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RESEARCH

Novel combination treatment of CDK 4/6 inhibitors with PARP inhibitors in triple negative breast cancer cells

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Abstract

Cyclin-dependent kinase 4/6 (CDK4/6) inhibitors provide promising results for treating hormone receptor-positive breast cancer. However, the efficacy of CDK4/6 inhibitors remains uncertain in triple negative breast cancer (TNBC) patients with particularly carrying RB-deficient tumors. Poly-(ADP-ribose) polymerase (PARP) inhibitors offer a therapeutic strategy for the treatment of *BRCA*-mutated TNBC patients. However, the acquired drug resistance, changes in the cell cycle regulation, and DNA damage repair have demonstrated the necessity for developing new combination strategies. This preclinical study assessed a combinatory treatment of the CDK4/6 inhibitor abemaciclib with PARP inhibitors talazoparib (TAL) in HCC1937 *BRCA*-mutated RB-deficient TNBC cells and TAL-resistant HCC1937-R cells through WST-1 analysis, annexin V, cell cycle, acridine orange/propidium iodide staining, RT-PCR, and apoptosis array. Our findings revealed that abemaciclib and TAL combination synergistically suppressed the growth of TNBC cells and overcame TAL resistance through G0/G1 arrest and the activity of both intrinsic and extrinsic apoptotic pathways. These preliminary results suggest that the combination of abemaciclib and TAL could expand the use of these inhibitors in *BRCA* mutated and RB deficient TNBC patients and potentially overcomes PARP inhibitors resistance.

Keywords Abemaciclib · Talazoparib · Triple-negative breast cancer · Apoptosis · Resistance

Introduction

Cyclin-dependent kinase 4/6 (CDK4/6) inhibitors are potent inhibitors in the treatment of cancer due to suppressing the phosphorylation of retinoblastoma (RB) in G1/S transition. In different types of cancer, the amplification and aberrant activation of the CDK4/6-cyclin D1-RB-E2F pathway have been observed. Therefore, selective CDK4/6 inhibitors (palbociclib, abemaciclib, and ribociclib) have been approved by FDA for the treatment of estrogen receptor (ER)-positive, human epidermal growth factor receptor 2 (HER2)-negative advanced or metastatic breast cancer in combination with endocrine therapy (Choo and Lee 2018; Murphy 2019). However, recent studies have focused on the therapeutic efficacy of CDK4/6 inhibitors in triple negative breast cancer (TNBC) which is the most aggressive subtype of breast cancer with limited treatment options (Matutino et al 2018; Hu et al 2021; Saleh et al 2021; Wang et al 2021). Actually, the inhibition of CDK4/6 is not potential targeted therapy for the treatment of TNBC patients due to the loss of RB and overexpression of cyclin E (Lanceta et al 2021). However, TNBC cells are sensitive to CDK4/6 inhibitors in preclinical studies (O'Brien et al 2018; Li et al 2019). Additionally, multiple ongoing clinical trials have evaluated the effectiveness of CDK4/6 inhibitors alone or in combination with other targeted therapies in TNBC patients (Lanceta et al 2021). Our previous study reveals that abemaciclib potentially inhibits the growth of MDA-MB-231 RB-proficient TNBC cells through apoptosis (Ozman et al 2021). On the other hand, Fassl et al. (2020) state that most of TNBC cells and especially MDA-MB-468 and HCC1937 cells carrying inactivating mutations of the *RB1* gene are resistant to CDK4/6 inhibitors especially palbociclib through enhanced

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lysosomal biogenesis. In the study of Lanceta et al. (2021), alterations of lipid metabolism in MDA-MB-231 cells lead to acquired palbociclib resistance. Additionally, Collins et al. (2021) note that CDK4/6 inhibitors may be worse in germline *BRCA* mutant metastatic breast cancer patients than patients with germline *BRCA* wild type and unknown germline *BRCA* status. In this context, there is controversial results for the effectiveness of CDK4/6 inhibitors in TNBC cells. Therefore, identifying new drug–drug interactions and combining treatment strategies will be crucial for the further improvement of targeted treatment modality and the use of CDK4/6 inhibitors in TNBC.

Poly (ADP-ribose) polymerase (PARP) inhibitors have been the most promising therapeutic strategy leading to impaired DNA damage repair in *BRCA1/2* mutant cancer types and are approved by the FDA for the treatment of breast and ovarian cancer patients with mutations of the *BRCA1/2* genes (Cortesi et al 2021; Vanacker et al 2021). Among them, olaparib and talazoparib (TAL) as targeted therapy have been successfully used to treat patients with *BRCA* mutant advanced breast cancer patients (Guney Eskiler et al 2018; McCann and Hurvitz 2021; Singh et al 2021). However, resistance to PARP inhibitors through the restoration of homologous recombination repair, DNA end resection, reversion mutations, protection of DNA replication fork, RAD51-single strand DNA filament and D-loop formation, epigenetic modification, and pharmacological alteration restricts the success of therapy response (Li et al 2020a, b; Singh et al 2021). Thus, new combination treatment strategies are needed to broaden the use of PARP inhibitors to overcome resistance in the treatment of TNBC.

In the literature, some recent studies have demonstrated the synergistic effects of CDK4/6 and PARP inhibitors on RB-proficient and deficient breast cancer as well as bladder cancer cells (Li et al 2020a, b; Klein et al 2021; Zhu et al 2021). In general, CDK4/6 inhibitors are ineffective in RB-deficient cancer cells (Fassl et al 2020; Zhu et al 2021). However, the combination of PARP inhibitors with CDK4/6 inhibitors exerts potential therapeutic effects on RB-deficient cells and could play a crucial role in CDK4/6 responsiveness in breast cancer cells (Li et al 2020a, b; Zhu et al 2021). Especially, the combination of CDK4/6 inhibitors (palbociclib) and PARP inhibitors (niraparib) in RB-deficient TNBC cells increases DNA damage through ROS ((Li et al 2020a, b). Thus, this combination could expand the use of CDK4/6 inhibitors and PARP inhibitors for the treatment of *BRCA* mutated and RB deficient TNBC patients.

