

# Differences of palbociclib and ribociclib in terms of cell death pathways; a cell culture assay

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## Research Article

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# Abstract

Differences of palbociclib and ribociclib in terms of cell death pathways; a cell culture assay The contribution of adding cyclin dependent kinase 4/6 (CDK4/6) inhibitors to hormone therapy in relation with the survival was indicated for the treatment of metastatic breast cancer which had positive hormone receptor and negative HER2. In this study, it was planned to indicate two different CDK4/6 inhibitors (palbociclib and ribociclib) providing CDK4/6 inhibition on the cell lines (MCF-7 and BT474) having specifications of luminal-A and luminal-B, the molecular sub-types of positive hormone receptor of breast cancer, and to reveal the molecular differences and to compare cytotoxicity data and necrosis mechanisms in the cell culture experiments. It was planned to determine the differences of resistance mechanism of new combinations. MCF-7 and BT474 cell lines were handled with Palbociclib and Ribociclib individually. The possible cytotoxicity, apoptosis and autophagy effects and the levels of apoptotic proteins were examined. It was indicated that Palbociclib and Ribociclib had cytotoxic effects in both breast cancer cells. In regard of comparing from the point of intracellular pathways, it was determined that the effect of Palbociclib decreased in the increased dosage but that the effect of Ribociclib on cell indicated by means of apoptosis. It was found that palbociclib induced autophagy in the autophagy experiment conducted on the decrease of apoptotic activity with increasing doses. Even though they worked on the same pathway, Palbociclib and Ribociclib utilized different mechanisms to kill the cells and new evidences were obtained that the resistance mechanism may be different from each other according to the treatment.

# Introduction

Breast cancer is a cancer type that affects women around the world at the very most and it takes place on the 2nd following lung cancer in cancer related deaths of women[1]. Approximately 5-10% of the cases are advanced stage breast cancer during diagnosis[2]. It is found as an estrogen receptor (ER)+ in approximately 60-75% of breast cancer cases[3]. Palbociclib and Ribociclib which are cyclin dependent kinase (CDK4/6) inhibitors have come into use in cases of advanced breast cancer and breast cancer that is resistant to anti-estrogen treatment or that develops relapse [4,5]. The cyclin-dependent kinases (CDK) are a subgroup of the serine/threonine kinases which play a key role in cell cycle regulation. When the cell cycle G1 and S phase transition is linked to D-type cyclins, they are checked by the activated CDK4 and CDK6 causing gene expression required for S phase entry [6]. The cyclin D1 (CCND1) is a direct transcriptional purpose of ER. Anti-estrogen treatment ceases the growth of ER+ breast cancer cells and it reduces the effectiveness of cyclin D1. When it is compared with other subtypes of breast cancer, ER+ breast cancer is frequently associated with hyperactivity of CCND1 – CDK4/6 [7]. At the same time, the CDK4/6 excessive expression and CCND1 amplification are generally seen in ER+ breast cancers and this plays a key role in the development of resistance to endocrine treatment [8]. It has been indicated in the studies conducted that the use of Palbociclib and Ribociclib in combination with estrogen receptor blockers (aromatase inhibitor or fulvestrant) have positive impacts on general survival without progression in both primary and secondary stage in postmenopausal patients with HER -2 negative, ER+

metastatic breast cancer [4,8]. More limited studies are available in premenopausal patients. In the study of MONALEESA-7, the contribution of ribociclib to non-progression survival was shown [9].

The cell culture test was created in cell lines of two breast cancer with positive ER expression and without HER-2 expression by using MCF-7 and BT474. MCF-7 (Michigan Cancer Foundation-7) was firstly isolated from pleural effusion of a woman with metastatic breast cancer who is at age of 69 with the characteristic of Caucasian race by Herbert D. Soule, a researcher at Michigan Cancer Foundation, in 1970. The MCF-7 cell line of breast cancer was selected as exemplary to Luminal-A molecular subtype [10]. BT474 cell line of breast cancer was obtained from solid breast tissue of a 60-aged Caucasian woman and it was invasive ductal carcinoma. This cell line has aneuploidy cell alignment and its chromosome number is 55. The BT474 cell line of breast cancer was selected as an example of Luminal-B subtype.

