

# Melatonin Enhances the Efficacy of Cdk4/6 Inhibitor in Breast Cancer Cells Via Down-Regulating the Expression of CyclinD1

Zhumei Zhuang<sup>1</sup>, Qing Tang<sup>2</sup>, Miaomiao Gong<sup>2</sup>, Jinjin Pan<sup>2</sup>, Xiaocheng Yuan<sup>2</sup> and Yuhui Yuan<sup>2\*</sup>

<sup>1</sup>Zhejiang University-University of Edinburgh Institute, Zhejiang University School of Medicine, Haining 310058, China; Dr. Li Dak Sum & Yip Yio Chin Center for Stem Cells and Regenerative Medicine, Zhejiang University School of Medicine, Hangzhou 310058, China; Key Laboratory of Tissue Engineering and Regenerative Medicine of Zhejiang Province, Zhejiang University School of Medicine, Hangzhou 310058, P. R. China

<sup>2</sup>The Second Affiliated Hospital, Institute of Cancer Stem Cell, Dalian Medical University, Dalian 116400, P. R. China

**\*Corresponding author:** Yuhui Yuan, The Second Affiliated Hospital, Institute of Cancer Stem Cell, Dalian Medical University, Dalian 116400, P. R. China

## ARTICLE INFO

**Received:** 📅 November 29, 2024

**Published:** 📅 December 12, 2024

**Citation:** Zhumei Zhuang, Qing Tang, Miaomiao Gong, Jinjin Pan, Xiaocheng Yuan and Yuhui Yuan. Melatonin Enhances the Efficacy of Cdk4/6 Inhibitor in Breast Cancer Cells Via Down-Regulating the Expression of CyclinD1. Biomed J Sci & Tech Res 59(5)-2024. BJSTR. MS.ID.009367.

## ABSTRACT

**Background:** Breast Cancer (BC) is the most common malignancy and cause of death among women. CDK4/6 inhibitor-Palbociclib (PD) isethionate is a new chemotherapeutic agent in the treatment of breast cancer that works through blocking retinoblastoma phosphorylation protein and regulating the retinoblastoma (RB)-E2F pathway to inhibit cell cycle in the G0/G1 phase. However, the toxicity and resistance of PD are the primary limiting factors of this therapeutic effect. Melatonin (MLT) exerts anti-tumor activity in BC and arrests the cell cycle in the G0/G1 phase.

**Materials and Method:** Cellular and molecular studies were used to explore the efficacy of PD and MLT in T47D/MCF7 cell viability, proliferation and migration. Flow cytometry was used to detect cell cycle arrest and Senescence  $\beta$ -Galactosidase Staining Kit detected cell senescence.

**Results:** In this study, we found that MLT enhanced the inhibitory abilities of PD on cell proliferation and colony formation in breast cancer cells by inhibiting the phosphorylation of RB protein and RB down-regulating the expression of E2F1. Flow cytometry results revealed that co-treatment with MLT and PD could enhance the cell cycle arrest at the G0/G1 phase. Furthermore, our data demonstrated that MLT might enhance the efficacy of CDK4/6 inhibitor by down-regulating the overexpression of cyclinD1 induced by PD. In addition, we confirmed that MLT and PD exerted synergistic anti-tumor effects in the xenograft mouse model. Conclusions: Our study demonstrated that the combination of PD and MLT may represent a valuable therapeutic option for breast cancer.

**Keywords:** Breast Cancer; Cdk4/6 Inhibitor; Melatonin; Cell Cycle; CyclinD1

**Abbreviations:** BC: Breast Cancer; PD: Palbociclib; MLT: Melatonin

## Introduction

Breast cancer is the most common type of cancers with high rates of morbidity and mortality, which is classified into three molecular subgroups primarily, including roughly 60%-65% of cases as hormone receptor-positive, 20%-25% HER2-positive and 15%-18% triple-negative in more than 1.5 million new breast cancer cases in the world each year [1]. MCF7 and T47D, a hormone receptor-positive,

have been utilized specifically as the object of this study. Presently, the numerous medical treatments are available for BC are surgery, chemotherapy and radiotherapy [2]. These are accompanied by several complications like fatal adverse effect, and rapid drug resistance. Therefore, it is particularly important to try to find safe and effective alternatives. Palbociclib (PD) was approved by the US Food and Drug Administration (FDA) in 2015, PD combined with letrozole were the first-line treatment for estrogen receptor (ER)-positive, human epi-

dermal growth factor receptor 2 (HER2)-negative advanced breast cancer [3]. CDK/RB-axis/E2F was shown in hormone-receptor (HR) positive cells primarily, HER2-positive and rare triple-negative BC cells [4], and could lead to cell division and proliferation [5]. PD could hinder the CDK/RB-axis/E2F signal pathway [6], which hampered the cell cycle through the G1 to the S phase transition. The role of phosphorylation of the retinoblastoma protein contributed to the cell cycle from G1 to S phase transition [7].

Presently, some studies have shown that PD preferentially inhibited growth in RB+ breast cancer but not in RB-BC cell lines, and enhanced sensitivity to the luminal ER-positive breast cancer cell lines [8]. Research data indicated that PD could cause neutropenia, back pain, fatigue, diarrhea and leucopenia through its toxicity [9]. Melatonin (N-acetyl-5-methoxytryptamine, MLT), an indoleamine derived from the pineal gland [10], could adjust the human circadian rhythm and maintain cytoskeleton modulatory function [11]. MLT possesses pharmacological properties at physiological concentrations [12] and had shown to function as an anti-cancer agent in the breast, osteoblastic, colon and lung cancer, etc [13]. MLT also obviously overcomes the chemotherapeutic resistance [14]. Some reports showed that MLT could suppress cellular proliferation, cell cycle and metastasis of cancer cells [15]. MLT also suppressed the development and growth of mammary tumors to inhibit mammary carcinogenesis [16]. Currently, most studies show that the anti-proliferative actions of MLT are carried out by repressing cell cycle and ER transcriptional activity [17]. In our study, we testified the combined effects of PD and MLT in BC cells. Our study revealed that MLT could increase the efficacy of PD in human breast cancer cells both *in vitro* and *in vivo*. For *in vitro* experiments, results showed that the combination of MLT and PD obviously enhanced the inhibition of PD on proliferation, colony formation, cell cycle and senescence. We also proved the combined effects of PD and MLT in the xenograft mouse model. Such a combinational treatment might potentially become a more effective method in breast cancer chemotherapy.