In this context, we, for the first time, assessed the combination of abemaciclib as CDK4/6 inhibitor and TAL as PARP inhibitor in *BRCA1* mutated RB deficient HCC1937 TNBC cells to reveal the therapeutic potential of CDK4/6 inhibitors in the sensitivity of PARP inhibitors. Furthermore, we evaluated the reversal of TAL resistance upon administration of abemaciclib in TAL resistant HCC1937 TNBC cells.

Materials and methods

Cell lines

HCC1937 and HCC1937-R cells were cultured from early passages in RPMI (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Gibco), 1% Pen/Strep (Gibco), at 5% CO_2 in a humidified incubator. Stock solutions of TAL (Selleck Chemicals LLC, Houston, TX, USA) and abemaciclib (Biovision, San Francisco, CA, USA) were prepared in DMSO and diluted with fresh medium. HCC1937-R (TAL resistant) cells were generated by 0.1 nM TAL exposure for 12 months in our previous study (Guney Eskiler et al 2021) and the IC_{50} value of HCC1937-R cells was nearly eight times increased that of the parent cells.

Cell viability

A total of 5000–20,000 cells per well were seeded in 96-well plates and treated with abemaciclib for 24 and 48 h or TAL for 7 days. The concentrations of abemaciclib or TAL were determined in our previous studies (Guney Eskiler et al 2021; Guney Eskiler and Ozturk 2022). To assess the combination treatment, the cells were firstly pre-treated with TAL for 7 days and then incubated abemaciclib and TAL for further 24 and 48 h. After the defined incubation, 100 µl of WST-1 dye (Biovision, San Francisco, CA, USA) was added to the cells and incubated for 45 min in the dark. Finally, the optical density at 450 nm was measured with a microplate reader (Allsheng). The most effective abemaciclib and TAL combination concentrations were selected for further experiments and compared with abemaciclib alone treatment.

Apoptosis analysis

Cells ($1–3 \times 10^5$/well) were seeded in 6-well plates and treated with abemaciclib (1 µM) alone or pretreated with TAL (0.1 and 1 nM) alone for 7 days and then combined with abemaciclib (1 µM) for further 24 h. Following treatment with the defined duration, the cells were washed and stained with Annexin V & Dead Cell Assay kit (Luminex Corporation, Austin, TX, USA) in the dark and the percentage of apoptotic cells was measured using Muse Cell Analyzer (Millipore, Germany).

Cell cycle analysis

The cells were seeded in 6-well plates and treated with abemaciclib alone or combination with TAL. After treatment

with the defined incubation time, the cells were harvested and fixed in pre-chilled 70% ethanol. The fixed cells were then collected and stained with Muse™ Cell Cycle Kit (Luminex Corporation) in the dark for 30 min and subjected to Muse Cell Analyzer (Millipore, Germany).

Acridine orange (AO)/propidium iodide (PI) staining

A total of $1–5 \times 10^5$/well cells were seeded in 6-well plates and incubated with abemaciclib alone or combination with TAL at the indicated times. Afterward, the cells were fixed with 4% paraformaldehyde (PFA) for 30 min and stained with AO (100 mg/mL) and PI solution (1 mg/mL) for 30 min. After washing with PBS, the cells were captured with EVOS FL Cell Imaging System (Thermo Fisher Scientific Inc.).

Acidic vesicular organelles (AVO) staining

Cells ($1–5 \times 10^5$/well) were seeded in 6-well plates and treated with abemaciclib (1 µM) alone for 12 and 24 h or pretreated with TAL (0.1 and 1 nM) alone for 7 days and then combined with abemaciclib (1 µM) for further 12 and 24 h. Following incubation, the cells were stained with AO (1 µg/ml) for 15 min at 37 °C. Afterward, the cells were washed with PBS and the formation of AVO was observed using EVOS FL Cell Imaging System (Thermo Fisher Scientific Inc.).

RT-PCR

Total RNA was obtained from cells using E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA). The quantified RNA concentration by Qubit 4.0, cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). *CCDN1* (*cyclin D1*) and *RB1* expression levels were analyzed in triplicate on a Step One Plus Real- Time PCR (Applied Biosystems, Foster City, CA). *ACTB* was used as an internal control. The $2^{-\Delta\Delta CT}$ method was used for normalization to *ACTB* levels.

Apoptosis array

To simultaneously assess the expression of multiple proteins associated with apoptosis, Human Apoptosis Array (43 targets) (RayBiotech Life) was performed according to the manufacturer protocol. After treatment with the most effective abemaciclib and TAL combination, protein isolation was conducted and an equal amount of total protein was loaded per array. Finally, membranes were incubated with detection buffers C and D (kit components) and visualized by ECL detection kit (Biovision) in a chemiluminescent system (Syngene, USA).

Statistical analysis

Data were analyzed by GraphPad Prism 8 software and $p < 0.05$ was accepted as statistical significance. Data were presented as the mean ± SD. The significance of differences between groups was assessed by one-way ANOVA post-Tukey analysis. The Chou–Talalay method was conducted to determine the combination index (Chou 2006).