In this study, it is intended to demonstrate whether potent differences of two different CDK4/6 inhibitors are available in ER+ cell cultures (MCF-7 and BT474) which include luminal-A and luminal-B, the most frequent two molecular subtypes of breast cancer, to reveal differences in the meaning of molecular despite of CDK4/6 inhibition in the same pathway, to compare cytotoxicity in these cell cultures and to determine resistance mechanisms which may cause new combinations and pathways of cell death.

## Materials And Methods

### Reagents

Palbociclib (PD0332991) was obtained from Selleckchem (Germany). Five mg powder of Palbociclib was dissolved in 1.7432 ml of dimethylsulfoxide (DMSO) and 5 mM of stock solution was prepared. Ribociclib (LEE011) was purchased from Selleckchem (Germany). Ribociclib stock solution (5 mM) was prepared by dissolving 5 mg powder of Ribociclib in 2.0411 ml of DMSO. Both stock solutions were sterilized by a 0.22 µm filter and stored at -80° C.

### Cell culture

ER+ human breast cancer cell lines MCF-7 and BT474 were obtained from ICLC (Interlab Cell Line Collection, Italy) and HPA (HealthProtectionAgency, UK), respectively. MCF-7 cells were maintained in RPMI-1640 medium with 1% non-essential amino acids, 10% heat-inactivated fetal bovine serum, 10000 units/ml penicillin in 37° C and 5% CO<sub>2</sub>, humidified incubator. For the BT474 cell line, Dulbecco's modified Eagle's minimal essential medium (DMEM/F12) was used by adding 20% fetal bovine serum, 10.000 units/ml penicillin and 10 mg/ml streptomycin at 37° C with 5% CO<sub>2</sub>.

### XTT cell viability assay

XTT assay (Roche, Germany) was used to determine the cytotoxic effects of Ribociclib and Palbociclib in breast cancer cells at 24, 48 and 72 h. XTT solution was prepared in a 50: 1 ratio and 100 µL of the solution was added to each well. Then, 96-well cell culture plates were incubated at 37 ° C for 4 hours.

After incubation, the absorbance value of each well was measured in the microplate reader (DTX 880 Multimode Reader, BeckmanCoulter, USA) in the reference wavelength range of 450-490 nm. The concentration required to inhibit 50% of cancer cell growth (IC<sub>50</sub>) values of all synthesized compounds were calculated via Biosoft CalcuSyn 2.1 software (Ferguson, MO, USA).

### **DNA Fragmentation analysis**

Cell Death Detection ELISA Plus Kit (Roche Applied Science, Germany) was used to assess apoptosis according to the manufacturer's instructions. This kit measures the amount of mono and oligonucleosomes from apoptotic cells. Briefly, cell lysates from cells treated as single and combined with drugs and control cells without drugs were added to streptavidin-coated plates contained in the kit. The plate containing 80 µl of the reagent containing antibodies against histone proteins and DNA fragments (Anti-histon biotin and Anti-DNA-Peroxidase) was incubated at room temperature for 2 hours. Absorbance was measured in the multimode plate reader at the reference wavelength of 405 nm and 490 nm (DTX 880 Multimode Reader, BeckmanCoulter, USA).

### **Autophagy assay**

The autophagy assay kit (ab139484) was used to evaluate autophagy. Rapamycin was used as an autophagy inducer in the positive control group. Fluorescence emitted by binding autophagy dye to pre-autophagosomes, autophagosomes and autolysosomes.

### **Fluorescence staining**

Cells were seeded in 6-well plates at 105 cells per well. After 24 hours, the cells were treated with drugs for the desired time and the medium on the cells was withdrawn at the end of the incubation. Cells were washed 2 times with 1X Assay buffer. 100 µL of Dual detection solution was added and the plates were incubated at 37 ° C for 30 minutes away from light. Cells were washed again with 100 µL of 1X Assay buffer. Then the cells were incubated with 4% formaldehyde for 20 minutes and washed again 3 times with 100 µL 1X Assay buffer. Imaging was done under fluorescence microscope with FITCH filter.