## Materials and Methods

### Cell Lines and Culture

The human breast cancer cell lines T47D and MCF7 were obtained from the global bioresource center (ATCC). T47D cells and MCF7 cell were cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) and Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) respectively, supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/ml of penicillin and 100 µg/ml streptomycin, and incubated at 37 °C under 5% CO<sub>2</sub>.

### Reagents and Antibodies

PD is an ethanoate purchased from Selleck Chemicals (Selleckchem, USA) and was dissolved in sterile water at 50 mg/ml (87.15 mM) as a stock solution. Melatonin, purchased from Sigma (St. Louis,

MO, USA), was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 1 M as stock solution. The Rabbit monoclonal antibodies against E2F1 (3742s), p-RB (ser780), CDK4 (12790), CDK6 (3136), CyclinD1 (2978), PCNA (2586) and anti-rabbit (7074) as the secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibodies of RB1 and GAPDH were from Proteintech (Proteintech, inc, USA).

### Cell Viability Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to detect the viability of cells. The cells were planted at a density of 2×10<sup>3</sup> cells per well in 96-well plates with three replicates. Overnight, cells were incubated with PD at different concentrations (0.03, 0.1, 0.3, 1, 3 and 10 µM) with 3% FBS for 48 h and MLT at a series of concentrations (10<sup>-6</sup>, 10<sup>-5</sup>, 10<sup>-4</sup>, 10<sup>-3</sup> and 5×10<sup>-3</sup> M) for 48 h respectively. Then, 10 µl MTT solution (5 mg/ml) was added to all the above wells for 4 h at 37°C. After removal of the added MTT culture medium, MTT formazan was lysed in 150 µl of DMSO per well, using an absorbance reader (Perkin-Elmer, Waltham, MA, USA) to measure the optical density (OD) at 490 nm. The concentration required to inhibit cell growth by 50% (IC<sub>50</sub>) was calculated from survival curves. According to the IC<sub>50</sub>, in addition to the indicated concentrations of PD (0.3 µM) and MLT (1 mM) with a new medium for 48 h as the combined treatment, cell viability was found to be the same as the above method.

### Colony Formation Assay

By using the colony formation assay to analyze the effects PD and MLT, the BC cells were seeded in 6-well plates and counted at 1×10<sup>3</sup> per well supplemented with fresh medium cultured for 24 h. Then, the cells were exposed to the indicated concentrations of PD (0.3 µM) and MLT (1 mM) and incubated for an additional 12-15 days. Then, the cells were washed with PBS and stained with crystal violet. Clusters of more than 80 cells were counted as a colony.

### Wound-Healing Assay

To detect the migration ability of cells, a wound-healing assay was used. Approximately, 10<sup>6</sup> cells were seeded in a 6-well plate as single cells. After overnight in the starvation medium, cells were scratched with a yellow pipette tip and washed with PBS. Then, cells were treated with PD (0.3 µM) and MLT (1 mM) in 0.2 % FBS medium for 48 h. A Leica DM 14000B microscope was used to observe the wound gap.

### Western Blot Assay

The human BC cell lines T47D and MCF7 were treated with PD (0.3 µM) and MLT (1 mM) for 48 h, and then lysed with the RIPA buffer (25 mM pH 7.6 Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% deoxy-sodium cholate, PMSF, 1 mg/ml and 0.1% SDS) for 30 min. After centrifugation at 12,000 rpm at 4 °C for 20-30 min, the liquid supernatant was collected to determine the protein concentration by the BCA as-

say kit (Thermo Fisher Scientific, USA). The proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide mini gel (SDS-PAGE), transferred to a NC membrane and blocked with 5% skim milk in TBST buffer for 2 h. The membranes were then incubated with specific primary antibodies overnight at 4 °C, followed by treatment with HRP-conjugated secondary antibodies. The protein bands were detected by ChemiDoc™ XRS + Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

### Cell Cycle Analysis

The BC cells were seeded in each well of 6-well plates and treated with PD (0.3 µM), MLT (1 mM) and combination for 48 h, which were treated with trypsin (up to  $5 \times 10^6$  cells) and washed with PBS. Then, the cells were fixed with chilled 75% ethanol and were kept at 4°C overnight. The cells were collected and resuspended in 500 µl buffer [PBS 480 µl + 10 µl TritonX-100 (10 %) + 5 µl RNase (10 mg/ml) + 5 µl PI (5 mg/ml)] at 37 °C for 30 min. After centrifugation, the cells were suspended in 300 µl PBS. The samples were analyzed using a FACS Flow Cytometer (BD Biosciences, San Jose, CA).

### Cell Senescence Measurement

Senescence was measured by the SA-β gal staining kit (Beyotime Biotechnology, China). First, cells were plated at an appropriate density of 200,000 cells in each well of 6-well plates and treated with the indicated concentrations of PD and MLT for 48 h. Then, cells were washed with PBS, fixed and stained with SA-β gal solution overnight. Finally, the images were captured by a Leica DM 14000B microscope and the cells were then quantitated.

