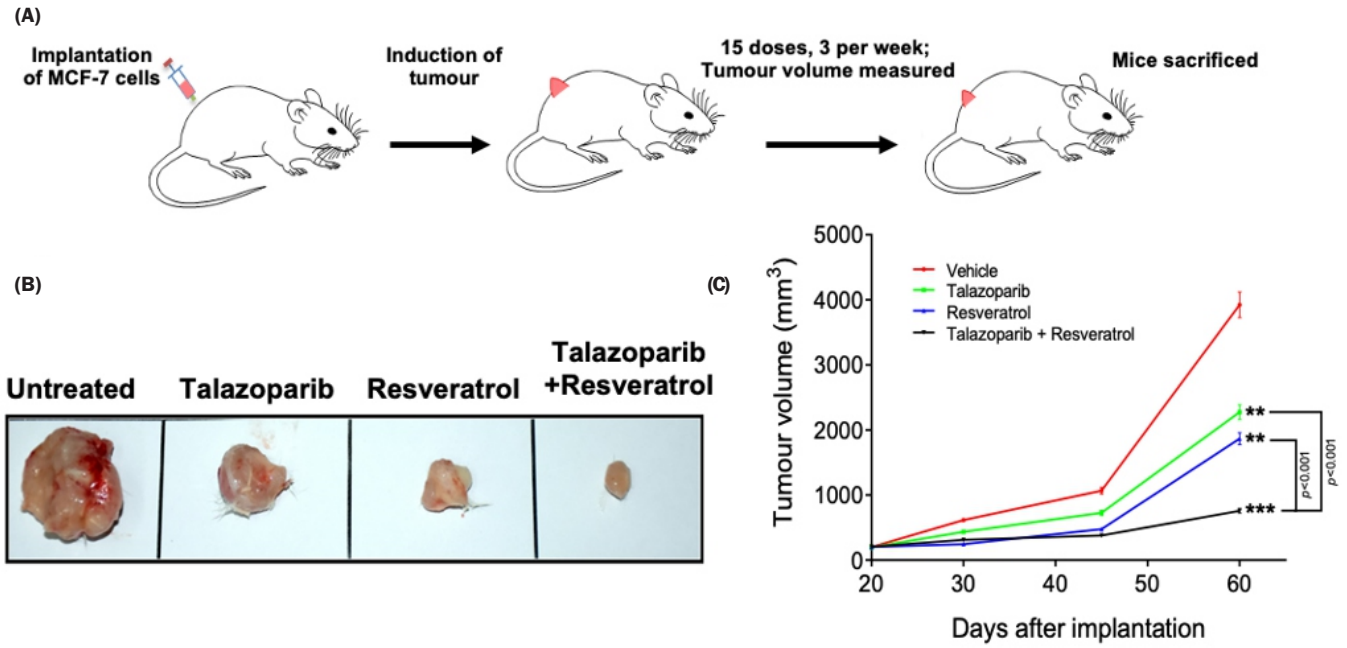

Fig.4: **Resveratrol is effective in sensitizing tumour xenografts in SCID mice to talazoparib treatment.** *(A) Scheme of the SCID mice experiment. (B, C) Representative images of the tumours excised from SCID mice bearing MCF-7 xenografts post treatment with 15 doses of talazoparib alone (1 mg/kg), resveratrol alone (50 mg/kg) or their combination. Quantification of tumour volume is indicated in C. (Adapted from Pai Bellare G and Patro BS, 2022 [7]).*

Conclusion

This work provides compelling evidence that the combination of talazoparib and resveratrol is effective in mitigating breast cancer *in vitro* and *in vivo*. Mechanistically, inhibition of late-stage autophagy i.e. fusion of autophagosomes with the lysosomes due to LMP induction by resveratrol in combination with talazoparib leads to extensive DNA damage induction and eventually, induction of breast cancer cell death. This combination can have potential implications in the treatment of homologous recombination proficient and PARPi resistant homologous recombination deficient breast cancers in the clinical setting.

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