### **Flow cytometric analysis**

Cells were seeded in 6-well plates at 106 cells per well. After 24 hours, the cells were treated with drugs for the desired time and the medium on the cells was withdrawn at the end of the incubation. Cells were removed by trypsinization and suspended at 106 cells per mL. After being centrifuged at 1000 rpm for 5 minutes, the pellet was washed with 1X Assay buffer and centrifuged again. The pellet was suspended with 250 µL of 5% FBS medium. 250 µL of dilue green solution was added to each sample and mixed. It was incubated for 30 minutes at room temperature and centrifuged at the end of the incubation. The pellet was washed with 1X Assay buffer and cells incubated for 20 min with 4% formaldehyde were washed 3 times with 1X Assay buffer and analyzed by flow cytometry with FL1 filter.

### **Statistical Analysis**

Three replicate samples were tested for each condition. The results were expressed as mean  $\pm$  S.D. and the data was analyzed by using one-way analysis of variance test (ANOVA) followed by *Dunnett's t-test* for multiple comparisons. Values with  $p < 0.05$  were considered as significant.

## Results

Effects of Palbociclib and Ribociclib on the viability of ER + breast cancer cells To determine the effect of increasing concentrations of Palbociclib (0.01 -100  $\mu$ M) on MCF-7 and BT474 cells at 24, 48 and 72 h, XTT cell viability assay was used. In both cell lines, the highest cytotoxicity was observed at 72 hours and a concentration of 100  $\mu$ M (Figure 1). IC50 values are calculated at 7h and found to be 10.6  $\mu$ M and 33.5  $\mu$ M for MCF-7 and BT474 cells, respectively. Similarly, to determine the effect of Ribociclib on cell viability, MCF-7 and BT474 cells were treated with the increasing concentrations of Ribociclib (0.5-20  $\mu$ M) for 24, 48 and 72 h and XTT assay was performed. The cytotoxic effect of Ribociclib was increased in a concentration and time dependent manner in both breast cancer cells ( $p < 0.05$ ) (Figure 1). The cytotoxicity of Palbociclib in MCF-7 and BT474 cell lines was 72% and 57.5%, respectively. In Ribociclib, the cytotoxicity ratios were 53.4% and 59.8%, respectively, at 72 hours (Table 1).

**Table 1. Cytotoxicity of palbociclib and ribociclib at MCF-7 and BT474 cell lines at 72 hours**

Cytotoxicity (%)	Palbociclib	Ribociclib
<b>MCF-7</b>	72	53,4
<b>BT474</b>	57,5	59,8

### Comparison of the apoptotic effects of Palbociclib and Ribociclib in ER + breast cancer cells

To investigate the effects of Palbociclib and Ribociclib on the induction of apoptosis, MCF-7 and BT474 cell lines were treated with different concentrations of the drugs for 72 h. At the end of 72 h, the mono-oligonucleosome fragments were detected using the Cell Death Detection ELISA Plus Kit (Roche Applied Science, Germany). To determine if the cytotoxic effect of Palbociclib in MCF-7 and BT474 cell lines is apoptosis-mediated 72. In Palbociclib treated MCF-7 cells, the DNA fragmentation percentages were 10.3% in the control group, 28% in the 1  $\mu$ M Palbociclib group and 47% in the 10  $\mu$ M Palbociclib treated group, as compared to kit positive control cells (100% apoptotic cells)( $p < 0.05$ ). However, the DNA fragmentation percentage was decreased to 10.5% in 100  $\mu$ M Palbociclib treated group. In BT474 cells, similar results were obtained. In the control group, the DNA fragmentation percentage was 8.2% in the control group, and 26.3% and 38.2% in 1  $\mu$ M and 10  $\mu$ M Palbociclib treated groups, respectively ( $p < 0.05$ ). In 100  $\mu$ M Palbociclib treated BT474 cells, the DNA fragmentation percentage was decreased to 9% (Figure 2).

