Dalpiciclib and Pyrotinib Exert Synergistic Antitumor Effects in Triple Positive Breast Cancer

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Abstract

Background: The therapeutic benefit of the standard combination of anti-HER2 and chemotherapy in triple-positive breast cancer (TPBC) is limited even after the addition of endocrine therapy to the regimen. Therefore, treatment optimization is required urgently.

Methods: Through the drug sensitivity test, the drug combination efficacy of anti-HER2 drug, endocrine drug and CDK4/6 inhibitor to BT474 cells were tested. The underlying molecular mechanisms were investigated using immunofluorescence, western blot analysis, immunohistochemical staining and cell cycle analysis. Potential biomarker which may indicate the responsiveness to drug treatment in triple positive breast cancer was selected out using RNA-sequence and tested using immunohistochemical staining.

Results: We found that pyrotinib combined with dalpiciclib showed better efficacy than pyrotinib combined with tamoxifen in BT474 cells. Degradation of HER2 could enhance ER nuclear transportation, whereas cell cycle blockers could reverse this process. This may be the underlying mechanism by which the addition of dalpiciclib was more beneficial than the addition of pyrotinib plus tamoxifen. Furthermore, CALML5 was revealed to be a potential indicator of responsiveness to anti-HER2 therapy plus CDK4/6 inhibition in triple positive breast cancer.

Conclusion: Our study provided evidence for the introduction of CDK4/6 inhibitor in the treatment of TPBC and indicated that the combination of anti-HER2 therapy and cell cycle blockers may be a better strategy for TPBC treatment.

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Keywords: triple-positive breast cancer, anti-HER2 therapy, cell cycle blockers

Introduction

Human epidermal growth factor receptor 2-positive ($HER2^+$) breast cancer is associated with an increased risk of disease recurrence and death (*Perou et al.*, *2000; Slamon et al.*, *1987; Tzahar et al.*, *1996*). HER2-overexpressing breast tumors have high heterogeneity, accounting partially for the co-expression of hormone receptors (HR) (*Loi et al.*, *2016*). Previous studies have demonstrated that extensive cross-talk exists between the HER2 signaling pathway and the estrogen receptor (ER) pathway (*Wang et al.*, *2011*). In addition, exposure to anti-HER2 therapy may reactivate the ER signaling pathway, which could lead to drug resistance (*Brandao et al.*, *2020*). Generally, however, HER2-positive patients are treated using the same algorithms, both in the early and advanced stages (*Moja et al.*, *2012*). Thus, novel therapeutic strategies are urgently needed for patients with $HER2^+/HR^+$ breast cancer.

Increasing evidence has confirmed that the intrinsic differences between $HER2^+/HR^+$ and $HER2^+/HR^-$ patients should not be ignored (*Carey et al.*, *2016*). Clinical outcomes have demonstrated that $HER2^+/HR^+$ breast cancer patients have a lower chance of achieving a pathologically complete response than $HER2^+/HR^-$ patients, when treated with neoadjuvant chemotherapy plus anti-HER2 therapy (*Cameron et al.*, *2017; Cortazar et al.*, *2014*). Nevertheless, the addition of concomitant endocrine therapy to anti-HER2 therapy or chemotherapy did not show any advantages in clinical trials, such as the NSABP B-52 and ADAPT $HER2^+/HR^+$ studies (*Harbeck et al.*, *2017; Rimawi et al.*, *2017*). Therefore, whether endocrine therapy is useful in $HER2^+/HR^+$ breast cancer treatment is questionable.

Recently, the synergistic effect of CDK4/6 (cyclin kinase 4/6) inhibitors and anti-HER2 drugs in $HER2^+$ breast cancer has been reported. The combination of anti-HER2 drugs and CDK4/6 inhibitors showed strong synergistic effects and high

efficacy in $HER2^+$ breast cancer cells (*Goel et al., 2016; Zhang et al., 2019*). The combination of CDK4/6 inhibitors and HER2-targeted therapy as an alternative strategy for $HER2^+/HR^+$ patients, warrants further exploration.

Herein, we investigated the combined effect of pyrotinib (anti-HER2 drug), tamoxifen (endocrine therapy), and dalpiciclib (CDK4/6 inhibitor) on the triple-positive breast cancer (TPBC) cell line BT474. We found that pyrotinib combined with dalpiciclib showed better efficacy than pyrotinib combined with tamoxifen. In addition, HER2-targeted therapy induced nuclear ER redistribution in TPBC cells, which could be reversed by the addition of a CDK4/6 inhibitor. Furthermore, CALML5 could be a potential indicator of responsiveness to HER2-targeted therapy combined with a CDK4/6 inhibitor. Our study provided a new function of the CDK4/6 inhibitor in TPBC cells treated with anti-HER2 therapy and suggested a novel strategy for improving the clinical response in TPBC.

