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Comparative Analysis of CDK4/6 Inhibitors (Ribociclib and Palbociclib) Combined with Enzalutamide in Triple-Negative Breast Cancer Cells

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ABSTRACT
Triple-negative breast cancer (TNBC) is recognized as a challenging subtype due to its poor prognosis. Recent molecular profiling studies have unveiled a significant subset expressing the androgen receptor (AR) subset which may respond to AR-blocking agents, offering a potentially effective treatment strategy. This study aims to investigate the potential synergistic cytotoxic and apoptotic effects of the AR antagonist enzalutamide (ENZA) in combination with CDK4/6 inhibitors palbociclib (PB) or ribociclib (RB) and compare the effectiveness of these combinations in TNBC cells. Results revealed that ENZA in combination with PB or RB induced synergistic cytotoxic cytotoxicity in all tested TNBC cell lines. While synergistic cytotoxic combinations of ENZA with PB did not induce apoptosis in any TNBC cell line, ENZA+RB combinations exhibited a synergistic apoptotic effect. This study suggests the ENZA+RB combination may be more favorable due to its apoptosis-inducing effect. However, these data need to be further supported by detailed in vivo and clinical studies.

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Introduction

Breast cancer stands as the most prevalent form of cancer among women globally, with one million new cases diagnosed annually [1]. Constituting 10-20% of all breast cancers, triple-negative breast cancer (TNBC) is identified by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) expression TNBC, known for its aggressive clinical behavior, bleak prognosis, and lack of targeted therapy, has become a focal point for researchers [3]. Recent molecular profiling studies have exposed significant molecular diversity within TNBC, revealing a subset that expresses the androgen receptor (AR), identified in 70–90% of breast cancer cases [4]. We now understand that this receptor plays a crucial role in the pathology and development of breast cancer [5]. This finding suggests that TNBC patients with a positive AR [AR(+)] subset may respond to AR-blocking agents, offering a potentially effective treatment strategy.

Enzalutamide (ED), an approved androgen receptor antagonist for prostate cancer, has shown potent anti-cancer effects in preclinical studies on TNBC cells, supporting the idea that AR inhibition is a promising target for TNBC [6]. Currently, clinical trials are in progress to explore the efficacy of neoadjuvant enzalutamide, both in combination with and without chemotherapy, for patients with TNBC. Palbociclib (PB) and Ribociclib (RB) stand out as highly selective inhibitors of cyclin-dependent kinase 4 and 6 (CDK4/6). They function by impeding the phosphorylation of the retinoblastoma (Rb) protein, subsequently arresting the cell cycle at the G1 phase. [7]. Hence, targeting CDK4/6 emerges as a crucial therapeutic approach for breast cancer owing to its pivotal role in the cell cycle and the proven efficacy of inhibitors in BC cases [8]. Additionally, the activation of androgen receptors (AR) contributes to enhanced cell survival by regulating the cell cycle; androgen deprivation induces G1 arrest [9]. It's worth mentioning that the expression of androgen receptor-dependent genes reaches its peak during the G1 phase and gradually decreases throughout the cell. These inhibitors present an effective therapeutic approach against breast cancer, where CDK4/6 activity is often dysregulated [10]. Literature indicates that ENZA enhances the cytostatic effect induced by PB and RB in AR-

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positive/RB-competent TNBC cells, suggesting that the combination of enzalutamide and CDK4/6 inhibitors may be a therapeutic strategy for AR(+)/RB-competent TNBC [11], [12].
The objective of this study is to investigate the potential synergistic effects of enzalutamide in combination with PB or RB and determine which combination is more effective in TNBC cells. The synergistic cytotoxic and apoptotic effects of these combinations were compared in AR-positive (MDA-MB-453 and BT-549), and AR-negative (MDA-MB-231 and MDA-MB-468) TNBC cell lines.

Material and Methods

Cell lines and cell culture

The cell lines utilized in this study were sourced from the Ege University Tülay Aktaş Oncology Laboratory cell line stock. Breast cancer cells were cultivated in RPMI 1640 medium containing with 10% (v/v) FBS and 2 mM L-glutamine. To prevent microbial contamination, penicillin-streptomycin solution (1%) was added to the medium. Cells were cultured in a 37°C incubator with 5% CO_2. During the study, cells were cryopreserved in liquid nitrogen for further analysis.