### Animal Experiments

Twenty-eight days female BALB/c nude mice (4-week-old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal procedures animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals under the approval of the SPF Laboratory Animal Center at Dalian Medical University. The mice were ovariectomized (OVX) and given free access to food and water and maintained in a room with a 12:12 hour light-dark cycle between 20 and 24 °C. A total of  $5 \times 10^6$  T47D breast cancer cells was suspended in 50 µl of 30% matrigel (BD Bioscience, USA) and injected into the right breast pad of each mouse to establish the orthotopic breast cancer model. The tumor burdened mice were randomly divided into 4 groups with 7 mice in each group (Control, PD, MLT and PD + MLT). The animals were treated with PD (125 mg/kg, orally, daily), MLT (40 mg/kg, intraperitoneally, 5 days/week), combination PD with MLT and propanediol (25% in PBS, intraperitoneally, 5 days/week) for 2 weeks once the tumors had reached 25 mm<sup>3</sup> approximately. Meanwhile, estrogen (40 µg/mice) was injected subcutaneously into the OVX mice once a week. The tumor volume (TV) was calculated every 4 days according to the following standard formula: TV (mm<sup>3</sup>) = length × width<sup>2</sup> × 0.5. At the end of the experiment, the mice were sacrificed

and the tumors were immediately dissected and weighed. For each mouse, half of the tumor was stored in liquid nitrogen, and the other half was fixed in 4% paraformaldehyde solution for paraffin section.

### Hematoxylin and Eosin (H&E) Staining

Paraffin-embedded T47D xenografted tumors were sliced into 4-µm sections, and tissue sections were deparaffinized by immersion in xylene. Prepared slides were stained using H&E (Sigma Aldrich, Corp.) staining method. All sections of tumor masses were investigated under an Olympus microscope.

### Immunohistochemistry (IHC) Assay

For immunohistochemistry examination, the tumor slices embedded in paraffin were cut into 4-µm-thick sections. Tissue sections were deparaffinized, rehydrated, had antigens retrieved, and then incubated with 1% H<sub>2</sub>O<sub>2</sub> in methanol for 15 min at room temperature to block endogenous per-oxidase. After blocked with 5% bovine serum albumin, sections were incubated overnight with anti-PCNA and anti-RB rabbit monoclonal antibodies (1:4000 dilution and 1:100) at 4°C. Immunoreactivity was visualized using diaminobenzidine. After, a light hematoxylin counterstain was applied. Images were obtained with a microscope (Olympus, Japan).

### Statistical Analysis

In this study, all experiments were performed at least three times. The analysis was performed using SPSS 13.0 software. Statistical analysis was conducted by students test of one way and the ANOVA test, and variance was used to evaluate significant differences when  $P < 0.05$ .

## Results

### MLT Enhanced the Inhibitory Effects of PD on the Proliferation and Survival of Breast Cancer Cell

We tested the efficacy of PD or MLT alone on cellular proliferation of T47D and MCF7 by MTT analysis (Figures 1A & 1B). After treatment with different concentrations (0.03, 0.1, 0.3, 1, 3, 10 µM) of PD for 48 h, the proliferation of cells was significantly suppressed in a dose and time-dependent manner (T47D: IC<sub>50</sub> = 0.373 µM, MCF7: IC<sub>50</sub> = 0.412 µM) (Figure 1A). MLT at 1 mM significantly inhibited the proliferation of BC cells (T47D: IC<sub>50</sub> = 1.102 µM, MCF7: IC<sub>50</sub> = 1.213 µM) (Figure 1B). To detect whether melatonin enhanced the inhibitory effects of PD on proliferation in BC, we combined the melatonin (1 mM) and PD (0.3 µM) to examine the cell viability (Figure 1C). We found that the combined treatment of MLT and PD markedly enhanced the inhibitory effects when compared to being treated with either MLT or PD alone. Colony formation assay was carried out to detect cellular survival. Compared to the BC cells treated with either melatonin (1 mM) or PD (0.3 µM) alone, the combined treatment markedly reduced the number of clones and the size of cells (Figures 2A & 2B). These results demonstrated that MLT synergized the effect of PD.

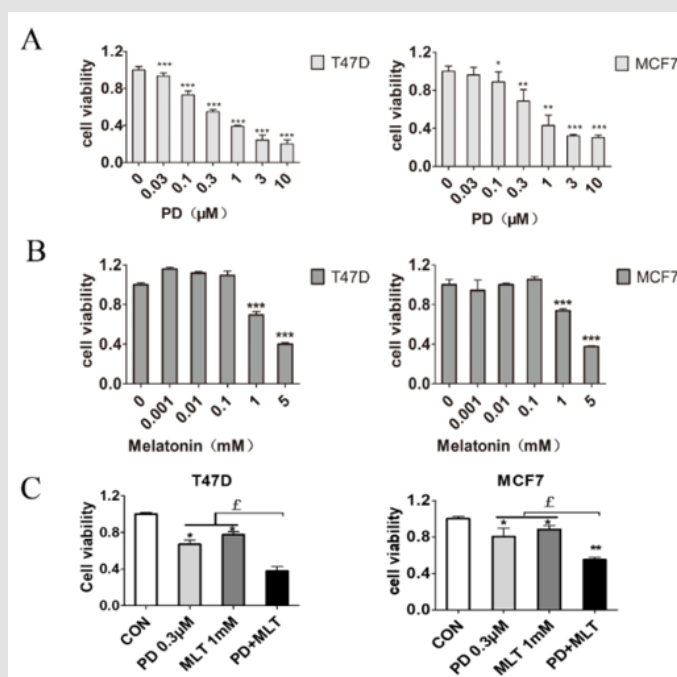


Figure 1: The combination of MLT and PD inhibited cell survival in BC cells by MTT assay.

a) PD was exposed to various concentrations (0.03, 0.1, 0.3, 1, 3, 10  $\mu$ M)-dependent manner. At 48 h after treatment.

b) Melatonin was treated at a time 48 h and dose (0.001, 0.01, 0.1, 1 and 5 mM).

c) BC cells were determined with PD (0.3  $\mu$ M) and MLT (1 mM) combination after treating 48 h. CON means to control, PD means palbociclib, MLT means melatonin and PD + MLT means the combination of palbociclib with melatonin. Data are presented as mean  $\pm$  SD (n = 4). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared with the control group. £P < 0.05 PD + MLT compared with the PD/MLT group in T47D/MCF7 cells.

