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Original Articles

CDK4/6 inhibitor palbociclib enhances the effect of pyrotinib in HER2-positive breast cancer

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ABSTRACT

Human epidermal growth factor receptor 2 (HER2) is amplified in about 20% breast cancers. Treat of HER2 positive breast cancers has been greatly promoted in last few years, but the accompany HER2 blockade has hindered the therapeutic effect. Pyrotinib is a pan-HER kinase inhibitor that suppresses signaling through the RAS/RAF/MEK/MAPK and PI3K/AKT pathways. Palbociclib is a CDK4/6 inhibitor that inhibits cell cycle progression and cancer cell proliferation in ER+ breast cancers. We hypothesized that the combination of pan-HER kinase inhibitors and CDK4/6 inhibitors would show synergistic antitumor activity in vivo in vitro. Our data show that a combination of palbociclib and pyrotinib was highly synergistic in inhibiting cancer proliferation and colony formation. The combined treatment also induced significant decreases in pAKT and pHER3 activation, induced G0-G1 cell cycle arrest, and increased rates of apoptosis. In the xenograft model, the combination treatment demonstrated greater antitumor activity than either agent alone, with no apparent increase in toxicity. Our results offer a preclinical rationale clinical investigation of the effectiveness of a combination treatment of palbociclib with pyrotinib for breast cancer treatment.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and a major cause of cancer mortality in women [1,2]. Approximately 20% of breast carcinomas show overexpression of HER2 (human epidermal growth factor receptor 2) [3–5]. HER2 overexpression is associated with the development and aggressive progression of HER2+ breast cancer, which contributes significantly to tumorigenesis, resistance to chemotherapy, thus associates with increased risk of disease recurrence and death in this breast cancer subtype [3,6,7]. The relationship between HER2 expression and the aggressive phenotype of HER2+ breast cancer has made this gene appealing for novel gene-targeting therapies, with many HER2-targeting drugs under development [8–10].

Several HER2-targeting therapies are currently available to treat *HER2*-overexpressing breast cancer [11,12]. Trastuzumab (Herceptin), a humanized anti-HER2 antibody targeting the extracellular domain of HER2, is an effective remedy for patients with HER2-positive breast cancers [13,14]. Another treatment, Lapatinib, is an orally reversible tyrosine kinase inhibitor (TKI) that specifically inhibits the activity of *HER1*, *HER2*, and *HER1/HER2*-dependent downstream signaling pathways [15]. Other innovative HER2-targeting drugs including the monoclonal antibody Pertuzumab [16], which binds to the intracellular domain of HER2, the antibody-drug conjugate (ADC) Trastuzumab-DM1 (TDM-1) [17], small molecule TKIs such as neratinib are newer agents available for clinical use [18]. And there is also pyrotinib [19–21], although not yet available in clinic, has been proved to be an effective orally administered, irreversible pan-HER family receptor tyrosine kinase inhibitor of HER1, HER2, and HER4. Preclinical data have indicated that pyrotinib can irreversibly inhibit pan-HER receptors and can thereby efficiently inhibit the proliferation of HER2-positive cancer cells both in vivo and in vitro. Further clinical trials has proven pyrotinib as an effective drug for HER2-positive breast cancer patients [21].

The cell cycle is often dysfunctional in breast tumor tissues [22]. The transition from the G1 to the S phase is controlled by cyclin-dependent kinases 4 and 6 (CDK4/6), and retinoblastoma protein (RB)

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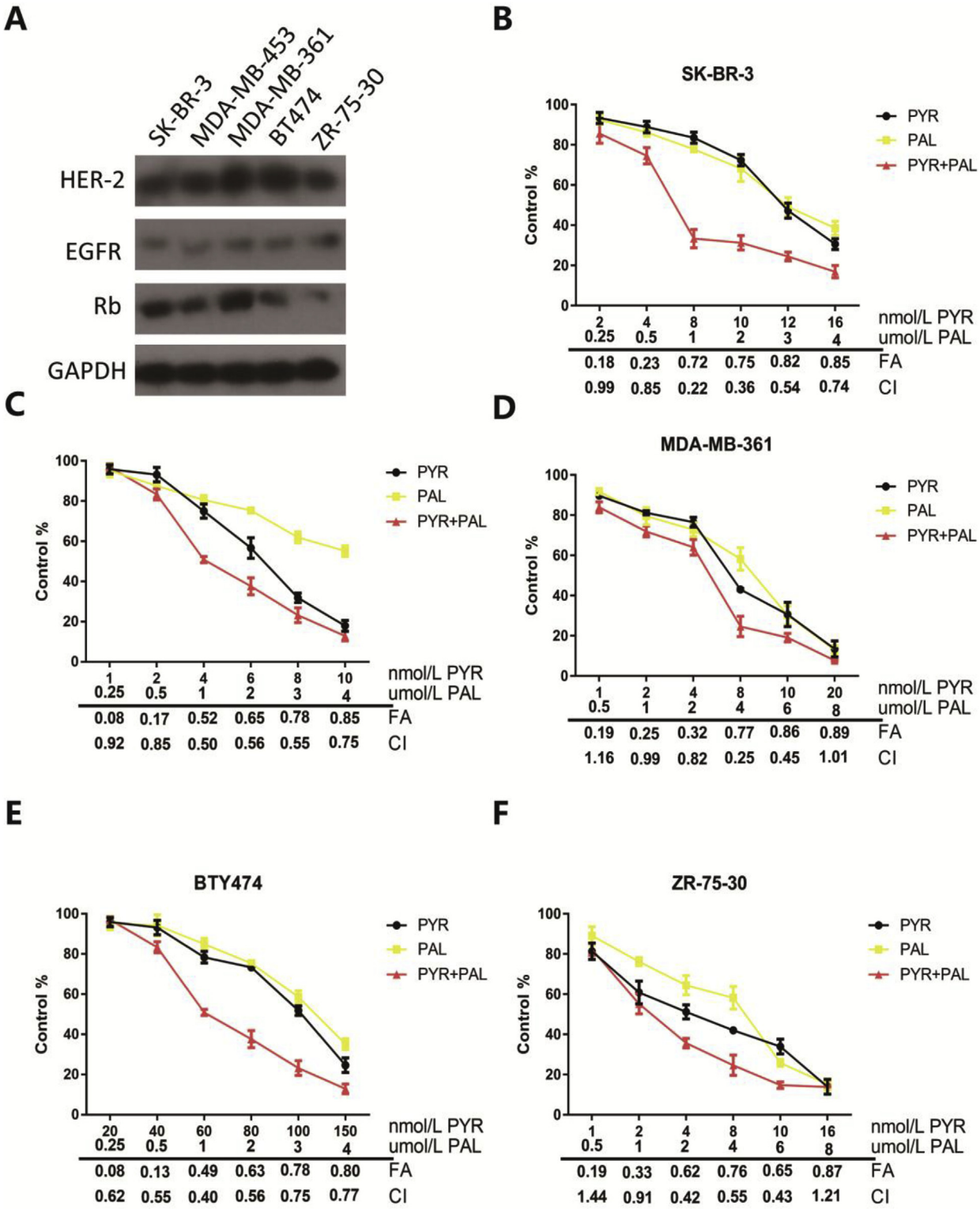


Fig. 1. Synergistic growth inhibitory effects of pyrotinib (PYR) combined with palbociclib (PAL) in a panel of HER2-amplified breast cancer cell lines. Growth inhibition was determined by MTS assay after 72 h. Five HER2-amplified breast cancer cell lines were exposed to increasing doses of PYR and PAL. Data points represent mean ± SD percent growth inhibition (from three independent experiments) compared to controls. The combined drug effect was calculated using the CI equation and was presented with FA for combinations. Synergy is defined as CI values < 1.0; antagonism as CI values > 1.0; and additivity as CI values = 1.0.