Preparation of drugs

A concentrated solution of ENZA was formulated with a concentration of 20 mg/mL by dissolving 40 mg of ENZA in 2 mL of dimethyl sulfoxide (DMSO). PB (PD0332991) and RB (LEE011) were procured from Sigma. To prepare a 5 mM stock solution, 5 mg of PB was dissolved in 1.7 mL of DMSO. 5 mg RB was dissolved in 2 mL of DMSO to obtain a 5 mM solution.

Assessment of cell viability via MTT assay

MTT (2,5-diphenyl-2H-tetrazolium bromide) assay was employed for the analysis of cell viability. For this purpose, breast cancer cells were seeded in 96-well culture plates (10.000 cells/well) and treated with drugs alone or in combination for 24, 48, and 72 hours. After adding 10 µl of MTT solution to each well, plates were kept at 37°C in a CO_2 incubator for 4 h. After the incubation period, cells were drained and 200 µL DMSO was added and mixed. Cell viability percentages were calculated based on the optical density determined with a multimode plate reader at 490 nm wavelength.
The IC_{50} values are calculated using cell viability percentages via GraphPad software. The Combination Index (CI) is the synergistic, additive, or antagonistic effects of drug combinations in pharmacology. CI values were calculated via CalcuSyn software. The interpretation of Combination Index (CI) values is as follows: CI < 0.1: very strong synergism, 0.1-0.3: strong synergism, 0.3-0.7: synergism, 0.7-0.85: moderate synergism, CI = 1: additivity, and CI > 1: antagonism [13].

DNA fragmentation analysis

Apoptotic cells after treatment with drugs and drug combinations were determined using a cell death detection ELISA kit (Merck). 100 µl of coating solution is pipetted into each well, covered, and incubated overnight at +4°C. After thorough removal of the coating solution, 200 µl of incubation buffer is added to all samples and kept at +25°C for 30 min. The solution is then removed, and wells are rinsed three times with 300 µl washing solution per well. Then, sample solution (100 µl) is pipetted into each well, and for background determination, 100 µl of incubation buffer is added to two wells. The microplate is covered and incubated for 90 min. at RT. After removing the solution and rinsing the wells, 100 µl of Conjugate solution is added, except for the blank position. Following a 90-minute incubation and subsequent rinsing, 100 µl of substrate solution is pipetted into each well. The microplate is then incubated on a shaker for 10 min. Well contents are homogenized, and measurements are taken at 405 nm for a substrate solution blank, or at 490 nm as the reference wavelength.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software (La Jolla, CA, USA). The data were assessed using a one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. Values with $p < 0.05$ were considered statistically significant.

Results and Discussion

Cytotoxic effects of ENZA, PB, and RB on human TNBC cell lines

ENZA was administered to breast cancer cells at concentrations of 1, 5, 10, 20, 40, and 80 µM throughout 24, 48, and 72 hours. PB and RB were administered at concentrations of 10, 25, 50, 75, and 100 µM. All tested drugs showed concentration and time-dependent cytotoxic activity against all tested breast cancer cells (Figures 1, 2, and 3). IC_{25} and IC_{50} values of drugs were calculated from viability plots at 72 hours and presented in Table 1.

ENZA reached its peak effectiveness at 72 hours in all breast cancer cells (Figure 1). ENZA was most effective in the MDA-MB-231 cell line with an IC_{50} value of 69.5 ± 0.8 µM, whereas it demonstrated the least efficacy in the MDA-MB-468 cell line with an IC_{50} value of 82.0 ± 2.1 µM.

MDA-MB-231 is a TNBC cell line that is proficient in RB but lacks AR expression. The effectiveness of enzalutamide in AR-negative MDA-MB-231 cells suggests that its anti-cancer properties may involve mechanisms beyond its primary action on AR. These alternative mechanisms could include targeting other receptors or signaling pathways that are involved in cancer cell proliferation, survival, or metastasis. Additionally, ENZA might induce cellular changes or alterations in gene expression profiles that lead to the inhibition of tumor growth or the induction of cancer cell death, irrespective of AR status. Further research is needed to fully elucidate the precise molecular mechanisms underlying efficacy of ENZA in AR-negative breast cancer cells.