Results

Pyrotinib combined with dalpiciclib shows better efficacy than when combined with tamoxifen

To explore the effects of pyrotinib, tamoxifen, and dalpiciclib in TPBC, we first evaluated the cytotoxic activities of these three reagents in BT474 breast cancer cells. The results showed that the IC50 doses for pyrotinib, tamoxifen, and dalpiciclib were 10 nM, 5 μM, and 8 μM, respectively (Figure 1-figure supplement 1a). To further investigate whether these three drugs could have a synergistic effect in BT474 cells, we assessed the efficacies of the combinations of pyrotinib and dalpiciclib, pyrotinib and tamoxifen, and tamoxifen and dalpiciclib on the inhibition of cell proliferation at different concentrations. We calculated the combination index for each combination using Compusyn software to determine if the antitumor effects were synergistic (*Chou*

and Talalay, *1984*). Synergistic effects were observed in the combination group of pyrotinib and dalpiciclib, as well as in the pyrotinib and tamoxifen groups; both with CI values of <1 at several concentrations (Figure 1a). However, in the combination group of tamoxifen and dalpiciclib, no synergistic effect was observed.

We also analyzed the effect of the three-drug combination, and it showed a stronger cytotoxic effect on TPBC compared with the effect of the other two-drug combinations (Figure 1b). As both dalpiciclib and tamoxifen showed synergistic effects in combination with pyrotinib, we sought the combination that showed better efficacy. Hence, we treated the BT474 cells with different combinations at EC50 or half EC50 concentrations. The three-drug combination and the combination of pyrotinib and dalpiciclib showed a stronger cell inhibition compared with that exerted by pyrotinib and tamoxifen as well as tamoxifen and dalpiciclib (Figure 1c). The colony formation assay also showed similar trends as the cell viability assay; the three-drug combination formed the least number of colonies, followed by the combination of pyrotinib and dalpiciclib (Figure 1-figure supplement 1b–c).

To verify the efficacy of endocrine therapy in $HR^+/HER2^+$ patients, we retrospectively analyzed the clinical data of 221 $HR^+/HER2^+$ patients who received adjuvant therapy at the Shengjing Hospital. Of these, 44 patients received anti-HER2 therapy plus chemotherapy, and 177 patients received anti-HER2 therapy combined with chemotherapy and endocrine therapy. A Kaplan-Meier analysis showed that the addition of endocrine therapy to adjuvant anti-HER2 therapy plus chemotherapy did not significantly alter disease-free survival (DFS; $P = 0.600$) or overall survival (OS; $P = 0.5276$) in $HR^+/HER2^+$ patients (Figure 1d).

Nuclear ER distribution is increased after Anti-HER2 therapy and could be

reversed by the introduction of a CDK4/6 inhibitor

The results of the drug sensitivity test showed that the combination of pyrotinib and tamoxifen was less effective than the combination of pyrotinib and dalpiciclib. Considering that anti-HER2 therapy may activate the ER signaling pathway, we performed immunofluorescence staining for ER distribution on the different drug-treated groups. We found that pyrotinib induced ER nuclear translocation in BT474 cells, which could be partially reversed by the addition of dalpiciclib, rather than tamoxifen (Figure 2a). To further investigate whether the expression of HER2 could affect the distribution of ER, we transfected MCF7 cells with HER2 overexpression plasmids. We found that ER in MCF7 cells (WT and NC) was mainly expressed in the nuclei, whereas in the HER2 overexpressing MCF7 cells, ER was distributed throughout the cell plasma. Treating HER2 overexpression MCF7 cells with pyrotinib could redistribute the ER into the nuclei (Figure 2b). Western blot analyses revealed although the nuclear ER levels increased considerably, the total expression of ER was reduced after the use of pyrotinib (Figure2-figure supplement 1a–b). The use of tamoxifen increased the expression of total ER and nuclear ER, significantly, while the expression of nuclear ER in combination with pyrotinib and dalpiciclib showed a decrease (Figure2-figure supplement 1a–b).

Based on our *in vitro* findings, we further explored the ER distribution in clinical samples from the different treatment groups. To this end, we collected the clinical information of 172 HER^+/HR^+ patients who received neoadjuvant therapy at the Shengjing Hospital (Table 1). We found significant elevations in the nuclear ER expression levels of patients who received chemotherapy and anti-HER2 therapy, compared with the levels in patients who only received chemotherapy (Figure 2c, d). However, in our ongoing clinical trial (NCT04486911), the nuclear ER expression

levels of patients did not show significant elevations after the HER2-targeted therapy combined with dalpiciclib (Figure 2d). These findings verified that the ER receptor may have shifted to the nucleus after anti-HER2 therapy, which could be reversed with the introduction of a CDK4/6 inhibitor.