[23–25]. The cyclin D-CDK4/6-RB-E2F signaling pathway is frequently disrupted in breast cancer, and CDK4 protein expression is correlated with lower rates of surviving breast cancer [26,27]. CDK4/6 inhibitors have been shown to be an impressive remedy for HER2 positive breast carcinomas in several clinical trials [28–30]. Palbociclib (PD0332991), a CDK4/6 inhibitor, has been shown to inhibit cell cycle progression and cancer cell proliferation [31,32]. However, HER2 overexpressing breast cancer is a molecularly heterogeneous disease characterized by genomic instability and high expression of cell cycle genes (including cyclins D1 and E1) [33]. Consequently, HER2 overexpressing breast

Table 1
Pyrotinib (PYR) combined with palbociclib (PAL) in five HER2-amplified breast cancer cell lines.

Cell Line	Protein Expression[a]			IC50 (72 h)[b]	
	EGFR	HER2	RB	PYR (nM)	PAL(uM)
SKBR-3	+	+ + +	+	3.7 ± 0.1	2.0 ± 0.4
MDA-MB-453	+	+ + +	+	5.3 ± 0.3	4.3 ± 0.1
MDA-MB-361	+	+ +	+ + +	5.9 ± 0.5	1.2 ± 0.5
BT474	+	+ + +	+ +	4.7 ± 0.4	6.6 ± 0.1
ZR-75-30	+ +	+ +	+	8.9 ± 0.2	7.3 ± 0.1

[a] Previously reported in this western bloting (Fig. 1).
[b] IC50 value (drug concentration required to inhibit growth by 50%) compared with vehicle treated controls. Calculations performed using Prism 7.0 (Graphpad). Values shown are the means of 3 independent experiments ± SD.

cancers have shown resistance to single-agent CDK4/6 inhibitors. Previous studies have reported that the CDK4/6 inhibitor Abemaciclib sensitized patient-derived xenograft (PDX) tumors to HER2-targeting therapies and postponed tumor progression in a transgenic model of HER2-amplified breast malignancy [34]. CDK4/6 inhibitors were also found to cooperate with multiple HER2-targeted agents to suppress the proliferation of residual HER2-positive tumor cells that survive treatment with T-DM1 [28].

Developing combination targeted therapies is critical for the future treatment of breast cancer. In HER2 positive breast cancer, pyrotinib has been proven effective, but it still has some limitations. Palbociclib, a CDK4/6 inhibitor, has been shown to inhibit cell cycle progression and cancer cell proliferation in ER+ breast cancer. Also, data from early human clinical trials have shown that no single CDK4/6 inhibitor has been found to be successful against ErbB2-overexpressing breast cancer, suggesting that combination therapies should be used. We therefore hypothesized that the combination of palbociclib and pyrotinib would show synergistic anticancer activities against ErbB2-overexpressing breast cancers both in vivo and in vitro. Accordingly, in the current study we investigated whether a combined treatment that included both pyrotinib and palbociclib could enhance cell growth inhibition, cell cycle arrest, and apoptosis in ErbB2-overexpressing breast cancer cells. Our results showed that enhanced cell growth inhibition and apoptosis following the combination therapy was closely associated with down-regulation of pHER2, pHER3, and pRB proteins along with the inhibition of PI3K-AKT signaling pathways. These findings suggest that therapeutic strategies combining ErbB2-targeted therapies with CDK4/6 inhibitors may effectively be used in the future to treat HER2-positive breast cancer patients.

2. Materials and methods

2.1. Cell lines and cell cultures

Human HER2-positive breast cancer cell lines BT474, MDA-MB-453, MDA-MB-361, SKBR3, and ZR-75-30 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). BT474 and SK-BR-3 cells were maintained in DMEM medium supplemented with 10% FBS. MDA-MB-453, MDA-MB-361, and ZR-75-30 cells were cultured in RPMI1640 supplemented with 10% FBS.

2.2. Chemicals and antibodies

Pyrotinib (SHR 1258) was obtained from Hengrui Medicine Co. Ltd. Palbociclib (PD0332991) was obtained from Pfizer. Agents were dissolved in dimethylsulfoxide (DMSO) at a concentration of 20 mM, and were then kept at −20 °C until further use. The following antibodies were purchased from Cell Signaling Technology (Beverly, MA): pHER2 (Tyr 1221/1222), HER2, pHER3 (Tyr 1289), HER3, pAkt (Ser473), AKT, P-4EBP1 (Thr37/46), 4EBP1,p-P70S6K (Thr 389), P70S6K, pRb (Ser 780), RB, CDK4, CDK6, Cyclin D1, caspase-3, caspase-9, PARP, cleaved PARP, cleaved caspase-3, and cleaved caspase-9. Antibodies against GAPDH were purchased from Proteintech, Wuhan, China.

2.3. Cell viability assay and drug combination study

MTS cell viability assays were carried out according to the manufacturer guidelines to identify the 50% inhibitory concentrations (IC50 values) of different treatments. Cells were seeded in 96-well plates at a density of 3000–10,000 cells/well and were treated the next day with DMSO, pyrotinib, palbociclib, or both drugs in combination; these treatments continued for 72 h. Combination index (CI) values were calculated using CompuSyn (ComboSyn Inc.) [35]. The fraction affected was determined according to the following formula: (FA) = (100 – growth inhibition)/100. CI values can reveal synergistic (< 1), additive (1–1.2), or antagonistic (> 1.2) effects of two-drug combinations.

2.4. Colony formation assay

Cells were cultured in 6-well plates at a density of 500–1000 cells/well. The cells were treated with DMSO (0.1%), pyrotinib (1 nM), palbociclib (1uM), or a combination of the two agents. During the assay the culture medium was renewed every three days. After growing for 7–14 days, colonies were fixed and stained with crystal violet. Drug-treated samples were then compared directly to the DMSO control group at 100%. This experiment was performed in triplicate for each cell line.

2.5. siRNA transfection

Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used to transfect targeted siRNAs or negative control siRNAs into the SKBR-3 and BT47 cell lines. Transfection was performed in 250 μl antibiotic-free opti-MEM (Gibco, Gaithersburg, MD) and cells were incubated afterward at 37 °C for 48 h.