To determine whether the cytotoxic effect of Ribociclib is mediated by apoptosis, DNA fragmentation percentages were measured at 72 h. In MCF-7 control group, the DNA fragmentation percentage was 6.5%, however, the DNA fragmentation percentages were 19%, 20.5% and 21% in 5, 10 and 20  $\mu$ M

Ribociclib treated groups, respectively ( $p < 0.05$ ). In BT474 cells, the DNA percentages were 7.5%, 18.10%, 19.2% and 25% in control, 7.5, 10 and 20  $\mu\text{M}$  Ribociclib treated groups, respectively ( $p < 0.05$ ) (Figure 2).

### **Comparison of autophagic effects of cdk4 / 6 inhibitors**

Palbociclib and ribociclib were applied separately to the autophagy kit (ab139484). Rapamycin was used as a positive control group. We found that palbociclib was treated with an autophagy kit at a dose of 100  $\mu\text{M}$ , and at this dose, it lumines at least as much as rapamycin in fluorescence, that is, it creates autophagic vacuoles. In our DNA fragmentation analysis, it is thought that palbociclib showed apoptotic effect up to this dose, but the decrease in DNA fragmentation was observed when the dose was increased to 100  $\mu\text{M}$ , caused by autophagy, another cell death pathway. On the other hand, the increase in DNA fragmentation detected with increasing doses in ribociclib suggests that it is not reflected in the autophagy kit, that is, it acts with a cell death mechanism from a single pathway (figure 3).

## **Discussion**

Cyclin-dependent kinases (CDK), as the family of serine-threonine kinases that transition from the G1 phase of the cell cycle to the S phase, are crucial for the control of eukaryotic cell proliferation. Cell cycle cyclins (CYC) are controlled by CDKs and cyclin-dependent kinase inhibitors (CDI). The levels of these proteins show differences at different phases of the cell cycle. Throughout the G1 phase, DNA replication is controlled. If DNA replication is appropriate, CDK is activated and the transition to the S phase starts with the creation of a positive feedback effect, which induces genome-wide transitional changes thereby triggering cell division.

CDK4 and CDK6 are the most studied CDKs, and are related to D-type cyclins (D1, D2, D3).

Retinoblastoma (Rb), which is a tumor suppressor gene not phosphorylated under normal conditions before transition from the G1 phase to the S phase, is bound to members of the E2F transcription factor gene family. Cyclins acquire the phosphorylation property when the CDK complex is formed, and by phosphorylating Rb, which is a tumor suppressor gene, the E2F transcription factor is expressed and the progression of the cell cycle is obtained [11, 12,13]. If the CYC-CDK 4/6 complex is inhibited with CDI, the cell cycle is arrested in the G1 phase, the arrested cells age, and apoptosis develops. Therefore, interest has increased, and many studies have been conducted on this subject in many cancer types and their treatments.

As cancer is a pathological manifestation of uncontrolled cell proliferation, over the past decades many studies have examined the control point of cell proliferation. Thus it has been revealed that CYCs and CDKs are an effective strategy in the targeting of many cancer types and treatment [14]. The increase in CDK 4/6 levels following endocrine treatment in hormone receptor positive (HR+) subtype of breast cancer and human epidermal growth factor receptor 2-negative (HER2-) advanced or metastatic breast cancer suggests that there may be a relationship between this pathway and resistance to endocrine treatment. Agents that show synergistic effects with anti-estrogen treatment have been developed with pre-clinical and subsequent clinical studies. The highly selective oral CDK4/6 inhibitors have been tested

in combination with endocrine therapy in Phase III studies in metastatic breast cancer. The results have led to the US Food and Drug Administration approval of palbociclib (PD0332991) and ribociclib (LEE011), and abemaciclib (LY2835219) is in development. The indications for these drugs in the treatment of HR+/HER2- advanced breast cancer include use with an aromatase inhibitor (AI) as initial therapy in postmenopausal women and with Fulvestrant in women who showed disease progression during endocrine therapy.