Bioinformatic analyses unravel the synergistic mechanisms underlying the dalpiciclib and Anti-HER2 therapy in TPBC

To further explore the synergistic mechanisms of the addition of CDK4/6 inhibitor treatment in $HER2^+/HR^+$ breast cancer, we first analyzed the gene expression profiles of the breast tumor cells treated with pyrotinib via RNA-seq. The signaling pathway enrichment analysis of the differentially expressed genes (DEGs) showed that majority of the DEGs were significantly enriched in the TNF signaling pathway and cell cycle, while steroid biosynthesis was also strongly active, suggesting that the steroid hormone pathway was activated by pyrotinib (Figure 3a-b). Similar results were obtained from the Gene Set Enrichment Analysis (GSEA). The administration of pyrotinib resulted in downregulation of the cell cycle and activation of the hormone pathway. The leading-edge subset of these pathways included the MITOTIC SPINDLE, G2M CHECKPOINT, and ESTROGEN RESPONSE EARLY (Figure 3c). These results showed good concordance with our *in vitro* findings.

We then investigated the alteration of the gene expression profiles between breast tumor cells treated with triple-combined drugs (pyrotinib, tamoxifen, and dalpiciclib) and those treated with the dual-combined drugs (pyrotinib and tamoxifen) via gene enrichment analyses. The results suggested that the addition of dalpiciclib markedly reduced cell cycle progression. This was characterized by the enrichment of the cell cycle and the DNA replication process (Figure 3e). The GSEA results further indicated that the progression of the cell cycle was impeded by the enrichment of the

gene sets, including MITOTIC SPINDLE and G2M CHECKPOINT (Figure 3f).

The inhibition of the ER pathway might be involved in the effect of pyrotinib plus dalpiciclib on TPBC cells; therefore, intersection analyses were performed to confirm this. As shown in Figure 3g, *CALML5*, *KRT15*, and *KRT19* are the common genes shared between the two sets, the upregulated genes treated with pyrotinib and the genes belonging to the estrogen signaling pathway. Since dalpiciclib is a cell cycle blocker, we also analyzed the common genes involved in the upregulation of the genes and the cell cycle progression after pyrotinib treatment. *CDKN1A* was the only shared gene in these two sets (Figure 3h). We then investigated whether any of the above-mentioned genes were upregulated with the use of pyrotinib and whether this could be reversed with the introduction of dalpiciclib, which may serve as a potential biomarker of the responsiveness to different treatments. The results showed that only one factor, *CALML5*, was the common gene (Figure 3i).

CALML5 is a potential indicator for the responsiveness to anti-HER2 therapy plus CDK4/6 inhibitor

Western blot analyses and bioinformatic analyses were conducted to verify the changes in the signaling pathways. The western blot analyses showed that while the introduction of tamoxifen did not significantly affect the expression of HER2 and partially inhibited the HER2 downstream pathway (AKT-mTOR signaling pathway), it did not significantly affect the phosphorylation of Rb (Figure 4a). In contrast, after the introduction of dalpiciclib, the activation of mTOR was partially inhibited, which relieved the negative feedback on the HER2 pathway, as evidenced by the slight increase in the HER2 and pAKT, which maintained the sensitivity of the HER2 pathway to pyrotinib. This was consistent with the findings of Goel et al (*Goel et al.*, *2016*). The combination of pyrotinib and dalpiciclib significantly reduced pRb

188 expression (Figure 4a). In addition, cell arrest analyses of the different drug
189 combinations were performed. As shown in Figure 4b, compared with the cells treated
190 with pyrotinib or tamoxifen, the introduction of dalpiciclib significantly increased the
191 number of cells arrested in the G1/S phase. This confirmed the synergistic inhibition
192 of cell proliferation by dalpiciclib and pyrotinib.

193 To verify whether CALML5 could be a potential predictor of treatment
194 responsiveness in clinical practice, clinical biopsy samples were collected from
195 $HER2^{+}/HR^{+}$ patients before and after neoadjuvant therapy (anti-HER2 therapy plus
196 chemotherapy or anti-HER2 therapy plus CDK4/6 inhibitor). The demographic
197 information is shown in Table 2. Immunohistochemical staining of CALML5 showed
198 that the CALML5-positive cells indicated better drug sensitivities and higher
199 probabilities of achieving pathological complete response (pCR) and PR in patients
200 receiving anti-HER2 therapy and a CDK4/6 inhibitor compared with anti-HER2
201 therapy plus chemotherapy (Figure 4c). Moreover, the positive rate of CALML5
202 decreased after anti-HER2 therapy plus a CDK4/6 inhibitor treatment (Figure 4d),
203 consistent with the results of the bioinformatic analyses.