Table 1 Calculated IC_{25} and IC_{50} values of Enzalutamide (ENZA), Palbociclib (PB), and Ribociclib (RB) on human TNBC cell lines at 72 h

	AR(+) TNBC cell lines				**AR(-) TNBC cell lines**			
	RB-negative		**RB-proficient**		**RB-negative**		**RB-proficient**	
	BT-549		**MDA-MB-453**		**MDA-MB-468**		**MDA-MB-231**	
	IC_{25}	**IC_{50}**	**IC_{25}**	**IC_{50}**	**IC_{25}**	**IC_{50}**	**IC_{25}**	**IC_{50}**
ENZA	35.4 ± 0.4	78.2 ± 0.2	32.8 ± 1.4	72.0 ± 0.4	42.4 ± 0.5	82.0 ± 2.1	30.4 ± 1.2	69.5 ± 0.8
PB	31.5 ± 1.2	78.0 ± 0.8	38.9 ± 0.2	82.0 ± 1.4	32.4 ± 1.1	78.0 ± 1.0	28.7 ± 0.4	71.0 ± 1.4
RB	24.8 ± 0.6	58.0 ± 1.2	21.7 ± 2.4	49.0 ± 0.6	29.8 ± 3.2	72.0 ± 3.6	28.2 ± 0.8	68.0 ± 2.7

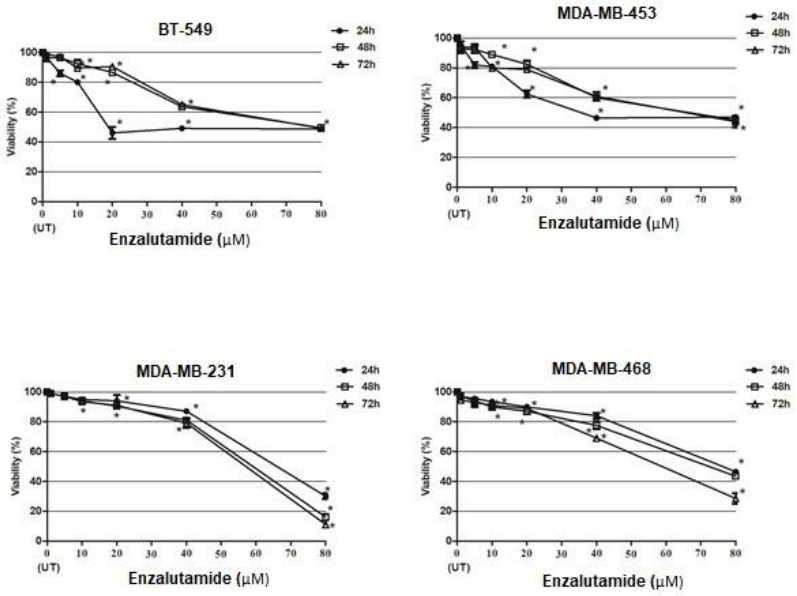

Fig 1 Effect of increasing concentrations of Enzalutamide on TNBC cells at 24, 48 and 72 h ($p<0.05$)

In the literature, the effect of ENZA was tested on TNBC cell lines and results revealed a dose- and time-dependent cytotoxicity in all breast cancer cells. IC_{50} values of ENZA were between 25 and 60 µM for tested breast cancer cells [11]. In another study, ENZA was tested on 11 different TNBC cell lines and the IC_{50} values ranged from 4 µM to >50 µM [14]. The reason for the significant variation in IC_{50} values of ENZA across different breast cancer cell lines may be because these cell lines exhibit different phenotypic characteristics from each other.

PB was also tested on both AR(+) and AR(-) TNBC cell lines and showed dose- and time-dependent cytotoxic activity against all TNBC cell lines (Figure 2). PB was most effective in the AR(-)MDA-MB-231 cell line with an IC_{50} value of 71.0 ± 1.4 µM, whereas it demonstrated the least efficacy in the AR(+)MDA-MB-453 cell line with an IC_{50} value of 82.0 ± 1.4 µM.