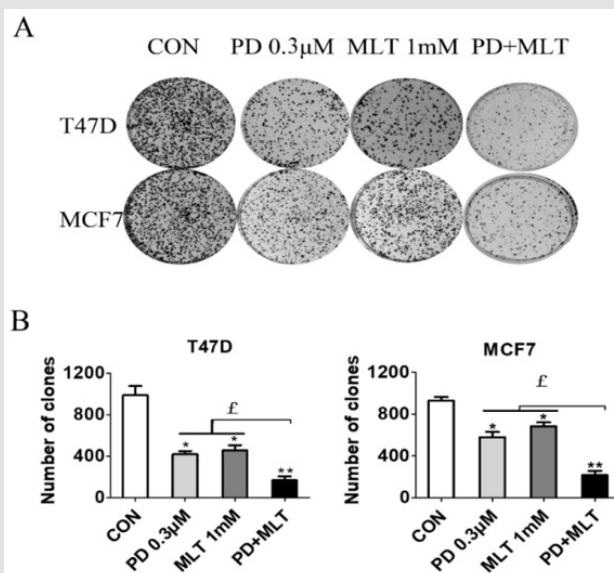


Figure 2: The combination of MLT and PD inhibited cell proliferation in BC cells by colony formation assay.

a) BC cells were treated with PD (0.3  $\mu$ M) or MLT (1 mM) alone or their combination. Colony-forming efficiency was detected 2 weeks later.

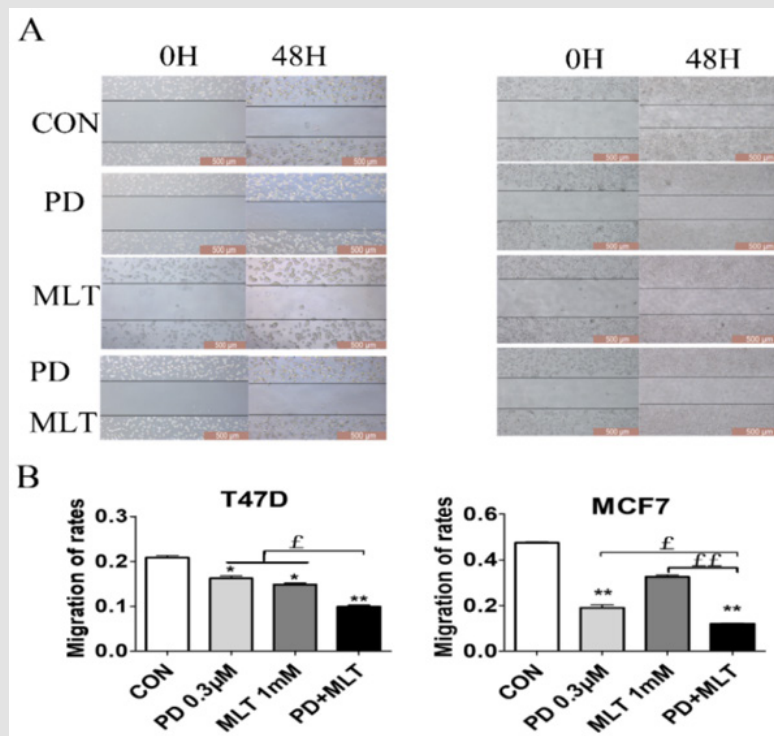
b) The data was quantitated. CON means to control, PD means palbociclib, MLT means melatonin and PD + MLT means the combination of palbociclib with melatonin. Data are presented as mean  $\pm$  SD (n = 4). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared with the control group. £P < 0.05 PD + MLT compared with the PD/MLT group in T47D/MCF7 cells.



### MLT Increased the Inhibitory Effect of PD on Cell Migration Of BC

To identify the combinational effect of MLT and PD on cell migration of T47D and MCF7 cells, wound-healing assay was used. The BC cell lines were treated with PD (0.3  $\mu$ M), MLT (1 mM) or both. After 48 h, the wounding space between cell layers was clearly occupied in the

control group of BC cells; however, the combined treatment of MLT and PD on BC cells failed to occupy the space between cell layers. The quantitative analysis showed that the combined treatment markedly inhibited BC cell migration compared with the treatment of MLT or PD alone (Figures 3A & 3B), which suggested that MLT increased the inhibitory effect of PD on cell migration.



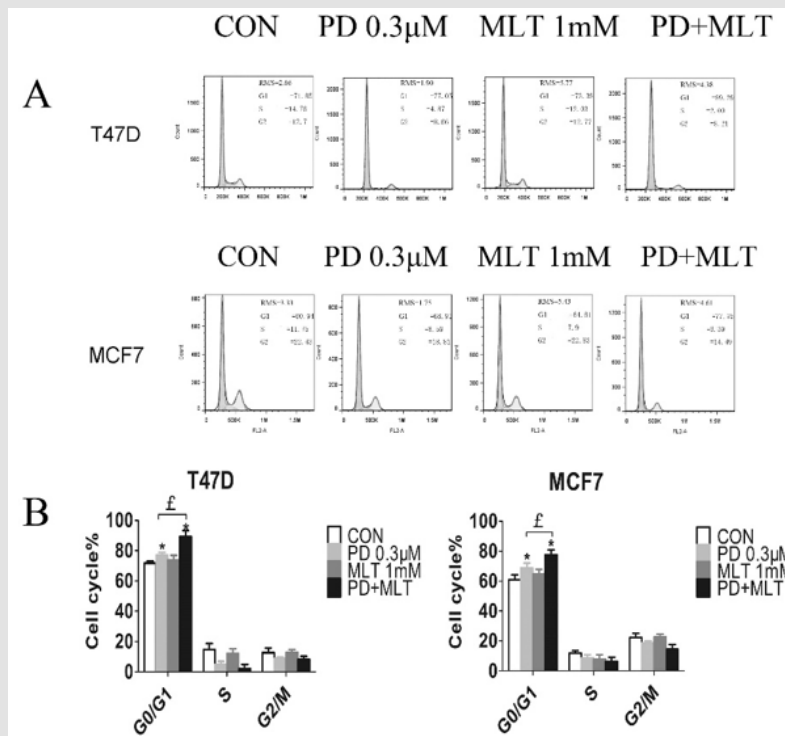
**Figure 3:** The combination of MLT and PD inhibited cell migration in BC cells by a wound-healing assay.