Results

The synergistic effects of abemaciclib and talazoparib combination on TNBC cells

To assess the potential anti-cancer activity of abemaciclib and talazoparib combination, WST-1 analysis was conducted. As shown in Fig. 1, abemaciclib alone significantly inhibited HCC1937 cells viability at concentrations ≥ 1.5 µM for 24 h (1 µM: 70.8 ± 1.2% and 1.5 µM: 51.9 ± 1.7%) ($p < 0.05$). Furthermore, abemaciclib and TAL combination synergistically suppressed both HCC1937 and HCC1937-R viabilities with the greatest effect at a concentration of 1 µM abemaciclib and 1 nM TAL for 24 h. The synergism between abemaciclib and TAL was summarized in Tables 1 and 2. According to CI values, 1 µM abemaciclib + 0.1 nM TAL and 1 µM abemaciclib + 1 nM TAL combinations for 24 h were accepted as the most effective combinations in both TAL sensitive and resistant TNBC cells. In our previous results, 0.1 and 1 nM TAL alone reduced the viability of HCC1937 cells to 92.2 ± 3.0% and 76.5 ± 2.0%, respectively for 7 days (Guney Eskiler et al. 2021).The combination of 1 µM abemaciclib + 0.1 nM TAL and 1 µM abemaciclib + 1 nM TAL considerably decreased the viability of HCC1937 cells to 55.5 ± 0.7% and 45.9 ± 1.7%, respectively compared with abemaciclib alone (70.5 ± 1.1%) ($p < 0.01$). Furthermore, 26.5 ± 2.5% and 33.7 ± 3.1% reduction were observed at 1 µM abemaciclib + 0.1 nM TAL and 1 µM abemaciclib + 1 nM TAL combination, respectively in the growth of HCC1937-R cell. On the other hand, abemaciclib treatment did not decrease the viability of TAL resistant HCC1937-R cells for 24 h (1 µM: 100.4 ± 2.9% and 1.5 µM: 100.6 ± 2.2%). The cytotoxic effects of TAL alone on these cells did not further analyzed in this study due to our previous findings. TAL alone did not induce any toxicity in TAL resistant cells due to acquired resistance (Guney Eskiler et al. 2021). Therefore, abemaciclib and TAL combination more significantly suppressed the proliferation of HCC1937 cells ($p < 0.01$) than abemaciclib or TAL alone treatment and could overcome TAL resistance at a certain degree.

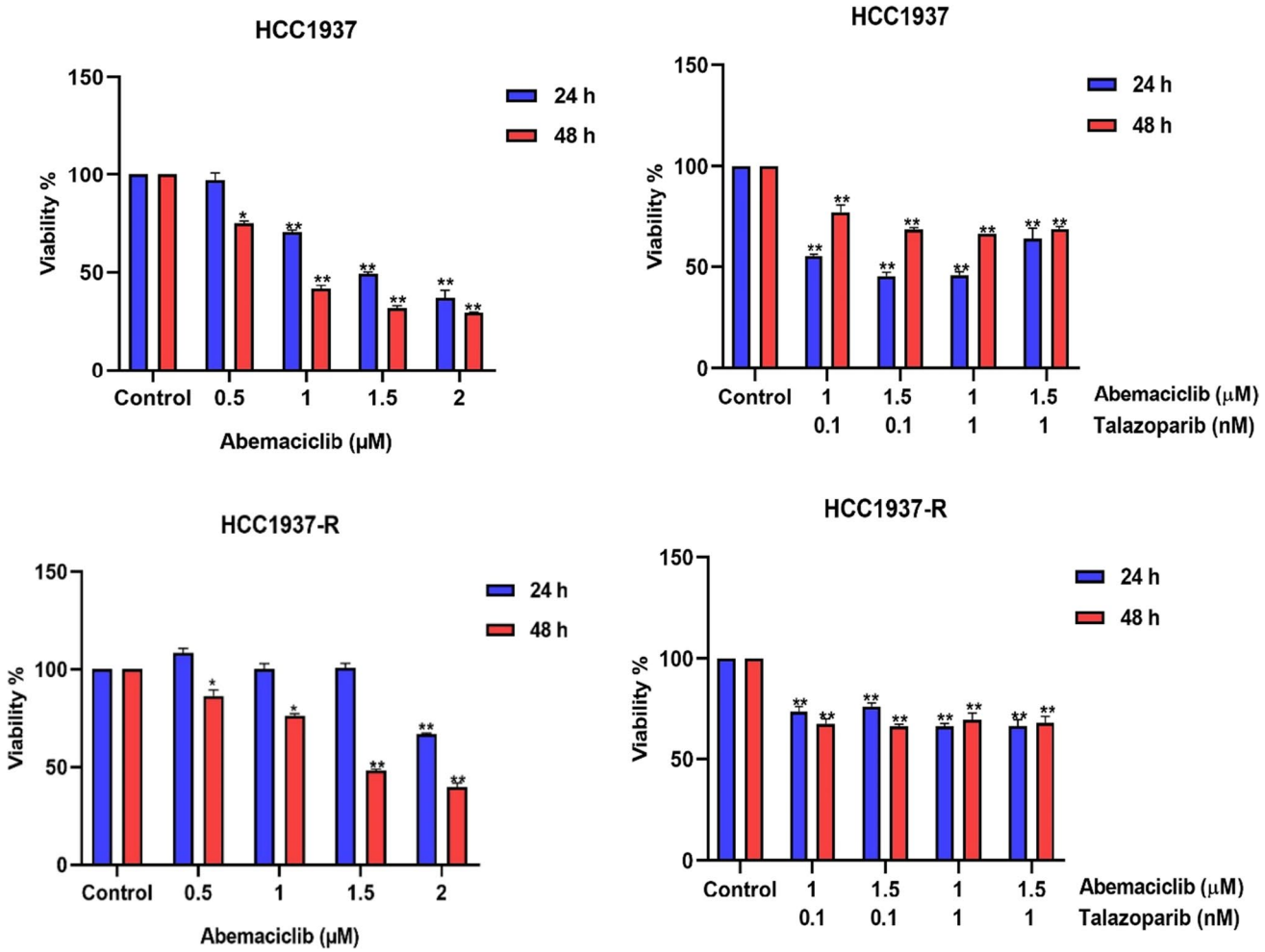


Fig. 1 Synergistic effects of abemaciclib and TAL on the growth of TNBC cells were determined by WST-1 analysis. The HCC1937 and HCC1937 cells were pre-treated with TAL for 7 days and further incubated with simulatenously abemaciclib and TAL for further 24 and 48 h compared with abemaciclib alone ($p < 0.05$*, $p < 0.01$**)

Table 1 CI values of abemaciclib (1 and 1.5 μM) and TAL (0.1 and 1 nM) combination in TNBC cells

	HCC1937		HCC1937-R	
TAL	0.1 nM	1 nM	0.1 nM	1 nM
CI at 1 μM abemaciclib	0.76	0.75	0.44	0.42
CI at 1.5 μM abemaciclib	0.92	1.56	0.61	0.70

CI, combination index; $CI < 1$, synergism; $CI = 1$, additive effect; $CI > 1$, antagonism