204 **Discussion**

205 Until now, the combination of antiHER2 therapy and chemotherapy have been
206 the major treatment strategies for treatment of TPBC, although sometimes this is
207 combined with endocrine therapy (*Gianni et al.*, *2012; Schneeweiss et al.*, *2013*).
208 Although pCR and DFS improve with the use of the combination of anti-HER2
209 therapy and chemotherapy, the strong adverse effects of chemotherapy cannot be
210 ignored (*Maguire et al.*, *2021*). Moreover, clinical data showed that the addition of
211 anti-estrogen receptor drugs in the treatment regimen of TPBC did not provide
212 additional advantages in the pCR rates and DFS (*Harbeck et al.*, *2017; Rimawi et al.*,

213 *2017*). Hence, with the rapid development of small-molecule drugs such as tyrosine
214 kinase inhibitors (TKIs) and CDK4/6 inhibitors, additional chemo-free strategies are
215 being developed for the treatment of $HER2^+/HR^+$ breast cancer (*Gianni et al.*, *2018;*
216 *Pascual et al.*, *2021; Saura et al.*, *2014*). In the present study, we investigated whether
217 the addition of CDK4/6 inhibitors to HER2-targeted and endocrine therapy could be
218 an alternative strategy for $HER2^+/HR^+$ patients.

219 In our study, we found that the combination of tamoxifen and pyrotinib was less
220 effective than the combination of pyrotinib and dalpiciclib in BT474 cancer cells. This
221 was anomalous since the two blocking agents of HER2 and ER were expected to
222 inhibit their crosstalk and achieve better responses. To explore the potential
223 mechanisms, we investigated the crosstalk between HER2 and the ER. After
224 degrading HER2 with pyrotinib, ER was found to relocate to the cell nucleus,
225 enhancing the function of ER which was consistent with the findings of Kumar et al
226 and Yang et al (*Kumar et al.*, *2002; Yang et al.*, *2004*). We believe that the anti-HER2
227 mediated ER redistribution caused the enhanced ER function, leading to the relatively
228 low efficacy of the combination of pyrotinib and tamoxifen in the treatment of
229 $HER2^+/HR^+$ cells. Moreover, we found that the introduction of dalpiciclib to pyrotinib
230 significantly decreased the total and nuclear expression of ER, reversing the ER
231 activation caused by pyrotinib (Figure2-figure supplement 1c). This may be the
232 underlying mechanism by which the addition of a CDK4/6 inhibitor was more
233 beneficial than the addition of pyrotinib and tamoxifen.

234 Furthermore, using RNA-seq and bioinformatics analyses, CALML5 was
235 selected as a potential biomarker for responsiveness to anti-HER2 therapy combined
236 with a CDK4/6 inhibitor. CALML5, known as calmodulin-like 5, is a skin-specific
237 calcium-binding protein that is closely related to keratinocyte differentiation (*Mehul*

238 *et al.*, *2001*). A previous study showed that the high expression of CALML5 was
239 strongly associated with better survival in patients with head and neck squamous cell
240 carcinomas (*Wirsing et al.*, *2021*). Misawa et al. (*Misawa et al.*, *2020*) reported that
241 the methylation of CALML5, led to its downregulation, and this showed a correlation
242 with HPV-associated oropharyngeal cancer. Moreover, the ubiquitination of
243 CALML5 in the nucleus was found to play a role in the carcinogenesis of breast
244 cancer in premenopausal women (*Debald et al.*, *2013*). Our results suggested that
245 TPBC patients with positive CALML5 may benefit from the addition of CDK4/6
246 inhibitors in neoadjuvant therapy. However, the underlying mechanism of CALML5
247 in breast cancer requires further investigation.

248 In conclusion, our study showed the novel role of the CDK4/6 inhibitor in TPBC
249 and provided evidence that CALML5 may be a potential biomarker in the prediction
250 of the responsiveness of $HER2^{+}/HR^{+}$ breast cancer patients to CDK4/6 inhibitors.
251

Materials and methods

Clinical specimens

A total of 221 $HR^+/HER2^+$ patients who received adjuvant chemotherapy + anti HER2 therapy with or without endocrine therapy at the Shengjing Hospital of China Medical University were enrolled for the analysis of DFS and OS. A total of 198 $HR^+/HER2^+$ patients who received neoadjuvant therapy were enrolled in this study to evaluate the status of ER and CALML5, of which 26 patients were from the clinical trial (NCT04486911), 41 patients received anti-HER2 therapy plus chemotherapy, and 131 patients only received chemotherapy. The sample size was calculated based on the four interrelated statistics in the Null Hypothesis Significant Test (NHST): sample size, effect size, alpha level, and statistical efficacy. The clinical information and specimens were analyzed to determine the impact of endocrine therapy on prognosis.