Metabolic changes that emerge following CDK4/6 inhibition are related to irreversible inhibition of the cell cycle, cellular aging and apoptosis induction [14].

MCF-7 and BT474 breast cancer cell lines were used in this study as examples of luminal A and luminal B, which are the most common molecular subtypes (HR+/HER2-) of breast cancer. These two cell lines were treated with the highly selective CDK 4/6 inhibitors palbociclib and ribociclib to determine the potential resistance mechanisms of these new combination treatments by revealing potency and molecular differences despite CDK4/6 inhibition in the same pathway of the cell cycle, and the determination and comparison of cytotoxic effects and cell death pathways.

The results of the current study showed the dose and time-dependent cytotoxic efficacy of in vitro palbociclib and ribociclib as single agents in ER+/HER2-breast cancer cell lines. In the potential cell death pathways, different responses developed in the two drugs in the MCF-7 and BT474 cell lines. With a dose increase in ribociclib there was a proportional increase in the rate of DNA fragmentation, while in palbociclib there was observed to be an increase in the rate of DNA fragmentation up to a certain dose, and at higher doses this rate fell. This finding showed that as the increase in the rate of DNA fragmentation was dose dependent in ribociclib, the cytotoxic effect was related to apoptosis. Despite the cytotoxicity in increasing doses in palbociclib, the decrease that develops in the DNA fragmentation rate suggests that cell death pathways other than apoptosis play a role.

There are studies in the literature showing that of the cell death pathways, autophagy is used by palbociclib. In an in vitro study of stomach cancer cells by Brown et al, LC3B-2 levels were shown to be increased as a marker of autophagy as a result of treating the stomach cancer AGS cell line with palbociclib. It was also shown that there was a decrease in the p62/SQSTM1 level which is a protein fragmented by autophagy. Thus, it was shown that palbociclib induced autophagy [15]. In a study of palbociclib by Capparelli et al, it was shown that CDK inhibitors induced autophagy and aging in fibroblasts related to cancer and there was increased expression of BNIP3, cathepsin B and ATG16L1, which are genes associated with autophagy [16].

Although there is limited information about cell death pathways induced by ribociclib, there are a few studies in the literature. To evaluate the apoptotic effect of ribociclib, HJ et al treated an aggressive thyroid cancer cell line (ATC) with ribociclib in an in vitro study, and using the immunoblot method showed that caspase-3-enzyme expression was increased, thereby demonstrating that ribociclib had an apoptotic effect [17].

Another mechanism in CDK4/6 inhibitor resistance is the activation of the P13K/AKT/mTOR pathway of breast cancer cells. mTOR is known to increase cell development and proliferation by suppressing autophagy [18]. Michaloglou et al (2018) suggested that in ER+ cancer cell lines resistant to CDK 4/6 inhibitors, sensitivity to CDK 4/6 inhibitors is regained by mTORC1/2 inhibition [19].

When other cell death pathways are considered, various studies are showing that there is a relationship between palbociclib and autophagy. There are various opinions on this issue. The results of the current study show that palbociclib inhibits the cell cycle through autophagy. Upon seeing that palbociclib uses the autophagy route in various studies, we set up an autophagy experiment considering that the reason for the decrease in apoptosis at 100 micromolar dose of palbociclib in our apoptosis experiment was due to palbociclib's use of the autophagy pathway. In this autophagy experiment, we found that palbociclib uses the autophagy pathway, but ribociclib does not use the autophagy pathway even at the highest doses. Both of these two CDK 4/6 inhibitors were found to have increased cytotoxicity depending on dose and time. Ribociclib, one of the CDK 4/6 inhibitors, only used the apoptosis pathway, while the other CDK 4/6 inhibitor, palbociclib, used the apoptosis pathway to a certain dose. However, with increasing doses, autophagy replaced apoptosis. The use of autophagy inhibitors in the treatment, especially in patients receiving palbociclib, suggests that it may have a positive effect on survival in the treatment of resistance to CDK 4/6 inhibition. At the same time, it should be kept in mind that this difference of both drugs may play a role in the differences that may develop in terms of side effect profiles. Considering the data in the literature, this study is the first study to compare the cell death pathways of drugs in breast cancer and should be supported by in-vivo studies.