2.6. Cell cycle analysis

Cells were starved for 24 h before treatments were added. Treatments included DMSO (0.1%), pyrotinib (1 nM), palbociclib (1uM), or both drugs in combination. After treatment for 24 h, cells in the logarithmic growth phase were trypsinized, washed with PBS, fixed in 75% ethanol, and kept overnight at −20 °C. Fixed cells were then collected, washed, and resuspended in PBS. Cell solutions were then adjusted to 5 × 10^5 cells/mL, incubated with RNase and propidium iodide (PI) solution for 30 min in the dark at room temperature, and finally analyzed using a flow cytometer (Beckman Coulter, CA) according to the manufacturer's instructions. All assays were independently performed in triplicate.

2.7. Apoptosis analysis

After treatment for 48 h, cells were washed, adjusted to 5 × 10^5 cells/mL, resuspended in binding buffer, and stained with 5 ml of FITC Annexin V and 5 ml of propidium iodide. Stained cells were then incubated in the dark at 37 °C for 30 min. Finally, apoptosis was determined by assessing cell counts using a flow cytometer (Beckman Coulter, CA) according to manufacturer's instructions. All assays were independently performed three times.

2.8. Western blot analysis

Cells and cancer tissues were prepared with cell lysis buffer (Beyotime, Shanghai, China), into which PMSF, protease, and

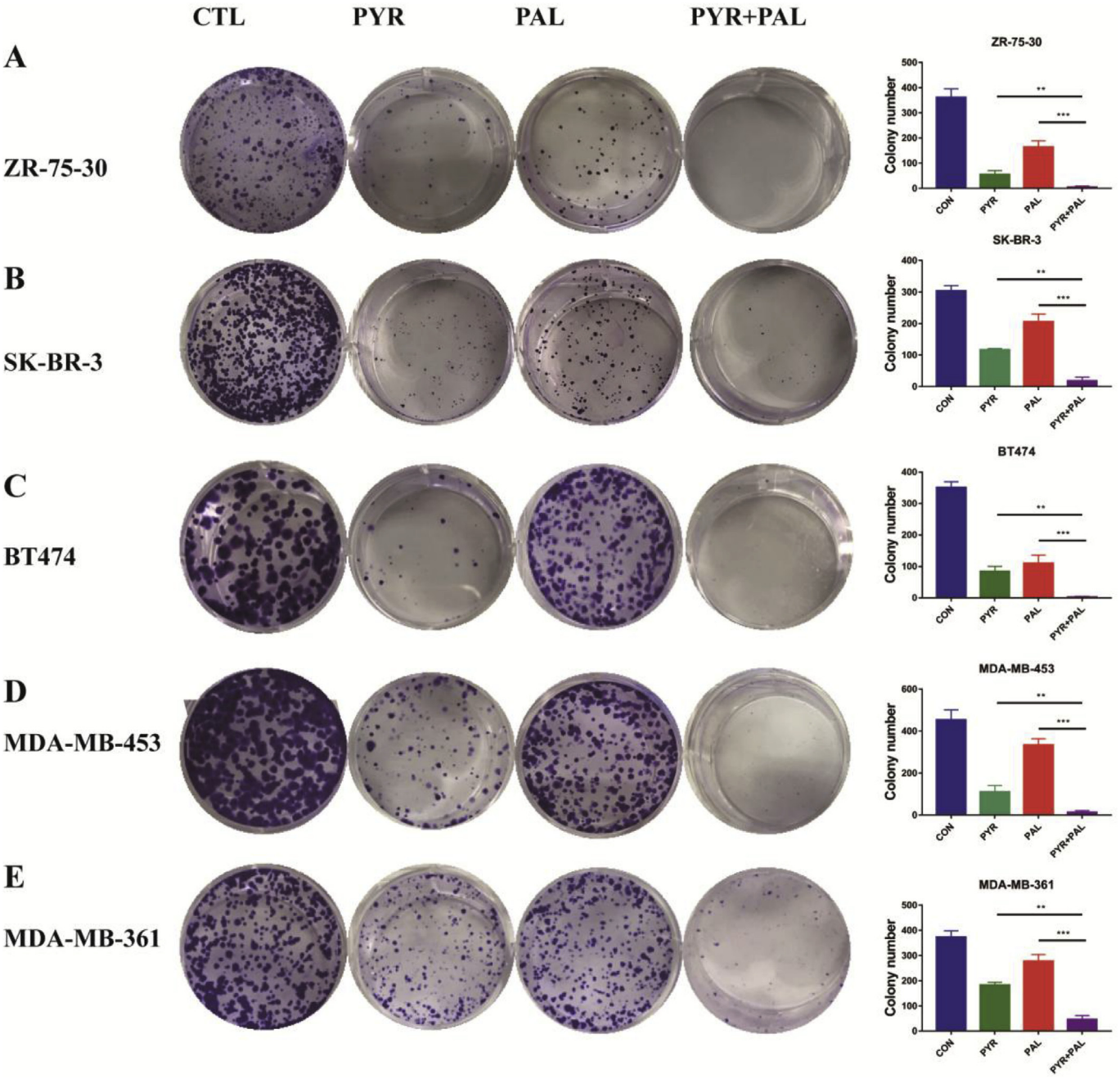


Fig. 2. A combination of HER2 and CDK4/6 inhibitors induces cell growth in vitro in a panel of RB1 wide type cell lines. (A), Colony assays for five representative cell lines treated with the HER2 inhibitor pyrotinib (PYR), the CDK4/6 inhibitor palbociclib (PAL), a combination treatment (PAL + PYR), or with a DMSO control for 10–14 days. (B) Summary colony assay results for a panel of HER2-amplified breast cancer cell lines treated with DMSO control, PYR, PAL, or PYR/PAL treatments.

phosphatase inhibitors were added. Protein concentrations were measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Proteins from cells and tissue lysates were separated by 10% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. Immunoreactive bands were detected using enhanced chemiluminescence (ECL).

2.9. Xenograft studies

Four-to five-week-old female athymic BALB/c nu-mice were raised and maintained in the animal husbandry facility of the SPF laboratory. All experiments were performed in accordance with the guidelines of the Regulations for the Administration of Affairs Concerning Experimental Animals and were approved by the Experimental Animal Ethics Committee of Sun Yat-sen University. BT474 cell suspensions (at 1×10^7 cells/100 μl) were injected into one site in the right back of each mouse. We observed 100% tumor incidence in both the BT474 and SK-BR-3 cell lines.

When tumor volumes reached nearly 100 mm^3, the mice were randomly assigned to one of four groups. Each group of mice (n = 7) was treated via daily oral gavage with vehicle, pyrotinib (10 mg/kg/d), palbociclib (100 mg/kg/d), or a combination of both drugs for 27 days. Tumors were measured 2 to 3 times each week, and tumor volume was calculated as $V = 1/2$ ($width^2 \times length$). Drug treatment continued for 28 days, after which the mice were humanely sacrificed by euthanasia. Samples of tumors were collected and analyzed by Western blotting and immunohistochemical staining.