The study was approved by the Institutional Ethics Committee and complied with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines of the National Medical Products Administration of China. Informed consent was obtained from all the participants.

Cell lines and cell cultures

BT474 and MCF7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human triple-positive breast cancer cell line BT474 was cultured in RPMI1640 culture medium supplemented with 10% fetal bovine serum (FBS), and MCF7 cells were cultured in DMEM culture medium supplemented with 10% FBS.

Chemicals and antibodies

Pyrotinib (SHR1258) and dalpiciclib (SHR6390) were kindly provided by Hengrui Medicine Co., Ltd. Tamoxifen (HY-13757A) was purchased from MCE

277 company. Compounds were dissolved in dimethylsulfoxide (DMSO) at a
278 concentration of 10 mM and stored at -20 °C for further use. The following antibodies
279 were purchased from Cell Signaling Technology (Beverly, MA, USA): ER, p-HER2
280 (Tyr 1221/1222), HER2, p-Akt (Ser473), AKT, p-mTOR, mTOR, pRb (Ser 780), Rb,
281 CDK4, CDK6, Lamin A, and GAPDH.

282 **Cell viability assays and drug combination studies**

283 CCK cell viability assays were (Cofitt life science) used to quantify the
284 inhibitory effect of the different treatments. Cells were seeded in 96-well plates at a
285 density of 5000 cells/well and treated the next day with DMSO, pyrotinib, tamoxifen,
286 dalpiciclib, or both drugs in combination for 48 h. The combination index (CI) values
287 of different drugs were calculated using CompuSyn (ComboSyn Inc.). The CI values
288 demonstrated synergistic (<1), additive (1–1.2), or antagonistic (>1.2) effects of the
289 two-drug combinations. The drug sensitivity experiments were performed three times
290 independently.

291 **Cell cycle analyses**

292 The cells were starved in culture medium supplemented with 2% serum for 24 h
293 before treatment. Treatments included DMSO (0.1%), pyrotinib (10 nM), dalpiciclib
294 (8 μM), tamoxifen (5 μM), or different combinations of drugs. After treatment for 24
295 h, cells in different treating groups were trypsinized, washed with PBS, fixed in 70%
296 ethanol, and incubated overnight at 4 °C. Next day, cells were collected, washed, and
297 re-suspended in PBS at a concentration of 5×10^5 cells/mL. The cell solutions were
298 then incubated with a RNase and propidium iodide (PI) solution for 30 min at room
299 temperature without exposure to light, and analyzed using a flow cytometer (BD
300 FACS Calibur) according to the manufacturer's instructions. This assay was
301 performed in triplicates.

Colony formation assays

Cells were seeded in 6-well plates at a density of 1000 cells/well. The cells were treated with DMSO (0.1%), pyrotinib (10 nM), tamoxifen (5 μM), dalpiciclib (8 μM), or a combination of the two or three agents. During the process, the culture medium was renewed every three days. After 14 days, the colonies were fixed and stained with crystal violet. Clusters of more than eight cells were counted as colonies. This assay was performed in triplicates independently.

Transfection of the human HER2 plasmids

MCF7 cells were cultured in DMEM supplemented with 10% FBS. Human HER2 plasmids were purchased from Hanbio. The plasmids were mixed well with lipo3000 and p3000, according to the manufacturer's instructions, and then added to the culture medium. Forty-eight hours after the transfection, the cells were fixed in formaldehyde and stained for the estrogen receptor.

Western blot analysis

Cells and cancer tissues were lysed using a cell lysis buffer (Beyotime, Shanghai, China). The total proteins were extracted in a lysis buffer (Beyotime, Shanghai, China), and the nuclear proteins were extracted using a nuclear protein extraction kit (Beyotime), in which PMSF, protease, and phosphatase inhibitors were added. Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The proteins from the cells and tissue lysates were separated using 10% SDS-PAGE and 6% SDS-PAGE, respectively, and then transferred to polyvinylidene fluoride (PVDF) membranes. The immunoreactive bands were detected using enhanced chemiluminescence (ECL). The western blot analysis was performed in triplicates independently.

Immunofluorescence assays

The cellular localization of different proteins was detected using immunofluorescence. Briefly, the cells grown on glass coverslips were fixed in 4% paraformaldehyde at room temperature for 30 min. Cells were incubated with the respective primary antibodies for 1 h at room temperature, washed in PBS, and then incubated with 590-Alexa-(red) secondary antibodies (Molecular Probes, Eugene, OR, USA). We used 590-Alexa-phalloidin to localize the ER. The nuclei of the cells were stained with DAPI and color-coded in blue. The images were captured using an immunofluorescence microscope (Nikon Oplenic Lumicite 9000). The distribution ratio of ER was calculated manually by randomly chosen 5 views in 400magnification. The immunofluorescence assay was performed in triplicates independently.