## **Declarations**

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### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Authors' contributions**

AAO, GB, HA and BK conceived and designed the study. HA performed the experiments. AAO and HA analyzed the data. All authors read and approved the final manuscript and agreed to be accountable for

all aspects of the research in ensuring that the accuracy or integrity of any part of the work was appropriately investigated and resolved.

### **Ethics approval and consent to participate**

Not applicable.

### **Patient consent for publication**

Not applicable.

### **Competing interests**

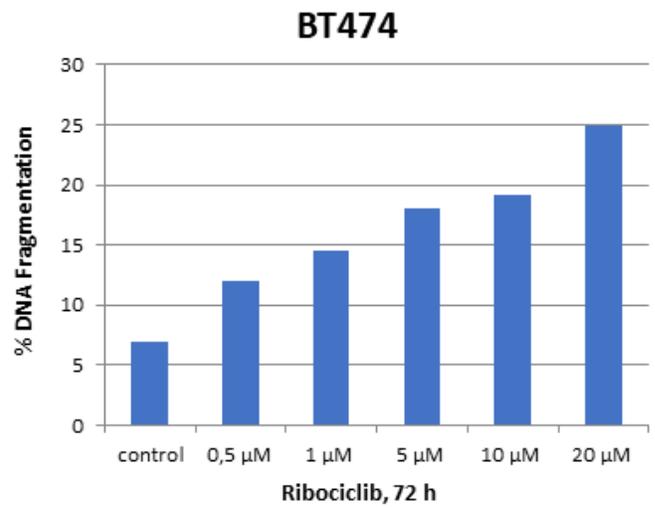
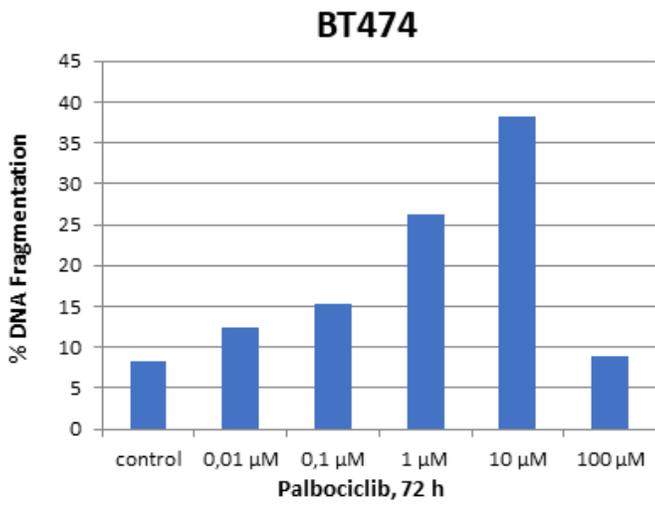
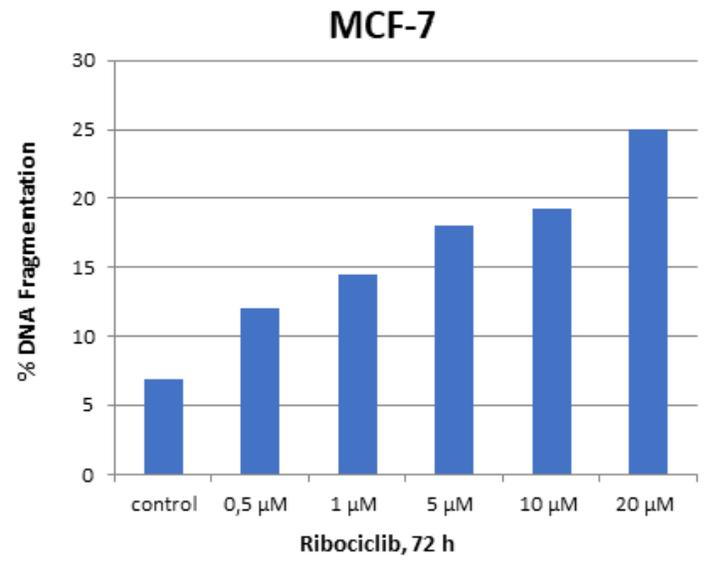
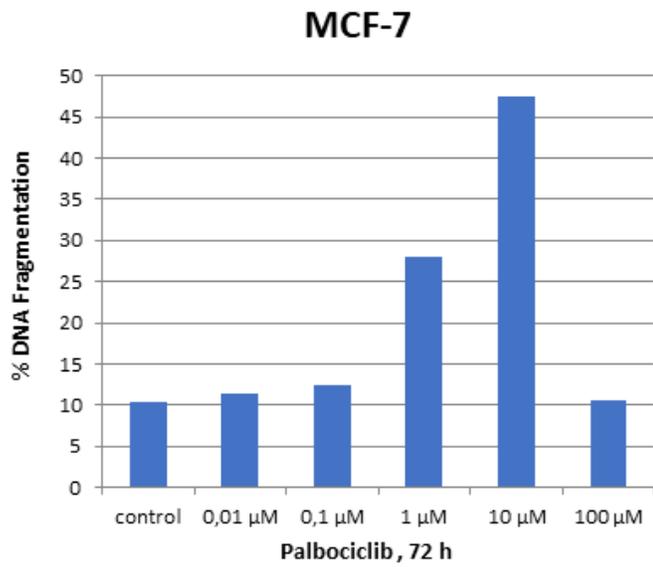
The authors declare that they have no competing interests.