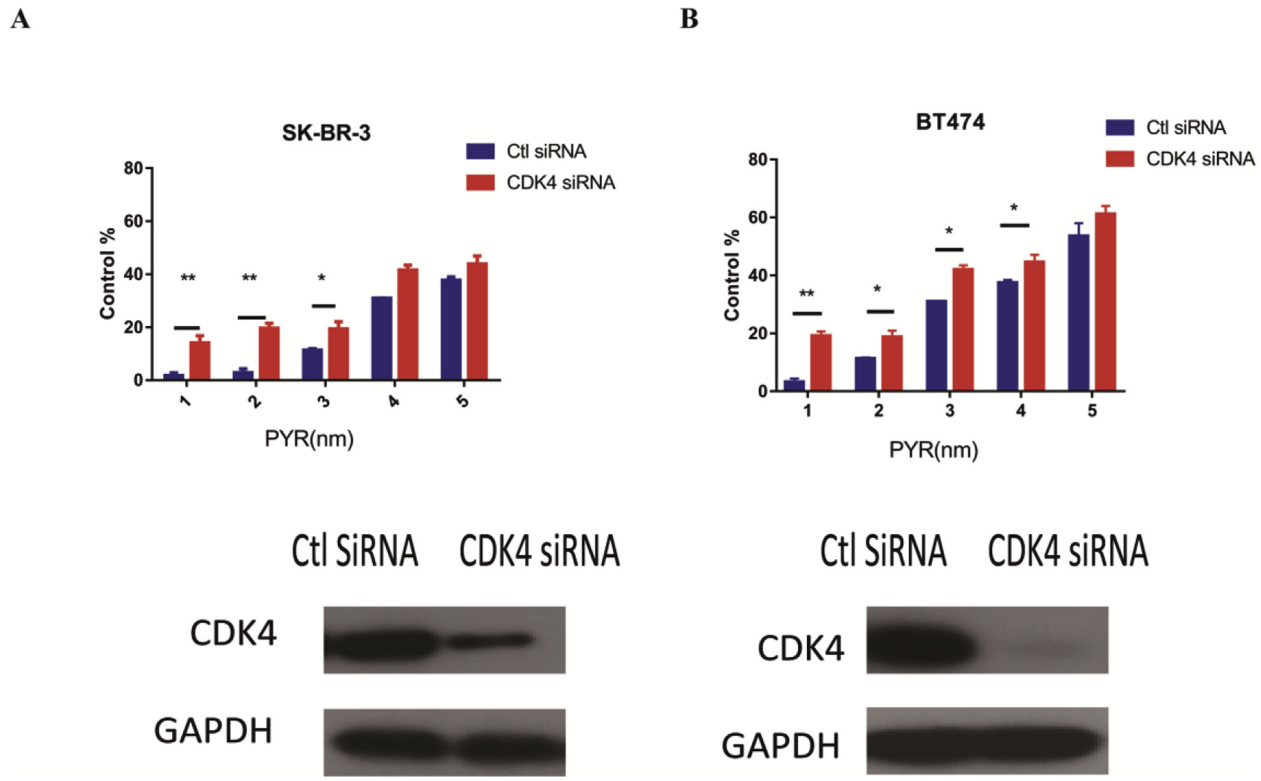


Fig. 3. CDK4 knockdown enhances sensitivity to pyrotinib. (A,B) After transfecting targeted siRNA CDK4 or negative control siRNA into SKBR-3 and BT47 cell lines, cells were seeded in 96-well plates and treated with pyrotinib at doses ranging from 1 to 100 mM for 72 h. The enhanced anti-proliferative effect of pyrotinib in CDK4 siRNA-transfected SKBR-3 and BT474 cells.

2.10. Immunohistochemistry

Tumors were fixed in 4% formalin overnight at room temperature before being embedded in paraffin. Sections were stained with anti-Ki-67, pHER3, and pAKT antibodies.

2.11. Statistical analysis

All descriptive statistics were presented as the mean ± standard deviation (SD). Analyses of variance models and student's t-tests were used to compare mean values between the test and control samples. Statistical analyses were performed with IBM SPSS version 22 (SPSS, Armonk, NY, USA) and GraphPad Prism version 7. Results were presented as the mean ± SD of three independent tests. The statistical significance of the difference between test and control samples was assessed at significance thresholds of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Pyrotinib and palbociclib synergistically inhibited the proliferation of breast cancer cells in vitro

We first examined HER2 and EGFR protein expression in a panel of HER2-positive breast cancer cell lines, and found that five of the cell lines analyzed (BT474, SK-BR-3, MDA-MB-453, MDA-MB-361, and ZR-75-30) showed high HER2 and RB expression (Fig. 1). We next evaluated the individual abilities of pyrotinib and palbociclib to inhibit the growth of the BT474, SK-BR-3, MDA-MB-453, MDA-MB-361, and ZR-75-30 breast cancer cell lines. Our results showed that pyrotinib suppressed the tumor proliferation of both trastuzumab-sensitive (BT-474 and SKBR-3) and trastuzumab-resistant (MDA-MB-453, MDA-MB-361, and ZR-75-30) cell lines (Table 1). Relative to the BT-474 and SKBR-3 cell lines, the MDA-MB-453, MDA-MB-361, and ZR-75-30 lines were more sensitive to pyrotinib (Table 1). Palbociclib also suppressed the tumor proliferation of HER2-positive cell lines (Table 1). Next, to determine whether a CDK4/6 inhibitor.in combination with a HER2 inhibitor has a synergistic influence on HER2 positive breast cancer cells, we assessed the efficacy of various concentrations of the combination of pyrotinib and palbociclib to inhibit the proliferation of BT474, SK-BR-3, MDA-MB-453, MDA-MB-361, and ZR-75-30 cells. To determine whether the antitumor effects were synergistic, we calculated the combination index for each cell line using the method of Chou and Talalay to establish drug CI values [35]. We observed that co-treatment with pyrotinib and palbociclib led to growth inhibition in all five HER2-positive breast cancer cell lines (Fig. 2). Moreover, these treatments revealed synergistic CI values that were consistently less than 1.

3.2. A pyrotinib and palbociclib combination treatment effectively reduced the ability of cells to form colonies in vitro

We performed colony formation assays to determine whether the combination of pyrotinib and palbociclib had an enhanced anti-tumorigenic effect in HER2-positive breast cancer cells beyond the effect of each drug on its own. Preliminary trials indicated that the IC50 dose for both drugs would have a strongly negative effect on colony formation. Then BT474, SK-BR-3, MDA-MB-453, MDA-MB-361, and ZR-75-30 cells were treated with both drugs, the number of colonies was significantly decreased compared with those cells treated with single drugs ($P < 0.001$). A similar reduction was seen in colony sizes (Fig. 2).