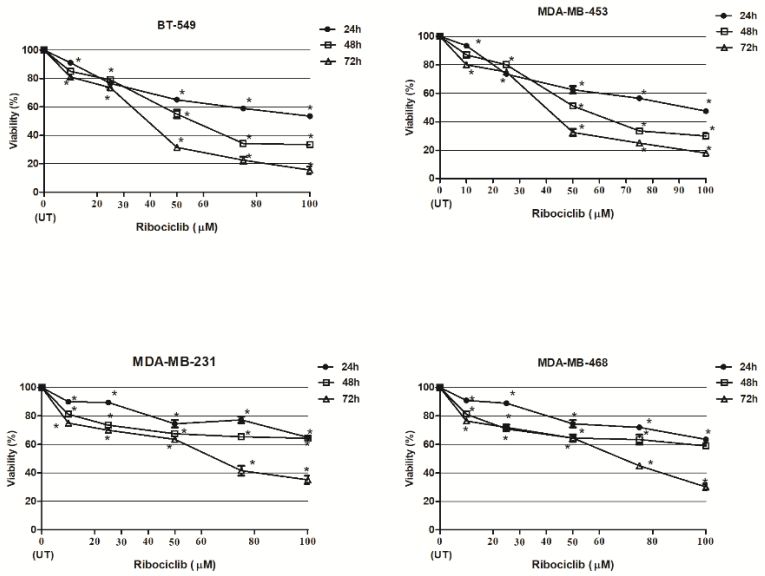

Fig 2 Effect of increasing concentrations of Palbociclib on TNBC cells at 24, 48, and 72 h ($p<0.05$)

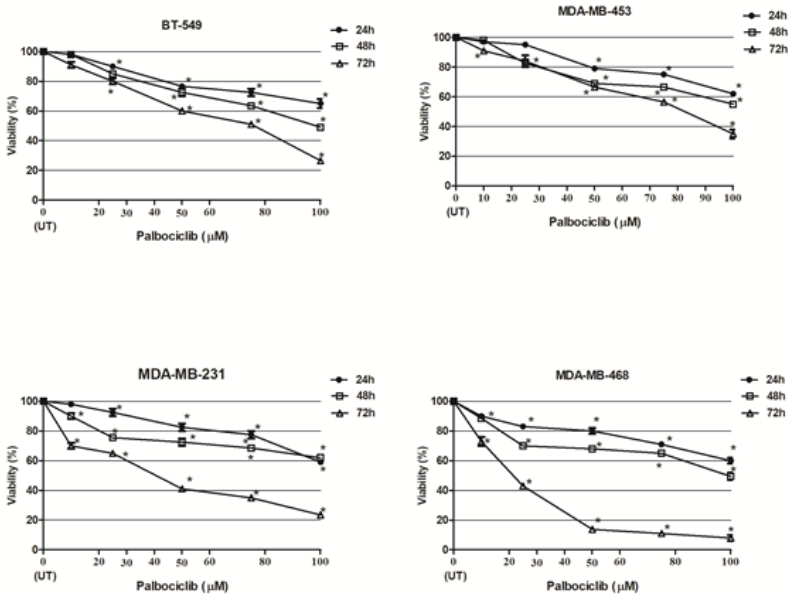

Fig 3 Effect of increasing concentrations of Palbociclib on TNBC cells at 24, 48, and 72 h ($p<0.05$)

RB reached its peak effectiveness at 72 hours in all breast cancer cells (Figure 3). RB was most effective in AR(+)MDA-MB-453 the cell line with an IC_{50} value of 49.0 ± 0.6 μM, whereas it demonstrated the least efficacy in the AR(-) MDA-MB-468 cell line with an IC_{50} value of 72.0 ± 3.6 μM. In the literature, it has been demonstrated in various studies that both PB and RB exhibit dose- and time-dependent cytotoxic effects in breast cancer cell lines [11], [12]. However, a study revealed that palbociclib markedly impeded cell growth in RB-proficient cells (MDA-MB-453 and MDA-MB-231), while exhibiting no significant impact on RB-negative cells (MDA-MB-468) [12].

Synergistic cytotoxic combinations of ENZA with PB or RB

After determining the individual cytotoxic effects of the drugs and calculating their IC_{50} values, the potential synergistic effects of ENZA in combination with PB or RB were investigated in TNBC cells. Various combinations were prepared with varying concentrations of drugs and applied to TNBC cell lines for a duration of 72 h.

CI value for the combination of 80 uM ENZA and 25 uM PB was calculated as 0.105 in BT-549 cells, indicating strong synergism and thus considered a highly synergistic cytotoxic combination (Table 2). In MDA-MB-231 cells, The CI value for the combination of 80 uM ENZA and 25 uM PB was calculated as 0.102 and considered strong synergism, whereas the CI value was 0.325 for the combination of 80 uM ENZA and

50 uM PB and considered synergistic cytotoxic (Table 2). In MDA-MB-453 cells, 80 uM ENZA and 25 uM PB resulted in synergistic cytotoxicity at 72 h (CI value: 0.310) (Table 2). In MDA-MB-468 cells, The CI value for the combination of 80 uM ENZA and 25 uM PB was calculated as 0.126 and considered strong synergism, whereas the CI value was 0.343 for the combination of 80 uM ENZA and 50 uM PB and considered synergistic cytotoxic (Table 2). In previous studies, it was shown that the combination of ENZA with PB amplifies the cytostatic effect in AR-positive/RB-proficient TNBC cells [12]. As shown in Table 2, ENZA in combination with PB induced synergistic cytotoxic effects in all tested TNBC cell lines.