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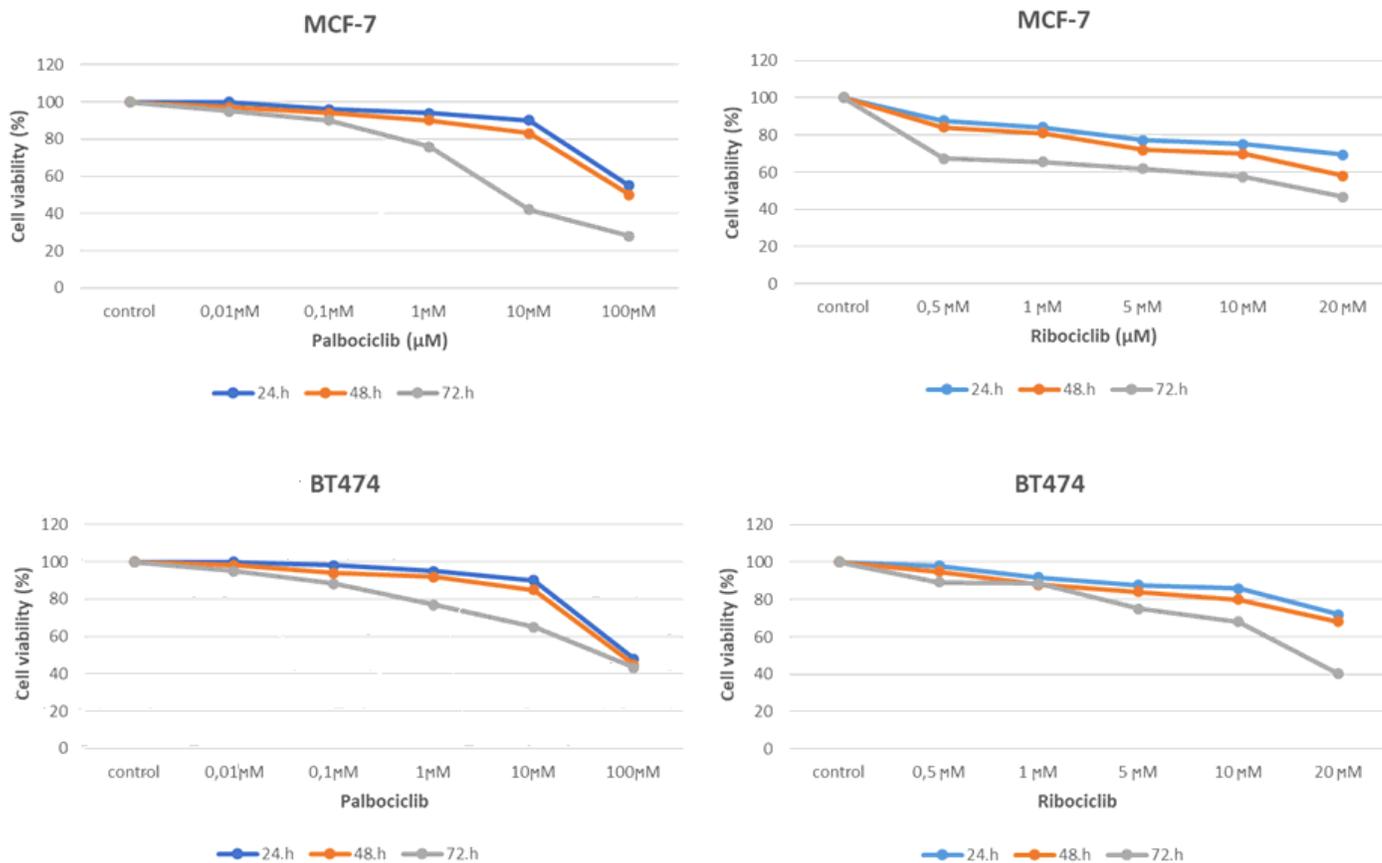
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## Figures