3.3. CDK4 knockdown enhances sensitivity to pyrotinib

We used siRNAs to knock down CDK4 in BT474 and SK-BR-3 cells, and treated the cells with various concentrations of pyrotinib

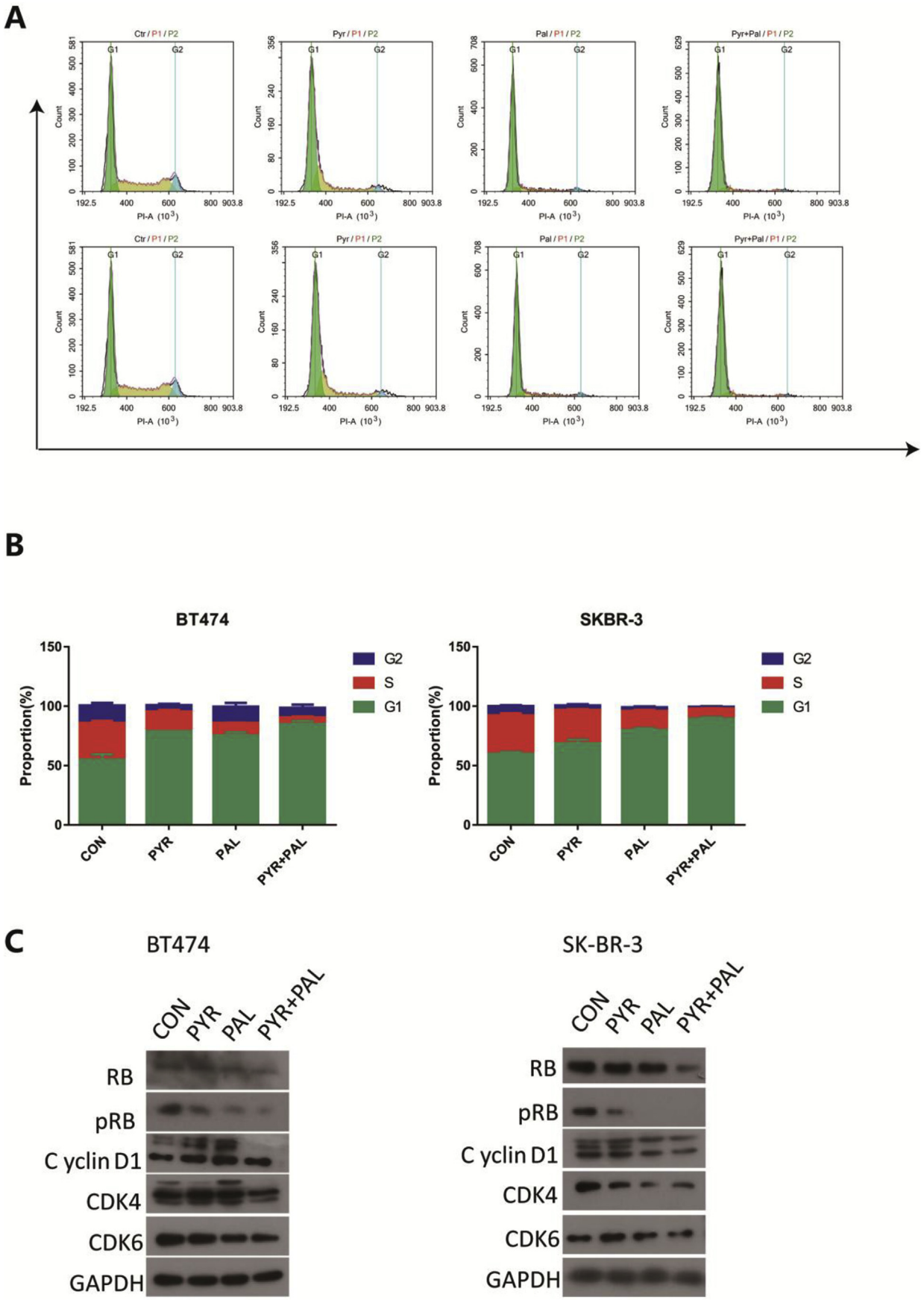
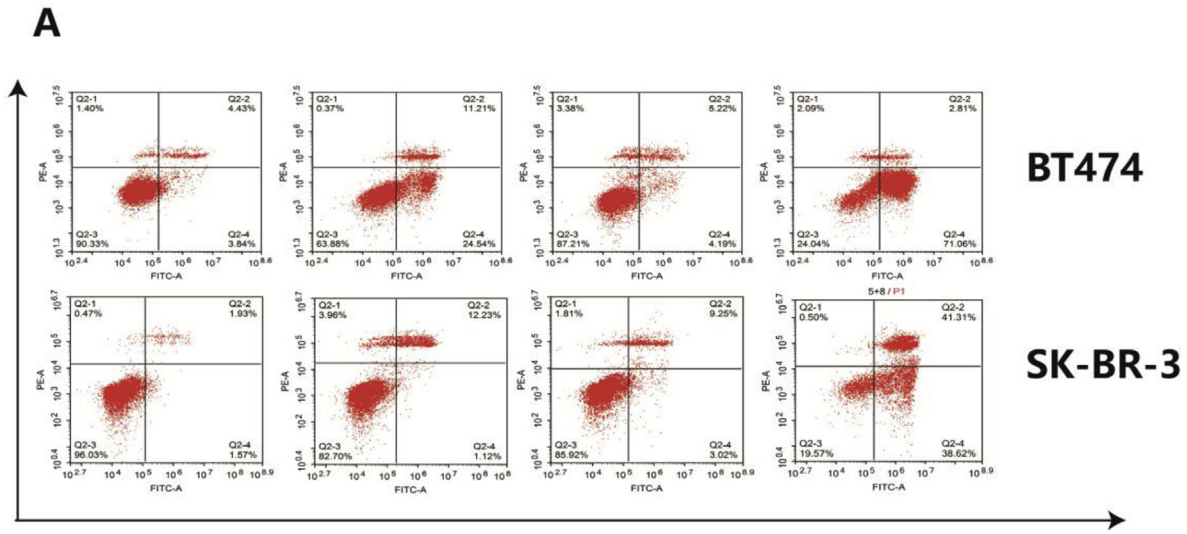
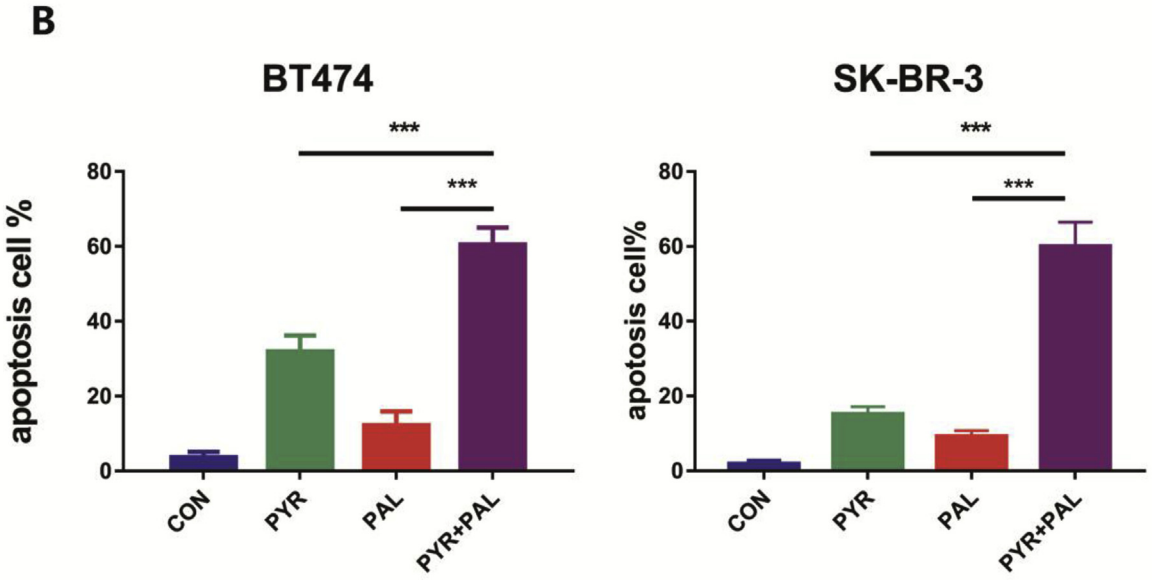