The combination of 80 uM ENZA and 25 uM RB in BT-549 cells was interpreted as strong synergism with a CI value of 0.119 (Table 2). In MDA-MB-231 cells, the CI value for the combination of 80 uM ENZA and 25 uM RB was calculated as 0.100 and considered strong synergistic cytotoxic, whereas the CI value was 0.352 for the combination of 80 uM ENZA and 50 uM RB and considered synergistic cytotoxic (Table 2). In MDA-MB-453 cells, the CI value for the combination of 80 uM ENZA and 25 uM RB was calculated as 0.110 and considered strong synergism, whereas the CI value was 0.302 for the combination of 80 uM ENZA and 50 uM RB and considered synergism (Table 2). In MDA-MB-468 cells, The CI value for the combination of 80 uM ENZA and 25 uM RB was calculated as 0.143 and considered strong synergism, whereas the CI value was 0.326 for the combination of 80 uM ENZA and 50 uM RB and considered synergistic cytotoxic (Table 2). In a study by Choupani et al., the cytotoxic effect of ENZA in combination with RB was investigated in AR- and AR+ TNBC cells, and all tested combinations reduced clonogenic proliferation and cell viability in both TNBC cells [11]. Understanding of the complex interaction between AR and CDK4/6 signaling pathways may pave the way for new therapeutic approaches in the treatment of TNBC, so the underlying molecular mechanisms of these combinations should be studied in detail.

Table 2 Combination index (CI) values of ENZA in combination with RB or PB in TNBC cells. CI < 0.1 indicates very strong synergism, 0.1- 0.3 indicates strong synergism, 0.3-0.7 indicates synergism, 0.7-0.85 moderate synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism. (*values indicating synergistic cytotoxic combinations) [13].

ENZA (µM)	PB (µM)	BT-549	MDA-MB-231	MDA-MB-453	MDA-MB-468
5	25	0.105*	0.924	0.933	1.102
5	50	0.912	0.855	0.978	1.025
80	25	0.836	0.102*	0.310*	0.126*
80	50	0.887	0.325*	0.899	0.343*
ENZA (µM)	**RB (µM)**				
5	25	0.981	1.247	0.958	1.240
5	50	0.945	1.025	0.896	1.139
80	25	0.119*	0.100*	0.110*	0.143*
80	50	1.056	0.352*	0.302*	0.326*

Detection of apoptosis in synergistic cytotoxic combinations

The measurement of DNA fragmentations was performed to determine whether combinations identified as synergistically cytotoxic induce apoptotic cell death in TNBC cells. While previous studies in the literature have demonstrated that PB does not induce apoptosis in TNBC cells [12], we investigated apoptotic cell death at synergistic concentrations obtained in our study. No apoptotic cell death was induced with the application of the ENZA+PB combination in any TNBC cells tested, parallel to the findings in the literature (Figure 4). However, despite previous findings indicating the synergistic effectiveness of combinations of ENZA with RB, the synergistic apoptotic effect has not been investigated. The androgen receptor (AR) influences the transcriptional activity of genes related to evading apoptosis and promoting cellular proliferation. Therefore, AR signaling leads to tumor growth [9].

Previously it was demonstrated that RB triggers apoptosis in the TNBC cell line MDA-MB-231 [15]. Similarly, there are studies indicating that ENZA also induces apoptosis in cancer cells [16], [17]. Based on this information, here we investigated for the first time the apoptotic effects of synergistic cytotoxic combinations of ENZA with RB in TNBC cells. Results revealed that ENZA in combination with RB resulted in synergistic apoptotic effect in all tested TNBC cells at 72 h (Figure 5).