The apoptotic effects of abemaciclib and TAL combination in TNBC cells

To assess abemaciclib and TAL combination mediated cell death, Annexin V and AO/PI staining were performed. As shown in Fig. 2A, 1 μM abemaciclib and 1 nM TAL combination considerably caused apoptosis in both cells. Compared with abemaciclib alone ($17.9 \pm 0.5\%$ and $6.5 \pm 0.3\%$), this combination significantly increased total apoptotic rate to $59.2 \pm 0.8\%$ and $21.2 \pm 1.2\%$ in HCC1937 and HCC1937-R cells, respectively ($p < 0.01$). Furthermore, co-treatment of abemaciclib and TAL led to the nuclear blebbing formation and chromatin condensation in these cells compared with abemaciclib alone (Fig. 2B). However, abemaciclib induced lysosomal biomass in these cells and this effect was less observed in HCC1937 cells than HCC1937-R cells following combination with TAL. To verify the association between autophagy and abemaciclib treatment, AVO staining by AO was performed in two different incubation time. Our results demonstrated that abemaciclib did not induce autophagy related cell death in both cells due to green fluorescence dots (Fig. 3).

Table 2 DRI values of abemaciclib (1 and 1.5 μM) and TAL (0.1 and 1 nM) combination in TNBC cells

TAL	HCC1937				HCC1937-R			
	0.1 nM		1 nM		0.1 nM		1 nM	
	ABE	TAL	ABE	TAL	ABE	TAL	ABE	TAL
1 μM ABE	1.35	49.12	1.57	8.68	2.29	1253.8	2.43	215.4
1.5 μM ABE	1.09	102.37	0.80	3.18	1.66	2520.2	1.45	82.8

DRI, dose reduction index; *DRI*: < 1, not favorable dose reduction; *DRI* = 1, no-dose reduction; *DRI* > 1, favorable dose reduction

The effects of abemaciclib and TAL combination on cell cycle arrest in TNBC cells

Cell cycle analysis and the mRNA levels of *cyclin D1* and *RB1* were conducted for the identification of the cell cycle distribution in these cells (Fig. 4). Compared with abemaciclib alone treatment, co-treatment of abemaciclib and TAL significantly induced G0/G1 phase arrest in both TNBC cells ($p < 0.05$). The combination of abemaciclib and 1 nM TAL caused a significant increase in the accumulation of cells in G0/G1 phase (78.4 ± 0.4% and 63.9 ± 0.5%) in HCC1937 and HCC1937-R cells, respectively unlike abemaciclib alone (58.3 ± 0.5% and 57.6 ± 0.5%, respectively) a ($p < 0.01$, Fig. 4A, B). Additionally, abemaciclib and TAL significantly inhibited the expression levels of *cyclin D1* and *RB1* in HCC1937 cells due to RB-deficiency ($p < 0.01$, Fig. 4C). However, this combination increased the mRNA level of *RB1* with the downregulation of *cyclin D1* in HCC1937-R cells. Therefore, the effectiveness of abemaciclib and TAL combination differentially affected cell cycle regulation in terms of TAL sensitivity or resistance. Additionally, abemaciclib and TAL combination were more induced apoptosis than abemaciclib alone in HCC1937 and HCC1937-R cells.

The molecular mechanism of abemaciclib and TAL combination mediated apoptosis in TNBC cells at protein level

We further analyzed the levels of multiple proteins in both intrinsic and extrinsic apoptosis pathways. Our findings showed that apoptosis-associated protein expression levels were different between HCC1937 and HCC1937-R cells upon administration of 1 μM abemaciclib and 1 nM TAL combination as summarized in Fig. 5. In HCC1937 cells, pro-apoptotic proteins Bax (1.8-fold), Bid (1.5-fold), Bim (2.0-fold), Cytochrome c (1.2-fold), Caspase-3 (5.3-fold), HTRA2 (1.3-fold) as well as p21 (1.9-fold), p27 (1.3-fold), and p53 (1.6-fold) were upregulated. On the other hand, anti-apoptotic Bcl-2 (1.3-fold), Bcl-w (2.0-fold), HSP27 (1.3-fold), HSP60 (1.2-fold), HSP70 (1.8-fold), and Livin (1.2-fold) were increased after abemaciclib and TAL combination treatment despite of the downregulation of cIAP-2 (0.9-fold), Survivin (0.9-fold), XIAP (0.6-fold), and TNFα/β. Furthermore, the expression levels of pro-apoptotic Bad (2.7-fold), Bax (2.4-fold), Bid (2.1-fold), Bim (1.6-fold), Caspase-3 (1.7-fold), Caspase-8, (1.7-fold), Cytochrome c (2.8-fold), and HTRA2 (2.1-fold) proteins were also more upregulated in HCC1937-R cells. However, abemaciclib and TAL combination induced the overexpression of anti-apoptotic Bcl-2 (2.1-fold), Bcl-w (2.3-fold), cIAP-2 (1.5-fold), XIAP (2.1-fold), Livin (2.8-fold), Survivin (1.3-fold), HSP27 (2.0-fold), HSP60 (1.8-fold), HSP70 (1.8-fold), and TNFα/β proteins upon combination treatment despite of increased p21 (4.0-fold), p27 (2.5-fold), and p53 (2.0-fold) protein levels. Taken together, abemaciclib and TAL combination induced cell apoptosis through both intrinsic and extrinsic apoptotic pathways in both TNBC cells. However, we detected an increased level of anti-apoptotic proteins in HCC1937-R cells due to TAL resistance.

Discussion

Herein, we first provided evidence that the combination of abemaciclib with TAL exerted a synergistic anti-cancer effect on HCC1937 *BRCA1*-mutated RB deficient TNBC cells and induced apoptotic cell death through activating mitochondrial apoptotic pathway. Notably, abemaciblib and TAL combination could potentially overcome TAL resistance at a certain degree. In this context, CDK4/6 and PARP inhibitors combination could expand the patient population that can not benefit from PARP or CDK4/6 inhibitors alone in patients carrying BRCA and RB defects.