Fig. 4. Pyrotinib combined with palbociclib induces G1/S arrest in HER2-positive breast cancer cells. (A,B) SKBR3 and BT-474 cells were treated with the indicated concentrations of pyrotinib (1 nm), palbociclib (1um) or a combination of the two compounds for 48 h. Treatments dramatically induced G1/S arrest in HER2-positive breast cancer cells after 48 h of treatment. Data are expressed as mean ± SD of three independent experiments. Proteins were analyzed by Western blotting with antibodies 48 h after treatment.





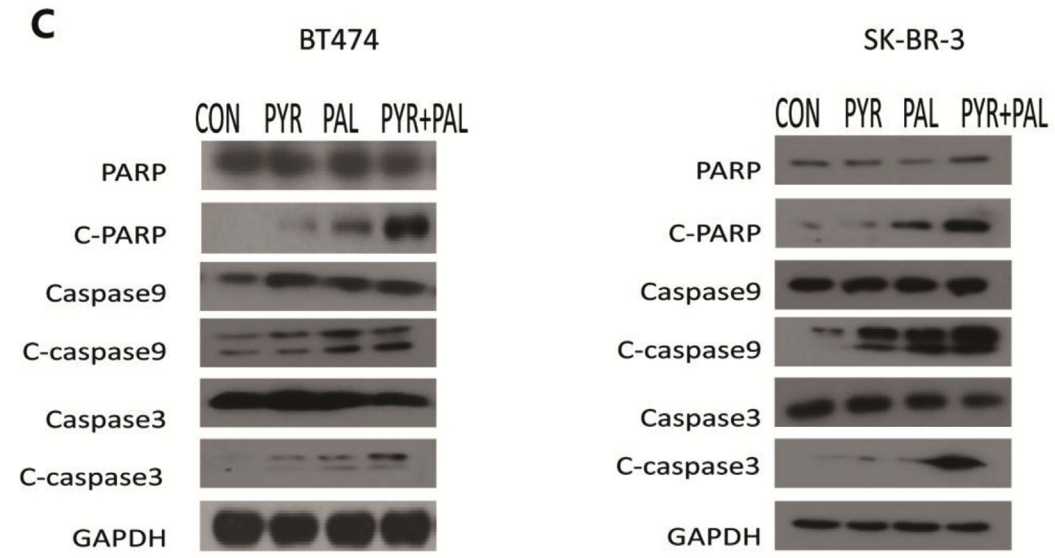


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Fig. 5. Pyrotinib combined with palbociclib induces apoptosis in HER2-positive breast cancer cells. SKBR3 and BT-474 cells were treated with the indicated concentrations of pyrotinib, palbociclib, or a combination of the two compounds for 48 h. (A) BT474 cells dramatically induced apoptosis in HER2-positive breast cancer cells after 24 h of treatment. Protein levels were analyzed by Western blotting with the indicated antibodies 24 h after treatment. A GAPDH antibody was used as loading control. (B) BT474 cells induced apoptosis in HER2-positive breast cancer cells 24 h after treatment. Protein levels were analyzed by Western blotting with the indicated antibodies.

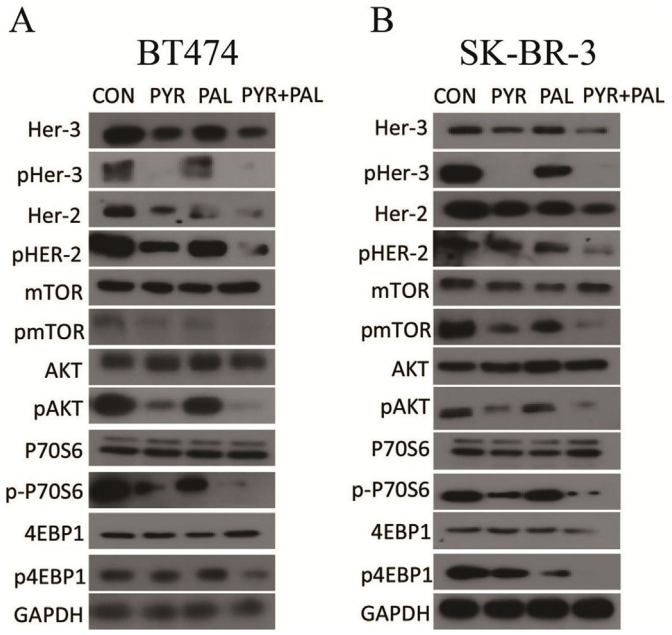


Fig. 6. A combination of CDK4/6 and HER2 inhibitors induces a greater inhibition of phosphorylation of HER2, AKT, and other growth factor signaling proteins. (A) Protein lysates were obtained from BT474 and SK-BR-3 breast cancer cell lines after treatment with DMSO control, pyrotinib (PYR) at a concentration of 1 nM, palbociclib (PAL) at a concentration of 1uM, or a combination (PAL + PYR) for 24 h.

(0–100 nM). The inhibition rate was found to be significantly higher in CDK4 siRNA-transfected cells than in control siRNA-transfected cells (Fig. 3A B). Thus, selective inhibition of CDK4 may enhance the effect of pyrotinib in RB1 wild-type BT474 and SK-BR-3 cells.

3.4. Pyrotinib and palbociclib induced G1/S arrest and apoptosis in HER2-amplified cell lines

Having determined that pyrotinib and palbociclib showed a very selective growth-inhibition effect on the five HER2-amplified breast cancer cell lines, we analyzed their effects on the cell cycle to identify the underlying mechanism responsible for this inhibition. The two most sensitive cell lines, BT474 and SK-BR-3, were incubated for 72 h h with either 1 nM of pyrotinib or 1 μM of palbociclib, after which the cells were analyzed by flow cytometry using PI-DAPI staining. The percentage of BT474 cells arrested in the G1 phase was significantly increased in response to the combined treatment (1uM palbociclib and 1 nM pyrotinib). A similar phenomenon was observed in the SK-BR-3 cells. A Western blot analysis revealed that pyrotinib and palbociclib potently suppressed CDK4, CDK6, cyclin D1, and pRB (Fig. 4AB). Similarly, the concomitant inhibition of CDK4/6 and HER2 signaling in both BT474 and SK-BR-3 cells enhanced apoptotic cell death compared to the inhibition of a single signaling pathway. A Western blot analysis revealed that pyrotinib and palbociclib potently stimulated the cleavage of PARP, caspase3, and caspase 9 (Fig. 5AB).