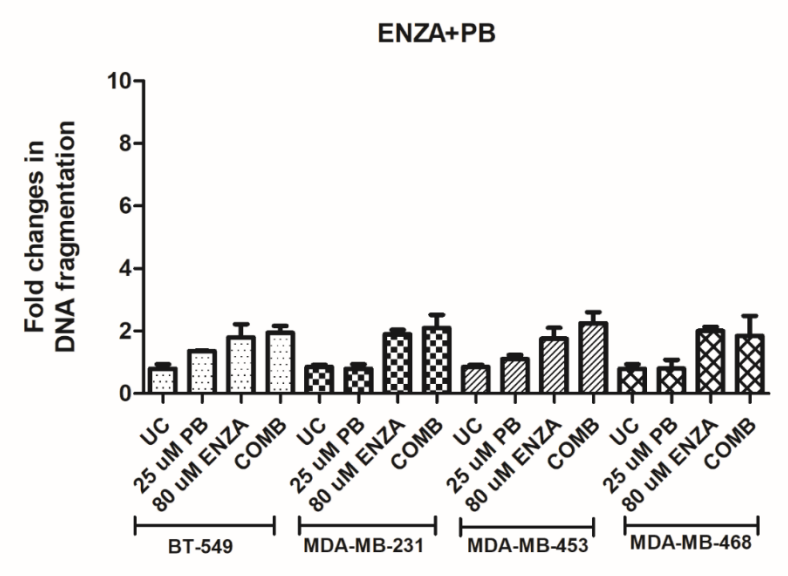

Fig 4 Evaluation of apoptosis in TNBC cell lines after treatment with 25 µM PB and 80 µM ENZA synergistic cytotoxic combination at 72 h. The combination treatment of ENZA and PB does not lead to apoptosis in TNBC cells ($p>0.05$) (UC: untreated control)

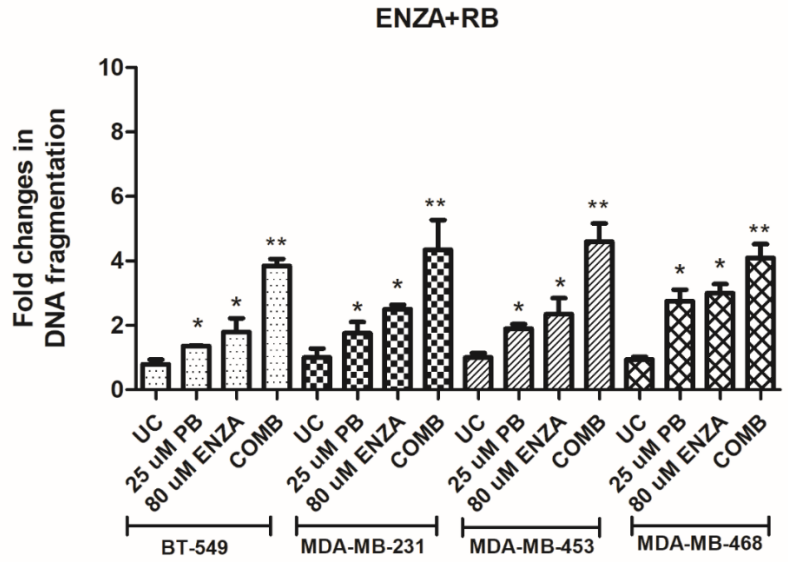

Fig 5 Evaluation of apoptosis in TNBC cell lines after treatment with 25 µM RB and 80 µM ENZA synergistic cytotoxic combination at 72 h. ENZA+RB combination treatment resulted in apoptosis in all tested TNBC cells at 72 h ($p<0.05$* as compared to untreated control (UC), $p<0.05$** as compared to single cells alone)

Conclusion

In conclusion the c,ombined application of ENZA with either PB or RB resulted in synergistic cytotoxicity across all tested TNBC cell lines. While synergistic cytotoxic combinations of ENZA with PB did not trigger apoptosis in any TNBC cell line, combinations of ENZA with RB demonstrated a synergistic apoptotic effect. This research implies that the ENZA+RB combination could be more advantageous owing to its apoptosis-inducing impact. However, these findings necessitate further support through comprehensive *in vitro* studies, including western blot analysis of target proteins. Additionally, additional *in vivo* and clinical studies are warranted to corroborate these findings. The present investigation has certain constraints; primarily, only four TNBC cell lines were employed, and there could exist additional protein profiles or mutants that play a role in the varying impacts of PB, RB, ENZA, and their combination in these particular cell lines.

Abbreviations

AR: Androgen receptor; CI: Combination index; DMSO: Dimethyl sulfoxide; ENZA: Enzalutamide; ER: Estrogen receptor; FBS: Fetal bovine serum; HER-2: Human epidermal growth factor receptor 2; IC50: Half-maximal inhibitory concentration; TNBC: Triple-negative breast cancer, PB: Palbociclib; PR: Progesterone receptor; RB: Ribociclib; Rb: Retinoblastoma;

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Availability of data and material
Please contact the corresponding author for any data request.

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