- Cells migration were detected.
- The cell migration of rates was calculated relative to the original gap. CON means to control, PD means palbociclib, MLT means melatonin and PD + MLT means the combination of palbociclib with melatonin. Data are presented as mean  $\pm$  SD (n = 4). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared with the control group.  $\epsilon$ P < 0.05 PD + MLT compared with the PD/MLT group in T47D/MCF7 cells.

### MLT Enhanced the Inhibitory Effect of PD on the Cell Cycle g0/g1 Phase Arrest of Breast Cancer Cell

To confirm the effect of PD combined with MLT on the cell cycle arrest, T47D and MCF7 cell lines were treated with PD (0.3  $\mu$ M) and

MLT (1 mM) for 48 h and confirmed by flow cytometry (FCM) (Figures 4A & 4B). The percentages of G0/G1 increased to 89.29%, 77.75% at the combination of MLT and PD, Meanwhile, the PD (0.3  $\mu$ M) treatment group and MLT (1 mM) treatment group were 77.05%, 68.91% and 73.38%, 64.81% for 48 h in T47D and MCF7 cells, respectively.



**Figure 4:** The combination of MLT and PD enhanced cell cycle G0/G1 phase arrest in BC cell by flow cytometry assay.

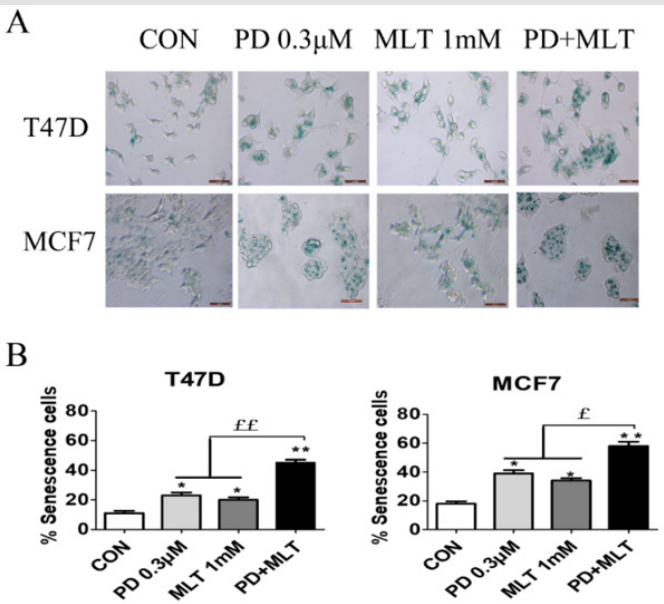
- a) T47D and MCF7 were treated with PD (0.3 μM) and MLT (1 mM) for 48 h respectively and stained with PI.  
 b) The column charts were used to show the different proportions in G0/G1, S and G2/M phases. CON means to control, PD means palbociclib, MLT means melatonin and PD + MLT means the combination of palbociclib with melatonin. Data are presented as mean ± SD (n = 4). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared with the control group. £P < 0.05 PD + MLT compared with the PD/MLT group in T47D/MCF7 cells.

### MLT Increased the Effect of PD on Cell Senescence of Breast Cancer Cell

By using the SA-β gal staining kit, we detected that the combined of PD and MLT had an influenced-on senescence in breast cancer cells. T47D and MCF7 cell lines were treated with the combination of PD (0.3 μM) and MLT (1 mM) for 48 h. SA-β galactosidase activity was observed with a Leica DM 14000B microscope (Germany) and senescence cells showed a deep blue (Figures 5A & 5B). The results suggested that MLT increased the percent of senescence cells of PD compared to PD treatment alone.

### The Combination of MLT and PD Enhanced the Inhibitory Effect on RB/E2F Signaling Pathway

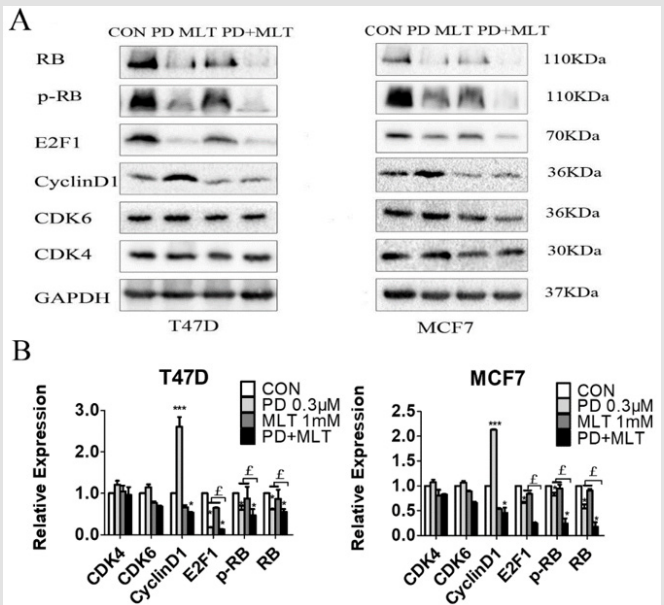
In order to further detect the efficacy of PD and MLT combination on the cell cycle, we analyzed the cell cycle-related proteins through western blotting (Figures 6A & 6B). We found that p-RB, total RB and E2F1 declined in T47D and MCF7 with the treatment of PD (0.3 μM) for 48 h; however, cyclinD1 protein was upregulated in BC cell lines, while CDK4 and CDK6 were not dramatically changed. Meanwhile, MLT (1 mM) decreased the expression of cyclinD1, CDK4, CDK6, p-RB and E2F1 in BC cells. The combined treatment of PD (0.3 μM) and MLT (1 mM) could have down-regulated the protein expression of p-RB, total RB, E2F1 and cyclinD1.



**Figure 5:** The combination of MLT and PD contributed to cell senescence in BC cell lines.

a) T47D and MCF7 cells were treated with PD (0.3 µM) and MLT (1 mM) alone or their combination for 48 h. Senescence β-Galactosidase staining Kit was used to detect the cell senescence.

b) The data was quantitated. CON means to control, PD means palbociclib, MLT means melatonin and PD + MLT means the combination of palbociclib with melatonin. Data are presented as mean ± SD (n = 4). The level of significance was indicated by \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. £P < 0.05 PD + MLT compared with the PD/MLT group in T47D/MCF7 cells.