3.5. The combination of pyrotinib and palbociclib potentiated the suppression of RB phosphorylation and the downregulation of PI3K-AKT

Next, we used Western blots to assess protein content of cell lines after treatment with DMSO, pyrotinib, palbociclib, or with a combination of the two agents. We found that the expression of phosphorylate-AKT was noticeably decreased by pyrotinib while the phosphorylation of RB was strongly inhibited. Palbociclib inhibited p-RB expression but had no obvious effect on the expression of p-AKT. However, the protein expression level of p-RB sharply decreased after the combination treatment, which coincided with a significant reduction in pAKT and its downstream signaling pathways (Fig. 6AB).

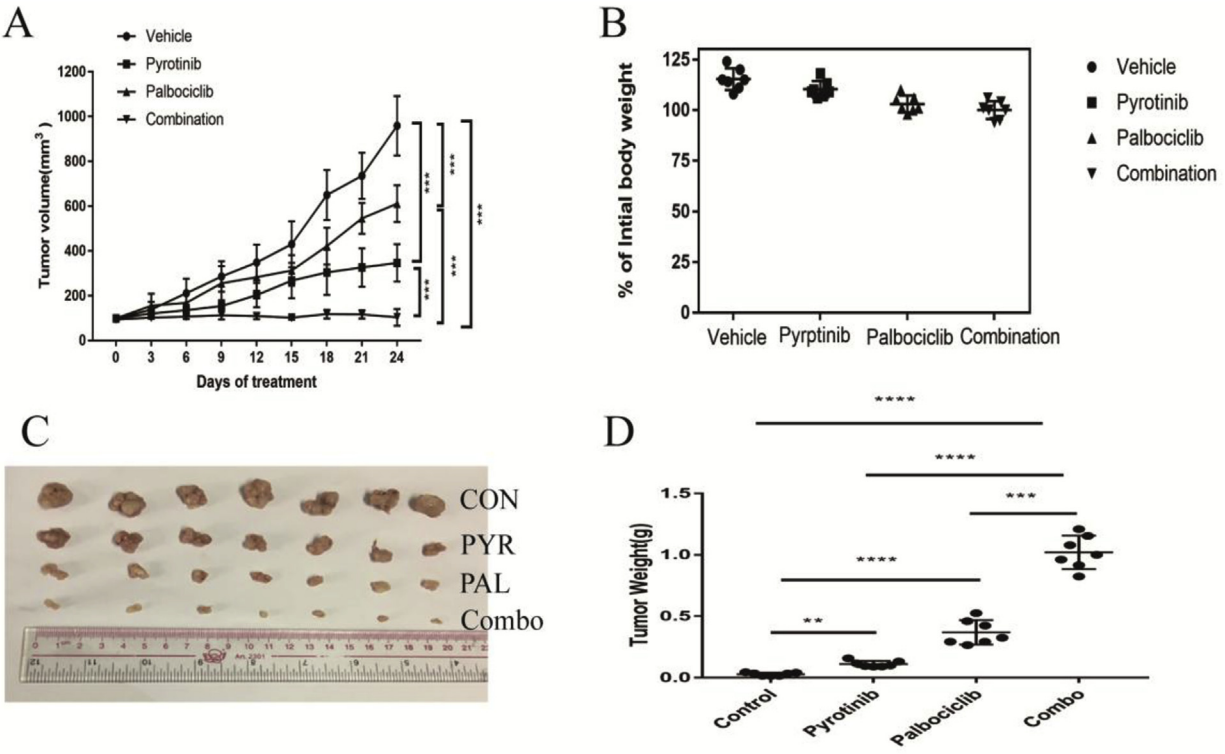
3.6. Pyrotinib and palbociclib suppressed the growth of HER2-positive breast cancer xenografts

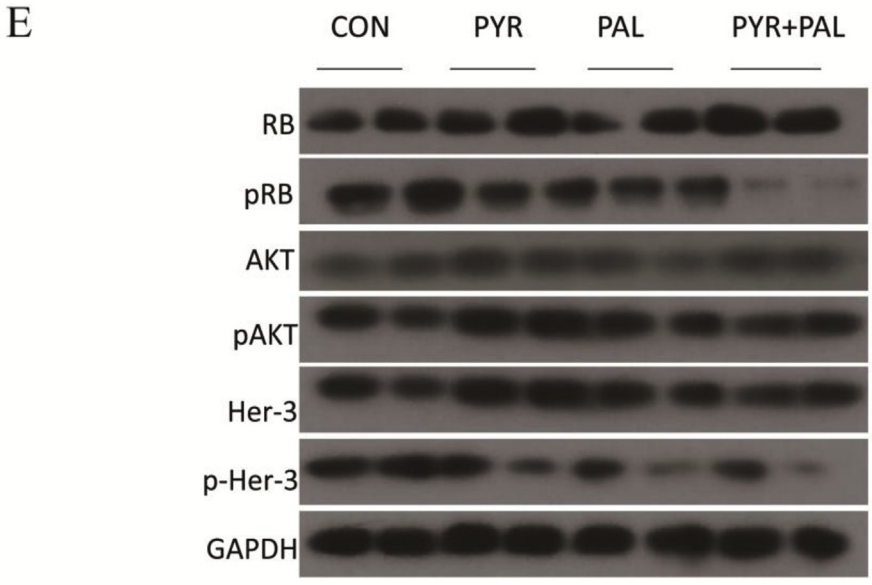
After confirming that the combination of pyrotinib and palbociclib reduced cell proliferation and cell growth in vitro, we examined whether these two drugs—either individually or together—could inhibit tumor growth in a xenograft mouse model of HER2-amplified breast cancer. Mice with BT474 xenografts were randomized into four treatment groups. These groups included a control group, as well as groups given pyrotinib (10 mg/kg every other day), Palbociclib (100 mg/kg weekly), or both pyrotinib and palbociclib. We found that pyrotinib was more effective than palbociclib at inhibiting BT474 tumor growth (Fig. 7A). However, the combination of pyrotinib and palbociclib exerted a much stronger inhibitory effect on BT474 xenograft growth than either drug exerted alone (Fig. 7C D). Pyrotinib showed the best safety profile with respect to variation in body weight, with the combination treatment group showing better body weights than the group treated only with palbociclib. These data demonstrate that a combined treatment including both palbociclib and pyrotinib can augment anticancer activity without increased toxicity (Fig. 7B).