Immunohistochemical staining

The clinical samples were fixed in 4% formaldehyde, embedded in paraffin, and sectioned continuously at a thickness of 3 μm. The paraffin sections were deparaffinized with xylene and rehydrated using a graded ethanol series. They were then washed with tris-buffered saline (TBS). After these preparation procedures, the sections of each sample were incubated with the primary anti-ER antibody (Abcam Company, ab32063), anti-HER2 antibody (Abcam Company, ab134182), and anti-CALML5 antibody (Proteintech, 13059-1-AP) at 4 °C overnight. The next day, they were washed three times with TBS and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Gene Tech Co. Ltd., Shanghai, China) at 37 °C for 45 min, followed by immunohistochemical staining using a DAB kit (Gene Tech Co. Ltd.) for 5–10 min.

Evaluation of the ER and HER2 statuses

The ER and HER2 statuses of patients who received neoadjuvant therapy were evaluated by a pathologist from a Shenjing affiliated hospital. The clinical specimens before and after the neoadjuvant therapy were evaluated. The analyses of the elevation or decline in ER statuses were based on these pathological reports.

Gene enrichment analysis

Gene annotation data in the GO and KEGG databases and R language were used for the enrichment analysis. Only enrichment with q-values less than 0.05 were considered significant.

GSEA

The hallmark gene sets in the Molecular Signatures Database were used for performing the GSEA; only gene sets with q-values less than 0.05 were considered significantly enriched.

Statistical analysis

All the descriptive statistics (except the drug sensitivity assay in Figure 1a and b) were presented as the means ± standard deviations (SDs). The drug sensitivity assay in Figure 1 a and b were presented as the means ± standard error of mean (SEM). The differences between the groups were analyzed by chi-squared or Student's t tests. Kaplan-Meier methods were used to compute the survival analysis and *P*-value was obtained by log-rank test. The statistical analyses were performed using IBM SPSS version 22 (SPSS, Armonk, NY, USA) and GraphPad Prism version 7. The statistical significance of the differences between the test and control samples was assessed at significance thresholds of $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

375 **Table 1. Demographic information of triple positive breast cancer patients who**

376 **received neoadjuvant therapy.**

Variables	Chemotherapy	Chemotherapy+antiHER2 therapy	*p*-value
No.of patients	131	41	
Age of year			ns
≤50	82(62.60)	25(61.00)	
>50	49(37.40)	16(39.00)	
T stage			ns
1	15(11.45)	5(12.20)	
2	90(68.70)	32(78.04)	
3	26(19.85)	4(9.76)	
ER status			ns
≤30%	31(23.66)	8(19.51)	
>30%	100(76.34)	33(80.49)	
PR status			0.0059
≤30%	80(61.07)	15(36.59)	
>30%	51(38.93)	26(63.41)	
HER2 status			0.0007
(++)	78(59.54)	12(29.27)	
(+++)	53(40.46)	29(70.73)	
Ki67 index			ns
<20%	51(38.93)	16(39.00)	
>20%	80(61.07)	25(61.00)	

377 **Table 2. Demographic information of triple positive breast cancer patients who**

378 **were tested for CALML5 before receiving neoadjuvant therapy.**

Variables	Chemotherapy+anti HER2 therapy	Pyrotinib+dalpiciclib+tamoxifen	*p*-value
No.of patients	41	26	
Age of year			
≤50	25(61.00)	16(61.53)	ns
>50	16(39.00)	10(38.47)	
T stage			
1	5(12.20)	2(7.70)	ns
2	32(78.04)	21(80.76)	
3	4(9.76)	3(11.54)	
ER status			
≤30%	8(19.51)	2(7.6)	0.0145
>30%	33(80.49)	24(92.4)	
PR status			
≤30%	15(36.59)	13(50)	ns
>30%	26(63.41)	13(50)	
HER2 status			
(++)	12(29.27)	10(38.5)	ns
(+++)	29(70.73)	16(61.5)	
Ki67 index			
<20%	16(39.00)	8(30.8)	ns
>20%	25(61.00)	18(69.2)	
CALML5			

positive	31(75.61)	19(73.08)	ns
negative	10(24.39)	7(26.92)	

ns, not significant.

Authors Contributions

J.B., Y.Z., L.S., X.Q., Y.W., X.J., D.W., H.L., and Q.M. conceptualized the study, performed the experiments, and analyzed the data. B.K. performed the bioinformatic analysis. Y.Z. and N.N. provided the clinical data and samples. C.L. designed the entire study and wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interests. H.L. is the employee of Jiangsu Hengrui Pharmaceuticals Co., Ltd, no other potential conflicts of interest were reported. The other authors have no competing interests to declare.