**Figure 6:** The treatment of PD (0.3 µM) and MLT (1 mM) had an effect on cell cycle in BC cell lines by western blotting assay.

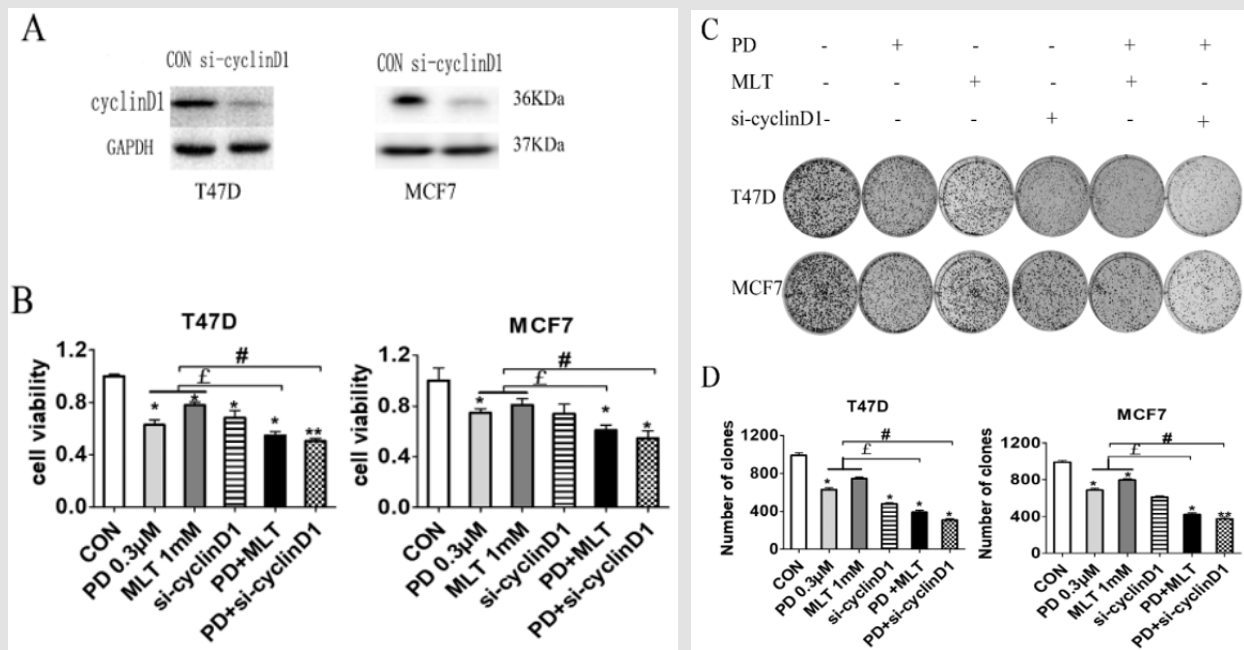
a) T47D/MCF7 was treated with PD (0.3 µM) and MLT (1 mM) alone or their combination for 48 h.

b) The levels of total and phosphorylated RB, E2F1, CyclinD1, CDK4 and CDK6 were quantified for GAPDH. CON means to control, PD means palbociclib, MLT means melatonin and PD + MLT means the combination of palbociclib with melatonin. Data are presented as mean ± SD (n = 4). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 compared with the control group. £P < 0.05 PD + MLT compared with the PD/MLT group in T47D/MCF7 cells.

## Si-RNA of CyclinD1 Down-Regulated the Expression of CyclinD1 and Enhanced the Efficacy of PD

To verify whether cyclinD1 plays an important role in enhancing the activity of PD, we knocked down cyclinD1 by using si-RNA in T47D and MCF7 cells; the expression of cyclinD1 was significantly declined (Figure 7A). In addition, the cells were treated with PD, MLT, cy-

clinD1-siRNA, PD plus MLT and PD plus cyclinD1-siRNA respectively. MTT assay showed that knocking down of cyclinD1 by cyclinD1-siRNA could inhibit the proliferation of BC cells (Figure 7B), and the colony numbers and sizes of BC cells with cyclinD1-siRNA were also decreased. The combination of PD with either MLT or cyclinD1-siRNA was more effective than PD, MLT or cyclinD1-siRNA alone (Figures 7C & 7D).