Recent studies have drawn significant attention for identifying molecular predictive factors to determine the sensitivity of TNBC to CDK4/6 inhibitors. Androgen receptor expression, the activation of Myc, MEK and Akt signaling pathway, *RB* expression status, *TP53* mutations, *CCNE1* amplification, and CDK2 and other cyclin dependent kinases activation play a crucial role in the response of CDK4/6 inhibitors in preclinical and clinical studies (Wang et al 2021). Therefore, combining CDK4/6 inhibitors with different targeted drugs could provide a promising treatment strategy for the treatment of TNBC (Saleh et al 2021). In Yamamoto et al. (2019) study, palbociclib

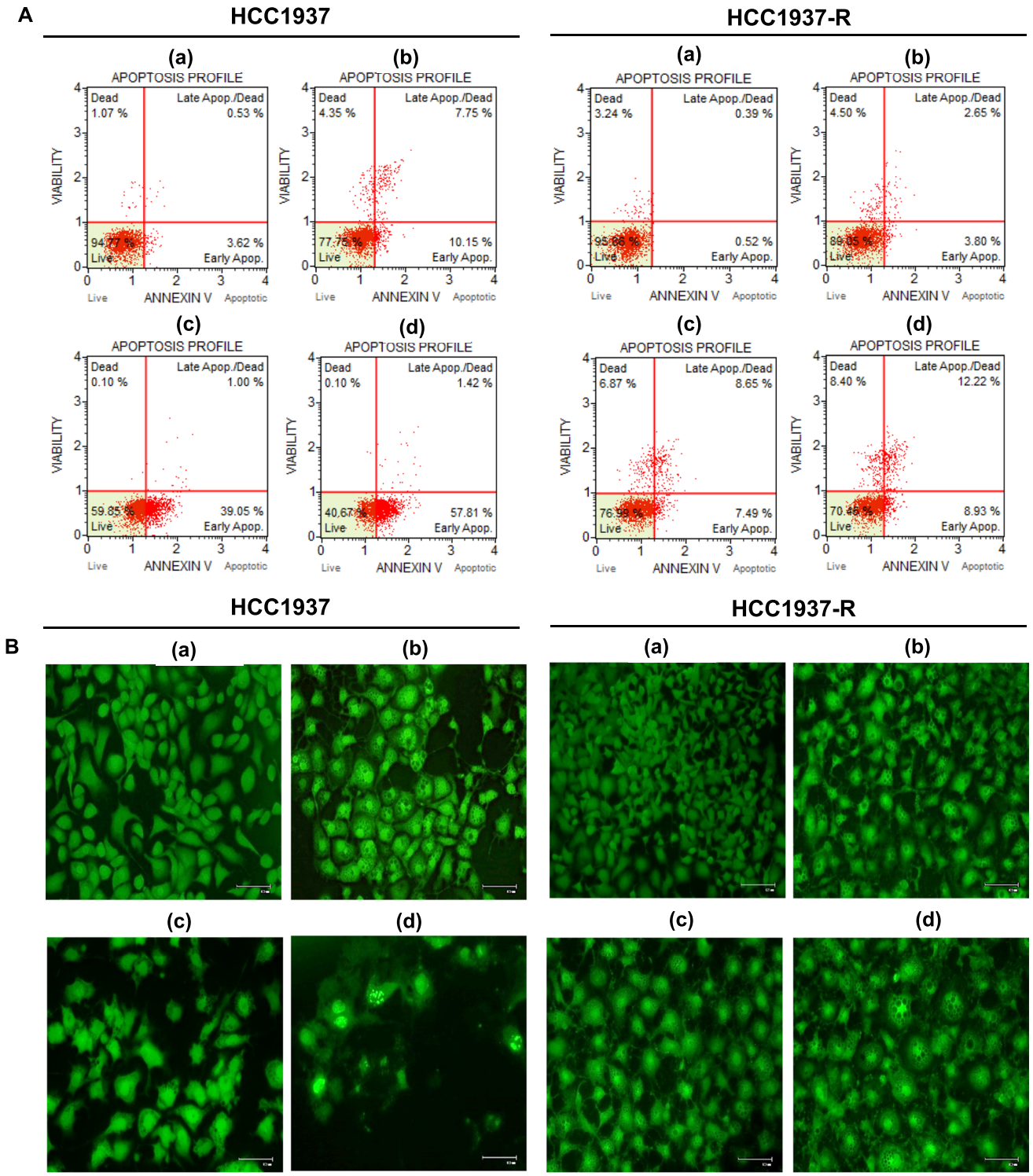


Fig. 2 The combined apoptotic effects of abemaciclib and TAL on TNBC cells were measured by (**A**) Annexin V assay and (**B**) AO/PI staining. (**a**) Control, (**b**) 1 μM abemaciclib, (**c**) 1 μM abemaciclib + 0.1 nM TAL, and (**d**) 1 μM abemaciclib + 1 nM TAL

and MLN0128 mTOR kinase inhibitor exert synergistic anti-cancer activity in both pRb + TNBC cell lines and PDX model (Yamamoto et al. 2019). Huang et al. (2020) state that pre-treatment palbociclib could enhance the efficacy of cisplatin on MDA-MB-231 TNBC in vitro and in vivo (Huang et al 2020) supported by Cretella et al. (2019) study. In this study, the efficacy of paclitaxel could be improved by a pre-treatment with palbociclib in TNBC