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Figure legends

Figure 1. Drug sensitivity test and cell cycle analysis of pyrotinib, tamoxifen, dalpiciclib and their combination on BT474 cells.

a-b: Drug sensitivity assay of BT474 cells to single drug and different drug combination (Data was presented as mean ± SEMs, all drug sensitivity assay were performed independently in triplicates).

c: Drug sensitivity assay of BT474 cells to different drug combination at EC50 concentration and 1/2 EC50 concentration. (Data was presented as mean ± SDs, *P<0.05, **P<0.01 and ****P<0.0001 using Student's t-test; all the assays were performed independently in triplicates).

d: DFS (P=0.600) and OS (P=0.5276) analysis of HER2+/HR+ patients received adjuvant therapy with (n=177) or without endocrine therapy (n=44). P-values were obtained by log-rank test.

Figure 2. Anti-HER2 therapy could lead ER shifting into cell nucleus in HER2$^+$/HR$^+$ breast cancer while CDK4/6 inhibitor could reverse the nuclear translocation of ER.

a: Distribution of estrogen receptor in BT474 cell line after different drug treatment. (The distribution ratio of ER was calculated manually by randomly chosen 5 views in 400magnification. All the assays were performed independently in triplicates).

b: Distribution of estrogen receptor in wild type MCF7 cells, MCF7 cells transfected with HER2 over-expression plasmids and MCF7 cells transfected with HER2 over-expression plasmids treated with pyrotinib. (All the assays were performed independently in triplicates).

c: Representative views of ER and HER2 expression in patients before and after anti-HER2 neoadjuvant therapy.

d: Ratio of patients with elevated ER expression and patients with unchanged or reduced ER expression in different kinds of neoadjuvant therapy groups. (***P<0.001 using Student's t-test)

574 **Figure 3. Bioinformatic analysis revealed dalpiciclib and pyrotinib blocking**
575 **HER2 pathway and cell cycle in BT474 cells synergistically**

576 a-b: Signaling pathway enrichment analysis of mRNA changes of BT474 cells treated
577 with pyrotinib compared to BT474 cells treated 0.1%DMSO

578 c: GSEA analysis of mRNA changes of BT474 cells treated with pyrotinib compared
579 to BT474 cells treated 0.1%DMSO.

580 d-e: Signaling pathway enrichment analysis of mRNA changes of BT474 cells treated
581 with pyrotinib+ tamoxifen+dalpiciclib compared to BT474 cells treated with
582 pyrotinib+ tamoxifen.

583 f: GSEA analysis of mRNA changes of BT474 cells treated with pyrotinib+
584 tamoxifen+ dalpiciclib compared to BT474 cells treated with pyrotinib+ tamoxifen.

585 g: Intersection of genes which was upregulated after pyrotinib treatment and belonged
586 to estrogen receptor signaling pathway.

587 h: Intersection of genes which was upregulated after pyrotinib treatment and belonged
588 to cell cycle genes.

589 i: Intersection of genes which was upregulated after pyrotinib treatment and was
590 downregulated after the introduction of dalpiciclib.

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592

Figure 4. CALML5 could serve as a predictor for responsiveness of anti-HER2 therapy plus CDK4/6 inhibitor.

a: Western blot analysis of HER2 signaling pathway and cell cycle pathway in BT474 cells treated with different drugs or their combination. (This assay was performed in triplicates independently).

b: Cell cycle analysis in BT474 cells treated with different drugs or their combination. (Data was presented as mean ± SDs, ***P<0.001 using Student's t-test; all the assays were performed independently in triplicates).

c: Representative views of CALML5 positive/negative tissue. The difference of PR+PCR ratio and PD+SD ratio in patients who received anti-HER2 therapy plus chemotherapy or anti-HER2 therapy plus CDK4/6 inhibitor regarding on their expression of CALML5. (****P<0.0001 using Student's t-test).

d: Representative views of CALML5 positive/negative tissue. Ratio of patients with elevated or decreased CALML5 after receiving anti-HER2 therapy plus chemotherapy, or anti-HER2 therapy plus CDK4/6 inhibitor. (****P<0.0001 using Student's t-test).

618 **Supplementary**

619 **Figure 1-figure supplement 1**

620 a: Drug sensitivity analysis of pyrotinib, tamoxifen and dalpiciclib in BT474 cells.
621 (Data was presented as mean ± SDs, all the assays were performed independently in
622 triplicates).

623 b-c: Colony formation assay of BT474 cells treated with different drugs. (Data was
624 presented as mean ± SDs, $^{**}P<0.01$ and $^{****}P<0.0001$ using Student's *t*-test; all the
625 assays were performed independently in triplicates).

626 **Figure 2-figure supplement 1**

627 a-b: Total ER expression and nuclear ER expression in BT474 cells treated with
628 different drugs. (This assay was performed in triplicates independently).