**Figure 7:** MLT suppressed the cyclinD1 upregulation caused by PD to enhance cell survival and proliferation.

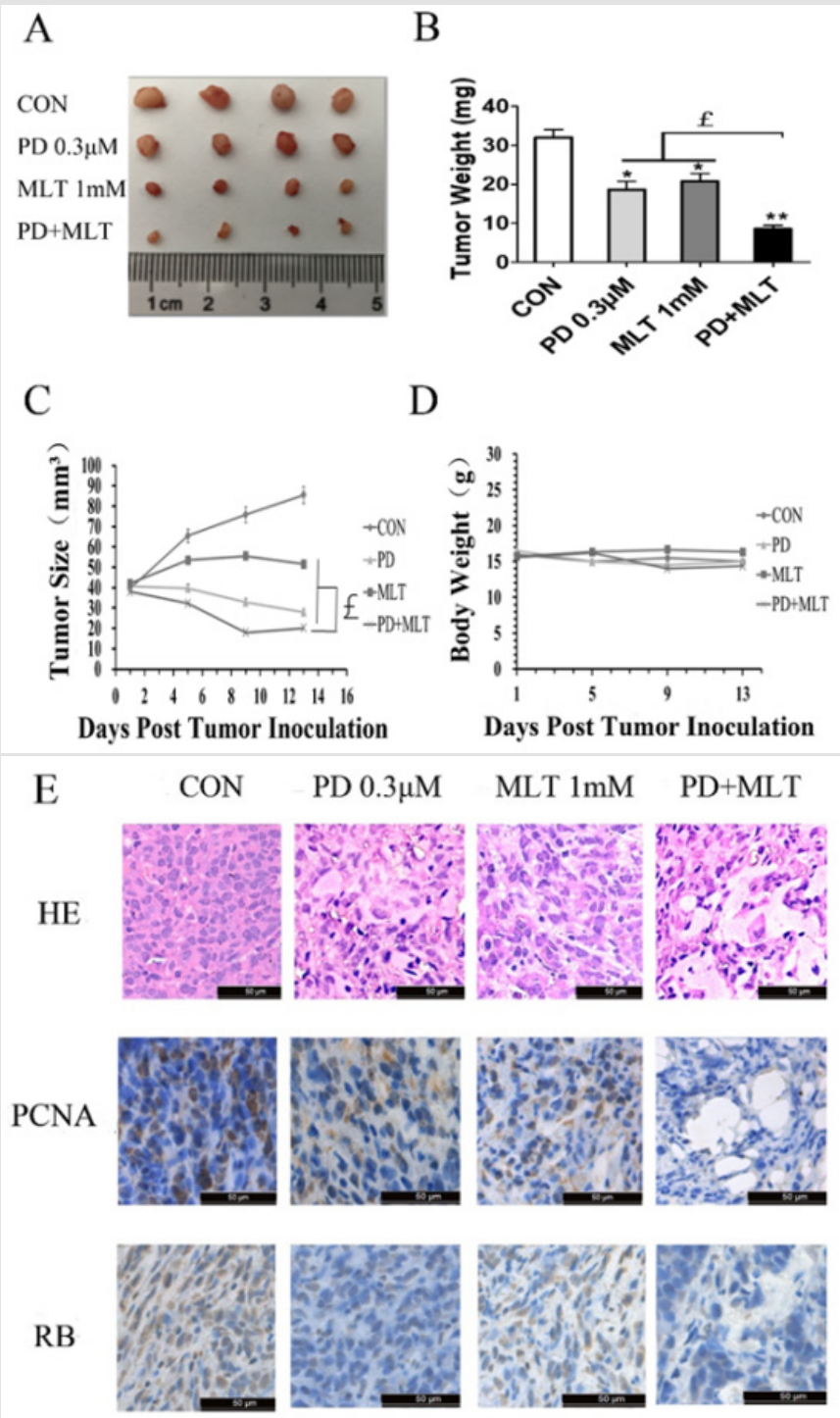
- The BC cells were knocked down of cyclinD1 by cyclinD1-siRNA.
- The cell viability was determined by MTT assay in CON, PD alone, MLT alone, cyclinD1-siRNA alone, the combination of PD with MLT and the combination of PD with cyclinD1-siRNA respectively.
- Colony-forming efficacy was detected after 2 weeks in all the above groups.
- Colony formation results were calculated. CON means to control, PD means palbociclib, MLT means melatonin and PD + MLT means the combination of palbociclib with melatonin. Data are presented as mean  $\pm$  SD (n = 4). \*P < 0.05 compared with CON in T47D and MCF7 cells.  $\#P$  < 0.05 PD + MLT compared with the PD/MLT group in T47D/MCF7 cells.  $\#P$  < 0.05 the combination of PD with cyclinD1-siRNA compared with PD/MLT alone group in T47D cell.

## The Combination of MLT and PD Inhibited Tumor Growth in Breast Cancer *in Vivo*

We further investigated the efficacy of the combination of PD and MLT *in vivo* (Figure 8D), the co-treatment of PD and MLT alone did not change the body weight of the mice. Moreover, co-treatment of PD with MLT significantly inhibited tumor volume and tumor weight when compared with the treatment of PD alone (Figures 8A & 8B). The tumor sizes were much smaller compared with PD or MLT alone

(Figure 8C). The morphological changes of tissues were observed by using HE staining. The tissue sections of tumors from the mice of the control group displayed hyperproliferative cellular formation with higher density, while those from the mice exposed to the combination of PD and MLT showed more disassembled or sparse cellular structures compared to PD or MLT treatment alone. Furthermore, the percentage of PCNA-positive and RB-positive cells in tumors from mice exposed to co-treatment was obviously lower than in those exposed to single treatment (Figure 8E).





**Figure 8:** The combination of MLT and PD significantly reduced xenograft tumor growth.

a) Tumor volume of each group of nude mice was measured.

b) Tumor volume of each group of nude mice was calculated the tumor volume.

c) The tumor sizes were calculated.

d) Body weight was not changed in the tumor reached 100 mm<sup>3</sup> after cell inoculation.

e) Tissue slides were analyzed by immunohistochemistry (IHC) using anti-PCNA and anti-RB antibodies. The level of significance was indicated by \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, EP < 0.05 PD + MLT compared with the PD/MLT group in T47D/MCF7 cells.

## Discussion

Breast cancer (BC) ranks second in causes of death induced by all cancers in the USA [18]. This high rate of BC-related deaths makes BC the most common cause of death in women. Treatment strategies that combined adjuvant chemotherapy with surgery could improve survival rates; however, patients eventually acquired resistance to chemotherapeutic agents, which is a common problem [19]. Palbociclib (PD 0332991) is one of the selected CDK4/6 inhibitors, which also included Ribociclib (LEE011) and Abemaciclib (LY2835219), and showed great potential in the treatment of ER+/HER2-metastatic breast cancer [20]. PD down-regulated the signal pathway of RB/E2F by reducing the RB protein phosphorylation and the release of the transcription factor E2F. Additionally, it restrained the up-regulation of the E2F-responsive gene which promotes cell proliferation with cell cycle G1/S transition. CDK4/6 inhibitors exhibited encouraging effects in clinical; however, there were some major limitations for adverse events, such as neutropenia (56%) and leukopenia (25.2%), which possibly limited the therapeutic benefits [21]. MLT has the function of regulating circadian rhythms, anti-inflammation and anti-oxidation, and inhibits the proliferation of cancer cells and pulmonary arterial smooth muscle cells [22]. MLT has an especially efficacy on hormone-dependent cancers, and it helps to prevent the side effects of chemotherapy and radiation therapy [23]. Meanwhile, MLT reduces the toxicity and side effects of anti-cancer drugs while increasing their efficacy.