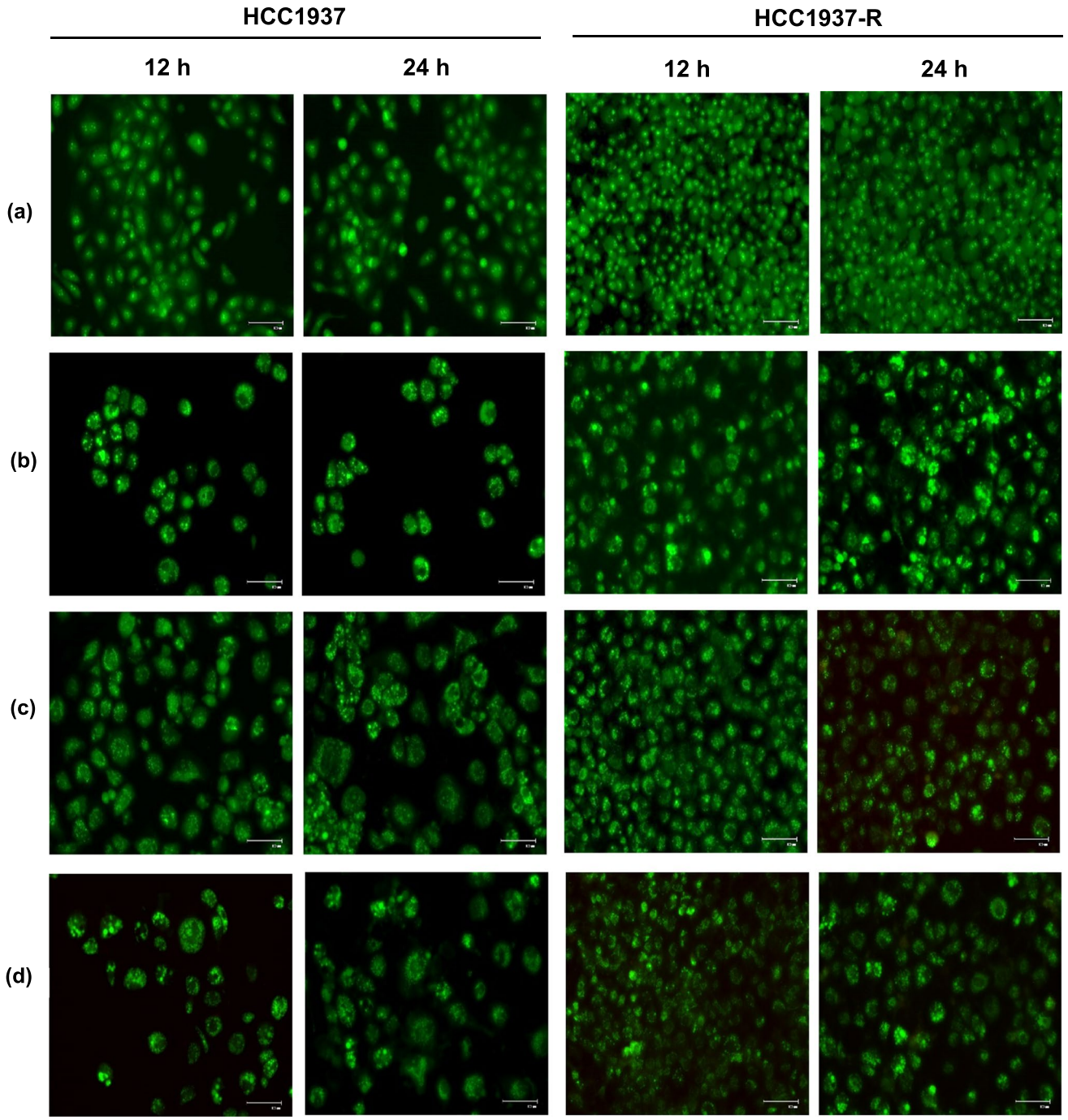
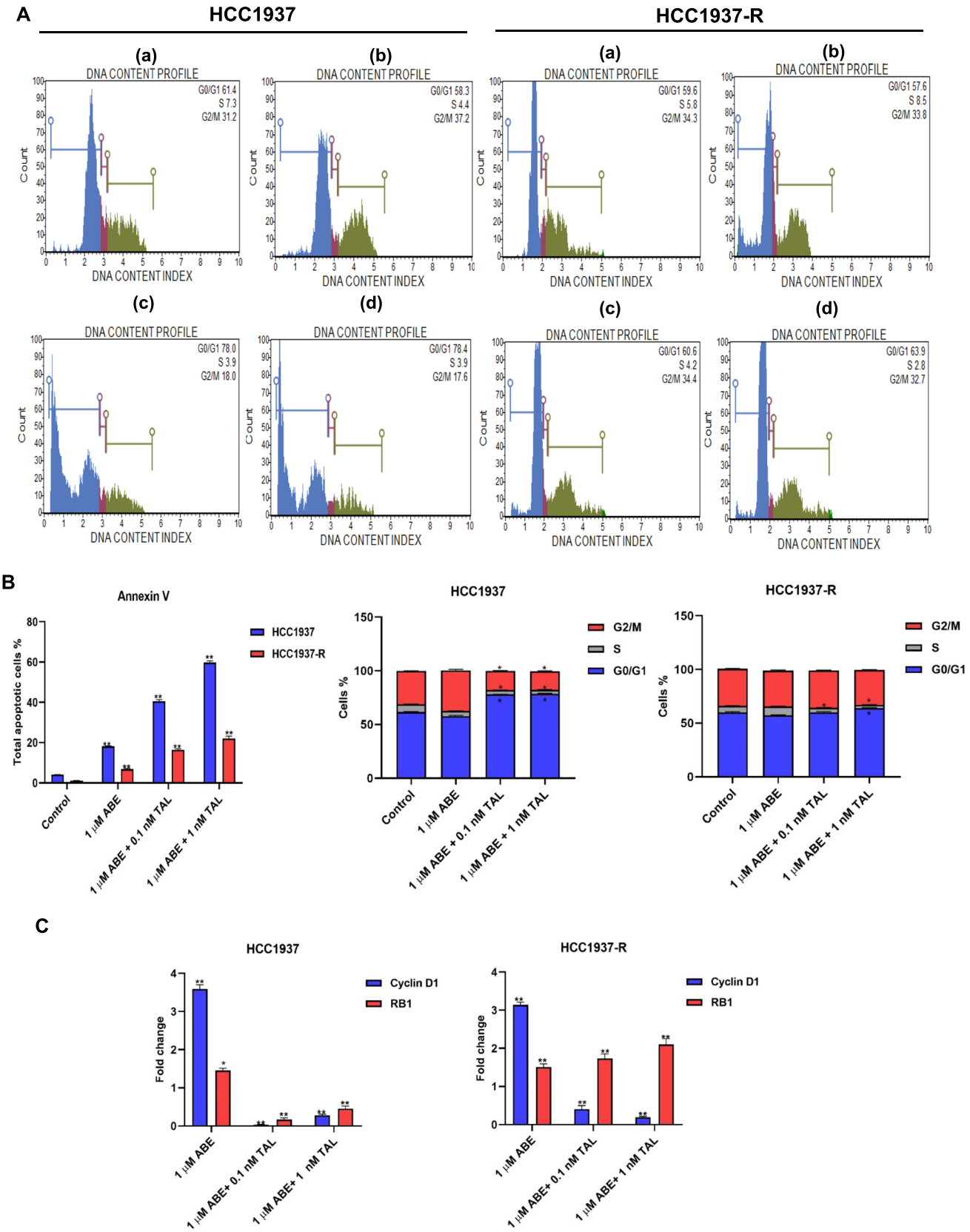