Protein analysis from tumor tissues also showed that total HER2, AKT, and p70S6K (as well as their phosphorylated forms) contents were significantly decreased in drug treatment groups (Fig. 7E). Our immunohistochemistry and Western blot results suggested that pyrotinib coupled with palbociclib was correlated with a distinct decrease in pHER2 expression in BT474 tumor tissue. This is indicative of a powerful synergetic effect of the combination of pyrotinib and palbociclib for HER2 treatment (Fig. 7E). Moreover, although immunohistochemistry and Western blot results both showed that pyrotinib-treated pHER3 was decreased in BT474 tumor tissue, pyrotinib and palbociclib treatment showed greater decreases in pHER2 and pHER3 expression (Fig. 7E and F). These data are consistent with the results obtained at the cellular level and confirmed the synergistic benefit of a combined approach to targeting CDK4/6-RB, HER2 and their downstream signaling pathways.

4. Discussion

To hamper or delay the development of resistance to chemotherapy and/or other targeted therapies, cancer patients are often treated with combinations of conventional cytotoxic agents that exhibit noncross resistance mechanisms of action. However, new targeted therapies are needed for treatment of breast cancers. Signaling kinases such as CDK4/6 and HER2 are activated in high grade HER2 amplified breast cancers. The main purposes of using combined drug treatments are to realize synergistic therapeutic effects, to reduce toxicity by lowering the dose,





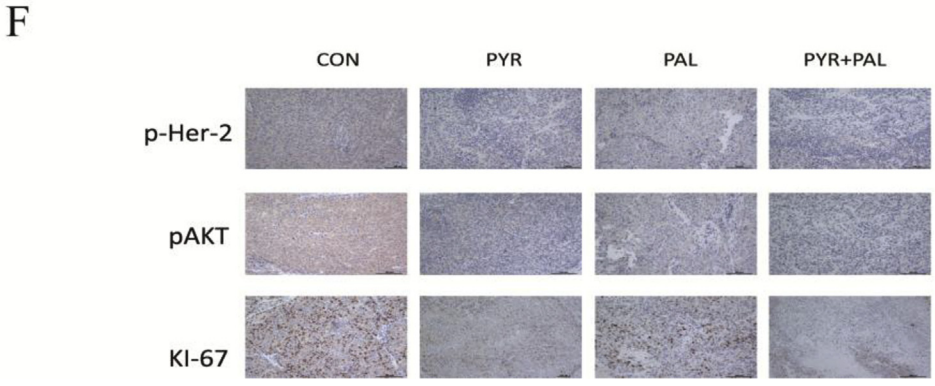


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Fig. 7. Antitumor activity of pyrotinib (PYR) and palbociclib (PAL) in BT474 xenograft models in vivo. Randomly grouped nude mice bearing BT474 tumors were treated with vehicle control, pyrotinib (10 mg/kg), palbociclib (100 mg/kg), or a combination treatment (PAL + PYR) for 27 days. (A.B) Tumor volume was determined every three days after the onset of treatment. (C.D) On day 27, tumors were carefully dissected from the mice. Body weight was also measured. (E) Harvested tumors were subsequently collected and Western blot analysis was performed to assess p-RB, RB, p-AKT, AKT, p-HER2, HER2, and GAPDH expression. (F) Representative immunohistochemistry results showing pHER2, pAKT, and KI-67 expression in BT474 tumors.

and to hamper the development of drug resistance.

Targeting CDK4/6 and HER2 is an attractive therapeutic strategy since both receptors promote cell growth and neoplasm survival. Here, we report that a combination of the pan-HER inhibitor pyrotinib and the CDK4/6 inhibitor palbociclib showed synergistic inhibition of tumor proliferation and enhanced antitumor effects in five HER2-positive breast cancer cell lines, both in vitro and in vivo. Our experimental data also showed that combined HER2 and CDK4/6 inhibition results in cell-cycle arrest at the G1 stage, thereby increasing tumor cell apoptosis and reducing cellular proliferation.

CDK and RB have been shown to have highly specific and indispensable roles in mediating anti-tumor responses to CDK4/6 inhibition. As previously reported, P70-S6K function is critical for G1 progression [36]. Inhibition of either HER2 or CDK4/6-RB-E2F signaling causes a decrease in Rb and P70-S6K/S6RP for S phase entry. This is consistent with distinctive feature studies that revealed both Rb and AKT/P70-S6K/4EBP1 are essential mediators of tumor proliferation and the transition from the G1 to S phase. Moreover, this transition is accompanied by enhanced reduction of pHER2 expression and its downstream signaling pathways.

Here, we performed a deeper investigation of the CDK4/6-HER2 relationship as well as of the mechanisms by which pRB and pAKT are affected. Our results show that synergism between CDK4/6 and HER2 inhibitors causes CDK4/6 and HER2 inhibition and prompts RB phosphorylation. Moreover, we found that the combined inhibition of CDK4/6 and HER2 induced a stronger suppression of pAKT and hence pP70S6K; indirectly, the CDK4/6 inhibitor caused a reduction in AKT phosphorylation, consequently causing cells to become more sensitive to pan-HER2 inhibitors. The result was a more effective suppression of p4EBP1 in the combination treatment group.

Our research shows that palbociclib encourages anti-tumor activity in HER2-overxepression breast cancers as a single agent inhibiting pHER2, but also that it has an added benefit of reducing activated HER3 phosphorylation (Fig. 7E). In addition, the combination of pyrotinib and palbociclib was able to block the upregulation of total and activated HER3 phosphorylation and exhibited synergistic enhancement of each drug's antitumor effects. Finally, our immunohistochemistry and Western blotting data showed that palbociclib-treated BT474 xenograft tissues had lower pHER2 expression compared to an untreated group, while pAKT expression was strongly decreased in both pyrotinib-treated and combination-treated groups (Figs. 6 and 7E). Based on these results, we speculate that combination treatments including both pyrotinib and palbociclib may be a useful strategy for realizing a blockade of pHER3 and its downstream signaling pathways.

Our findings also provide deeper insight into the toxicity in mices. In addition, mices subjected to the combinatorial treatment of our in vivo experiment did not show worse body weights than the patients in the groups treated with palbociclib or pyrotinib alone (Fig. 7B). Thus, our data show that a combination of palbociclib and pyrotinib results in enhanced antitumor activity without extended toxicity. The next phase of this avenue of research would be a randomized trial of palbociclib in patients with HER2-positive disease to prevent disease recurrence. Specifically, we recommend a clinical trial in which patients are given a pyrotinib-palbociclib combination treatment, which would yield the benefits of an effective anticancer therapy but would also avoid the toxicity associated with higher doses of pyrotinib and/or palbociclib alone. Further research should also investigate the mechanisms by which pyrotinib and palbociclib have a combined effect against HER2-therapy resistant breast cancers, and assess whether these tumors can retain dependency upon CDK4/6. This is important insofar as inhibiting CDK4/6 may be a promising therapy for preventing or delaying the recurrence of HER2-positive breast cancer.

Conflicts of interest

No author has financial or other contractual agreements that might cause conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.005.

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