Some studies indicated that melatonin could enhance anti-proliferation ability on synoviocytes, prostate cancer and breast cancer [24]. Our previous study has demonstrated that melatonin synergized with the chemotherapeutic effect of 5-FU to inhibit the growth of colon cancer by enhancing the anti-proliferative, migration inhibitor, and pro-apoptotic activities [25]. Furthermore, we also found that melatonin inhibits the proliferation of breast cancer cells induced by bisphenol A via targeting estrogen receptor-related pathways [26]. MLT down-regulated cyclinD1, and arrested cells to G0/G1 phase and G2/M phase [10]. In our current study, the results indicated that MLT also down-regulated the p-RB and E2F1 protein expression. In addition, it had been proved that MLT could reduce the serum estradiols level and estrogen receptor expression [27], leading to a lower rate of cyclinD1-CDK4/6 compound. Meanwhile, MLT could decrease CDK4 and cyclinD1 to arrest the cell cycle. We investigated the combined anti-tumor activity of PD and MLT in BC cells *in vitro* and *in vivo*. We found that MLT in combination with PD had a stronger effect than PD alone on BC tumor cell proliferation, survival and migration. Flow cytometry analysis demonstrated that MLT enhanced the cell cycle to be arrested in the G0/G1 phase. Western Blot results showed that MLT could enhance the p-RB/E2F signaling pathway of PD, and lead to the decreased expression of p-RB, total RB and E2F1. SA- $\beta$  gal staining also showed that MLT could enhance the BC cells senescence of PD.

Furthermore, the combined treatment of PD and MLT significantly enhanced the inhibitory effect on tumor growth of PD. These re-

sults suggested that MLT could increase the efficacy of PD *in vitro* and *in vivo*. It has been demonstrated that cyclinD1 plays an important role in the progression of cancer. Elevated cyclinD1 promotes breast cancer growth and occurrence [28,29]. Down-regulating the level of cyclinD1 inhibits cell proliferation and enhances cell cycle arrest [30]. In this study, we found that cyclinD1 expression showed a distinct difference with the treatment of PD and MLT respectively in BC cell lines. PD could up-regulate the expression of cyclinD1 protein; however, MLT down-regulated its expression. MLT has shown promising anti-cancer effects against a variety of cancer types correlated with the overexpression of cyclinD1, which is one of the important oncogenes [31]. In order to demonstrate that MLT downregulated the increased cyclinD1 to influence the efficacy of PD, cyclinD1 was knocked down for further study. Knocking down of cyclinD1 by cyclinD1-siRNA enhanced the inhibitory effects of PD on proliferation and survival by using cell viability assay and colony formation assay in breast cancer cells. Based on our experimental data above, the mechanism of MLT increasing the efficacy of PD might be associated with down-regulating cyclinD1 by MLT. Previous studies have documented that MLT had very low toxicity compared to most drugs used to treat cancer [32] and could enhance the efficacy of chemotherapy drugs, such as tamoxifen and 5-Fluorouracil [25].

Our research revealed that the combination of PD with MLT obviously enhanced the proportion of cell cycle arrest in the G0/G1 phase and improved the anti-tumor capability of PD. In addition, we found that MLT in combination with PD had a more obvious effect on BC tumor cell migration. A key for sensitivity to PD in breast cancer may be linked to RB protein [33]. By comparing the effect of CDK4/6 inhibition on the invasive potential of RB-high and RB-negative cells, it had been found that PD significantly impaired invasion of RB-high cells [34]. Meanwhile, it's reported that MLT could also repress the metastasis of Her2-positive human BC cells by suppressing RSK2 expression [35]. Through SA- $\beta$  gal staining, we also found that MLT increased the effect of PD on breast cancer cell senescence. Cellular senescence is usually representative of cell proliferation-specific inhibition [21]. It had been reported that PD could induce cellular senescence to prevent tumorigenesis [36]. PD-triggered senescence was partially RB-independent, as depletion of RB decreased senescence [37]; reactive oxygen species were also associated with senescence as another stress response signal [38]. Meanwhile, MLT could induce cell senescence by inhibiting the overgrowth of cancer cells [39]. It has been reported that MLT had effects on apoptosis in some tumor cells [40], while our results showed that the co-treatment of PD (0.3  $\mu$ M) and MLT (1 mM) did not induce a higher proportion of cleaved PARP, cleaved caspase-3 than the treatment of PD or MLT alone.

Our research demonstrated that MLT could increase the efficacy of PD to inhibit the growth of breast cancer by enhancing the inhibiting effect on proliferation, cell viability, migration, and cell cycle activity and so on, *in vitro*. The increased efficacy of combination may be closely related to the down-regulated expression of cyclinD1.

Meanwhile, MLT and PD exerted a synergistic antitumor effect in the xenograft mouse model. In conclusion, our findings indicated that the combination of PD and MLT may be a useful potential therapy in clinical practice for the treatment of breast cancer.

## Acknowledgments

We thank that the Institute of Cancer Stem Cell at Dalian Medical University for her support for FACS service.

## Funding Statement

The National Natural Science Foundation of China (No. 81472492), Postdoctoral Fellowship Program of CPSF (GZC20232306) and Zhejiang Province postdoctoral research project (ZJ2023043) offer support to this article.

## Author Contributions

The authors confirm contribution to the paper as follows: Z. Zhuang was the first author. Z. Zhuang and Y. Yuan designed the study, Z. Zhuang performed the experiments and analyzed the results. Z. Zhuang and Y. Yuan wrote the manuscript. All authors commented on the manuscript.

## Availability of Data and Materials

The all data and materials are available from the corresponding author.

## Ethics Approval

Use of laboratory animals under the approval of the SPF Laboratory Animal Center at Dalian Medical University.

## Conflicts of Interest

The authors declare that they have no conflict of interest.

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ISSN: 2574-1241

DOI: 10.26717/BJSTR.2024.59.009367

Yuhui Yuan. Biomed J Sci & Tech Res



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