Fig. 3 The combined effects of abemaciclib and TAL on the formation of AVO by AO staining in TNBC cells for 12 and 24 h. **a** Control, **b** 1 μM abemaciclib, **c** 1 μM abemaciclib + 0.1 nM TAL, and **d** 1 μM abemaciclib + 1 nM TAL

cells (Cretella et al 2019). Furthermore, niraparib or olaparib and palbociclib combination show potentially synergistic effects on RB-deficient TNBC cells (MDA-MB-468, MDA-MB436, BT549, and HCC1937) in a ROS-dependent manner (Li et al. 2020a, b). These findings are supported by Zhu et al. (2021) study. In this study, olaparib and palbociclib combination exhibit the synergistic effects on *BRCA* mutant TNBCs cells (olaparib-sensitive (MDA-MB-436) and olaparib-resistant *BRCA*mut/TNBC cell lines (HCC1937and SUM149)) (Zhu et al 2021). In this context, we, for the first time, demonstrated that the combination of abemaciclib and TAL exhibited a strong synergism in both *BRCA* mutant and *RB* deficient HCC1937 TNBC cells and improved TAL sensitivity.


A
HCC1937
HCC1937-R
(a)
(b)
(c)
(d)
DNA CONTENT PROFILE
DNA CONTENT INDEX
Count
G0/G1 61.4
S 7.3
G2/M 31.2
G0/G1 58.3
S 4.4
G2/M 37.2
G0/G1 59.6
S 5.8
G2/M 34.3
G0/G1 57.6
S 8.5
G2/M 33.8
G0/G1 78.0
S 3.9
G2/M 18.0
G0/G1 78.4
S 3.9
G2/M 17.6
G0/G1 60.6
S 4.2
G2/M 34.4
G0/G1 63.9
S 2.8
G2/M 32.7
B
Annexin V
Total apoptotic cells %
HCC1937
HCC1937-R
Control
1 μM ABE
1 μM ABE + 0.1 nM TAL
1 μM ABE + 1 nM TAL
Cells %
G2/M
S
G0/G1
C
Fold change
Cyclin D1
RB1
1 μM ABE+ 0.1 nM TAL
1 μM ABE+ 1 nM TAL

◂**Fig. 4** The synergistic effects of abemaciclib and TAL combination on the regulation of cell cycle. (**A**) Cell cycle distribution of TNBC cells. (**a**) Control, (**b**) 1 μM abemaciclib, (**c**) 1 μM abemaciclib + 0.1 nM TAL, and (**d**) 1 μM abemaciclib + 1 nM TAL. (**B**) Statistical analysis of the percentage of total apoptosis and G0/G1, S, and G2/M phase in TNBC cells. (**C**) The mRNA levels of *Cyclin D1* and *RB1* were measured by RT-PCR analysis in TNBC cells. ($p < 0.05$*, $p < 0.01$**)

The dysfunction of RB is observed in nearly 30% of TNBC cases and the loss of RB occurs in 7–20% of TNBC patients and leads to CDK4/6 inhibitors resistance (Wang et al 2021). In the study of Patel et al. (2020), 51% of 180 TNBC tissues are Rb positive expression and there is an association between AR expression, lower histologic grade, a lack of a germline *BRCA* mutation, and bone metastases and RB expression. In the literature, the combination of olaparib or niraparib and palbociclib exerts synergistic effects on RB deficient TNBC cells (Li et al 2020a, b; Zhu et al 2021). However, MDA-MB-468 and HCC1937 cells with inactivating mutations in the *RB1* gene show a lack of palbociclib response in Fassl et al. (2020). In our study, higher concentrations (1, 1.5, and 2 μM) of abemaciclib significantly suppressed the growth of HCC1937 cells, unlike HCC1937-R cells. Additionally, co-treatment with abemaciclib and talazoparib enhanced the efficacy of abemaciclib and TAL alone and reversed TAL resistance.

Furthermore, increased mRNA level of *RB1* was detected in particularly HCC1937-R cells. Therefore, higher mRNA level of *RB1* could correlate with abemaciclib and TAL response rate. However, further studies are required to identify the molecular mechanism underlying the overexpression of *RB1* in HCC1937-R cells. Additionally, post-transcriptional and/or translational mechanisms define protein abundance (Greenbaum et al 2003). Therefore, further molecular experiments including western blot and proteomics analysis should be performed for the identification of RB and associated signaling pathway in HCC1937-R cells.

In the literature, abemaciclib treatment causes atypical cell death based on swollen lysosomes in the A549 lung cancer cell line (Hino et al 2020). Additionally, Fassl et al. (2020) state that increased lysosomal mass leads to the intrinsic resistance of TNBC and a subset of hormone receptor positive breast cancer tumors. In the current study, co-treatment with abemaciclib and TAL treatment did not induce autophagy due to non-acidic vesicles as in our previous study (Ozman et al 2021). However, the number of lysosomal biomass or swollen lysosomes mentioned in the previous studies (Fassl et al 2020; Hino et al 2020) was more pronounced in HCC1937-R cells. Therefore, the underlying molecular mechanisms of abemaciclib and lysosomal biomass and their associations with drug resistance need further investigation.