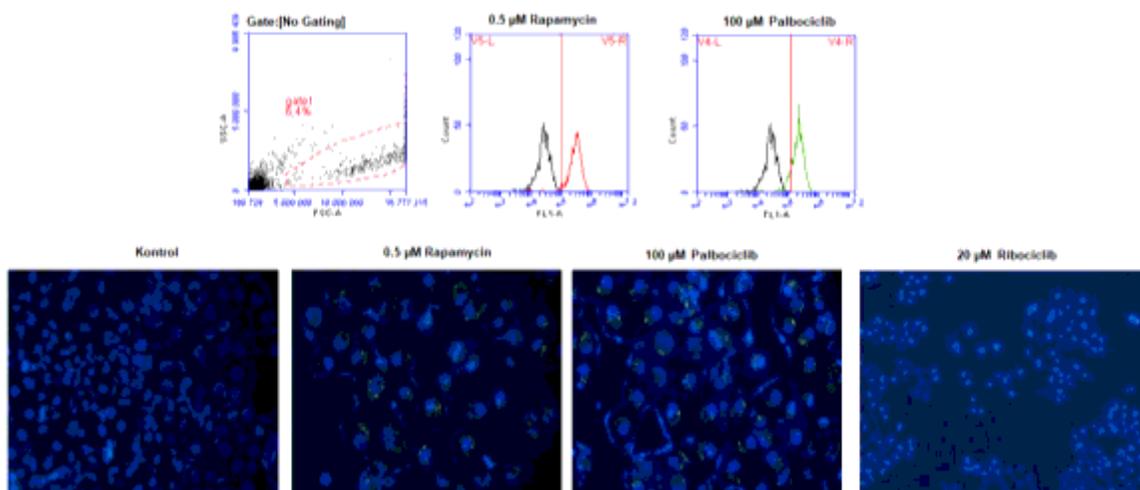
**Figure 1**

Comparison of apoptotic effects of palbociclib and ribociclib on MCF-7 and BT474 cell lines.



**Figure 1**

Concentration and time-dependent effects of palbociclib and ribociclib on the viability of MCF-7 and BT474 cell lines.  $p < 0.05$ ; significant as compared with the control group.



**Figure 1**

Comparison of autophagic effects of cdk 4/6 inhibitors