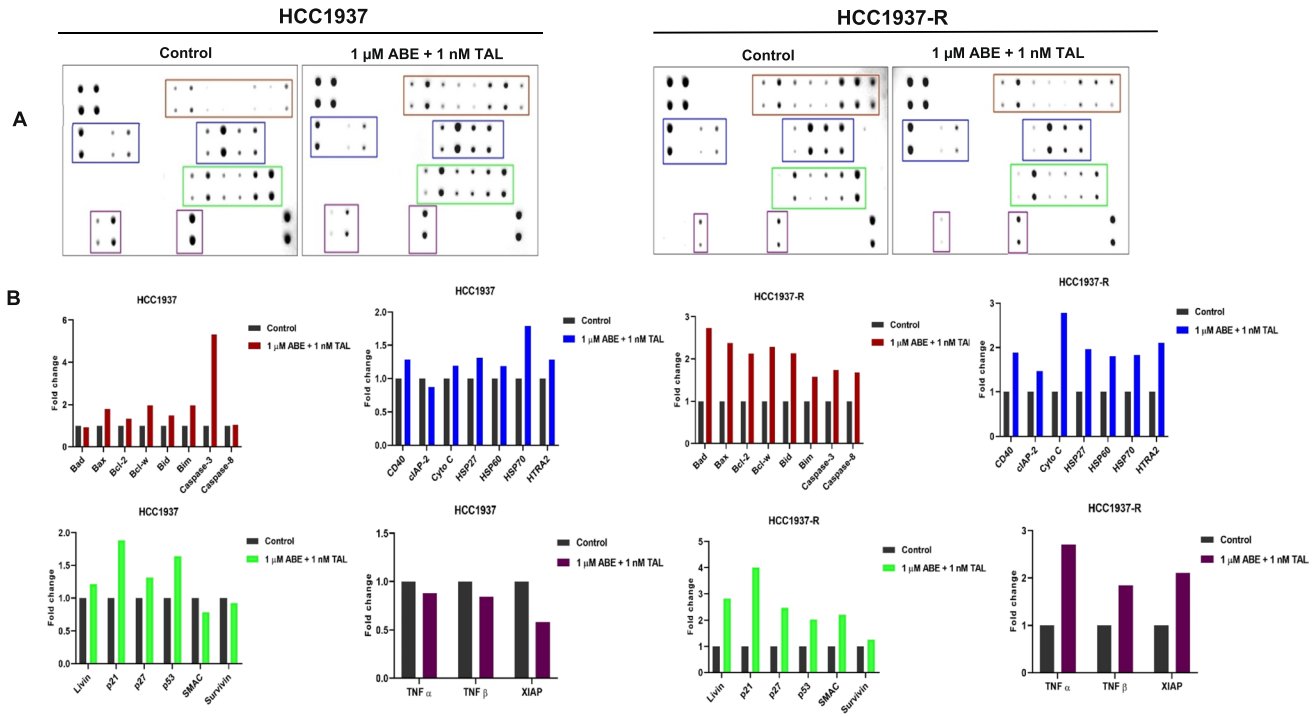


Fig. 5 The combinatory effects of abemaciclib and TAL on the expression of multiple proteins associated with apoptosis. **A** Apoptosis array of HCC1937 and HCC1937-R cells following the administration of abemaciclib and TAL combination treatment. **B** The expression levels of pro-apoptotic and anit-apoptotic proteins in co-treated TNBC cells compared with control

Furthermore, abemaciclib and TAL combination significantly induced G0/G1 arrest in TNBC cells. Changes in DNA damage and homologous recombination (HR) repair affect the cell cycle distribution. Typically, the inhibition of PARP treatment leads to G2/M accumulation in especially TNBC cells due to repair of DNA damage by HR (Chuang et al 2012; Eskiler et al 2018). We speculate that increased DNA damage leads to G0/G1 phase accumulation due to decreased *cyclin D1* level and pre-treatment of TAL may cause DNA damage. Thus, the molecular mechanism of each cell cycle phase will be further investigated upon abemaciclib and TAL combination treatment.

Finally, we revealed changes in the pro-apoptotic and anti-apoptotic protein levels by apoptosis array. Our findings showed that abemaciclib and TAL combination treatment caused over-expression of pro-apoptotic protein and these effects were more pronounced in HCC1937-R cells than HCC1937 parental cells. However, the expression of some anti-apoptotic proteins including Bcl-2, Bcl-w, Livin, and heat shock proteins was increased in both TNBC cells. Bcl-2 and Bcl-w are members of anti-apoptotic Bcl-2 proteins, and upregulation of these proteins leads to survival and chemotherapy resistance in different types of cancer (Inao et al 2018). Additionally, higher expression or activity of HSP27, HSP60, and HSP70 is associated with increased tumorigenicity, metastatic potential, and resistance to chemotherapy, resulting in poor prognosis (Albakova et al 2021). Furthermore, over-expressed XIAP and Livin levels are associated with lymph node metastasis, tumor size, and TNM stage in patients with TNBC (Han et al 2017; Hussain et al 2017). Despite apoptosis through intrinsic and extrinsic pathways induced by abemaciclib and TAL combination, the overexpression of anti-apoptotic proteins and increased pro-inflammatory cytokines (TNFα and TNFβ) (Pileczki et al 2013) may cause aggressiveness and apoptotic resistance in HCC1937-R cells. Therefore, co-treatment of abemaciclib with TAL could overcome TAL resistance at certain degree. In this context, further sequentially or simultaneously combination strategies should be improved for targeting multiple pathways and overcoming TAL resistance in vitro and in vivo.

Conclusion

Collectively, our preliminary findings provide a preclinical rationale for identifying the synergistic therapeutic effects of abemaciclib and TAL on *BRCA* mutant RB deficient TNBCs cells and the reversal of TAL resistance. However, further studies will focus on validating the efficacy of CDK4/6 inhibitors and PARP inhibitors combination and broadening the utility of these inhibitors for the prevention and/or treatment of drug-resistant TNBC patients. Additionally, in vivo experiments should be performed to validate the combined anti-cancer effects of CDK4/6 inhibitors and PARP inhibitors on TNBC treatment.

Author contribution GGE and ZO conceived and designed research. ZO and AH conducted experiments. GGE and DCD analyzed data and wrote the manuscript. All authors read and approved the manuscript.

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

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