629 c: The introduction of dalpiciclib to pyrotinib could significantly decrease the total
630 and nuclear expression of ER, thus reversed the ER activation caused by pyrotinib and
631 CALML5 could be served as a potential marker of ER activation after the treatment of
632 pyrotinib.

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643 **Source data**

644 **Figure 2 source data 1**

645 The clinical pathological data of patients who received neoadjuvant chemotherapy +
646 anti HER2 therapy

647 **Figure 2 source data 2**

648 The clinical pathological data of patients who received neoadjuvant chemotherapy
649 only.

650 **Figure 3 source data 1**

651 RNA-sequencing data of BT474 cells treated with different drug combination.

652 **Figure 4 source data 1**

653 Original files of western blot analysis in Figure 4 a.

654 **Figure 2-figure supplement 1 source data 1**

655 Original files of western blot analysis in Figure 2-figure supplement 1 a-b.

656

Fig 1

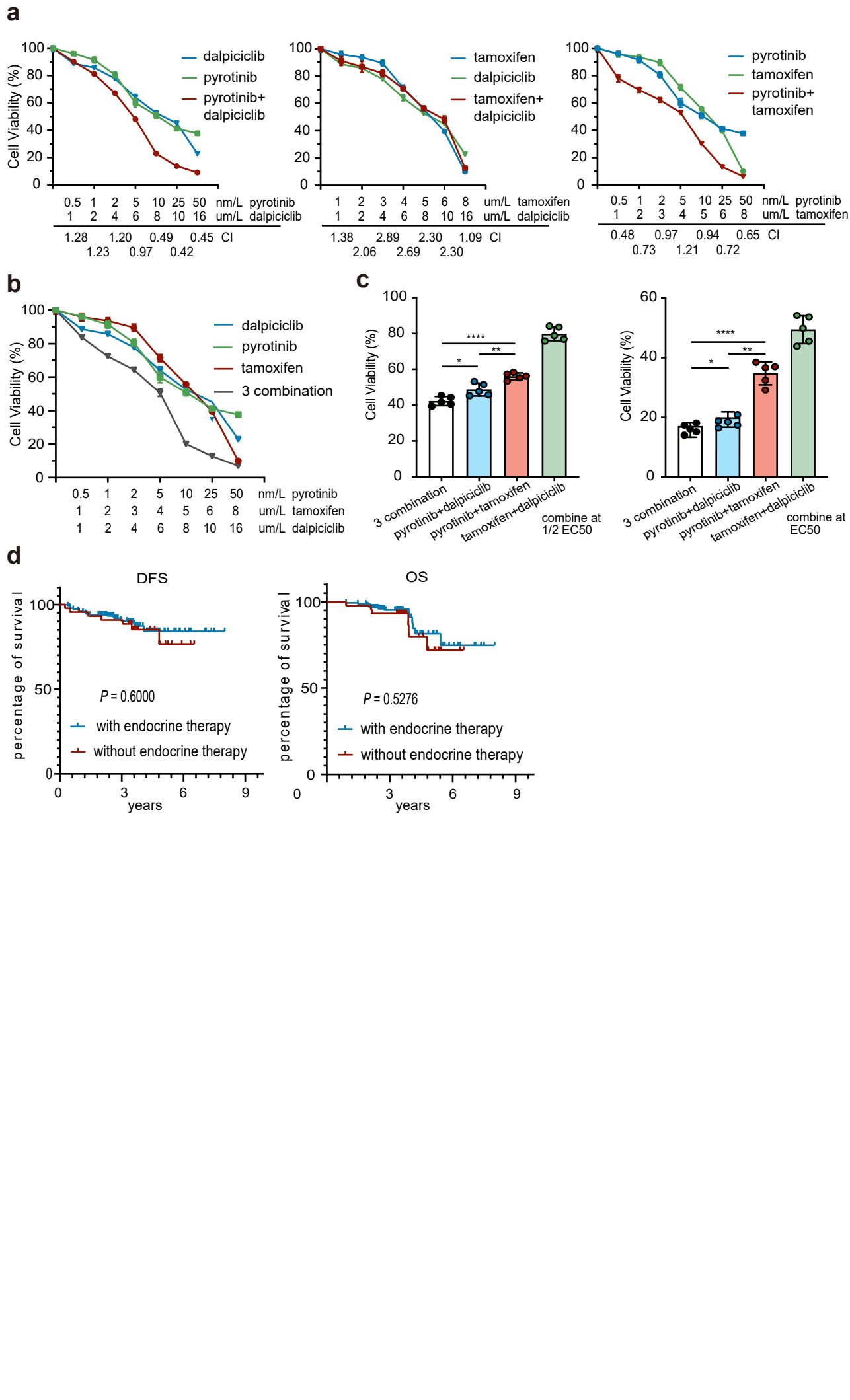
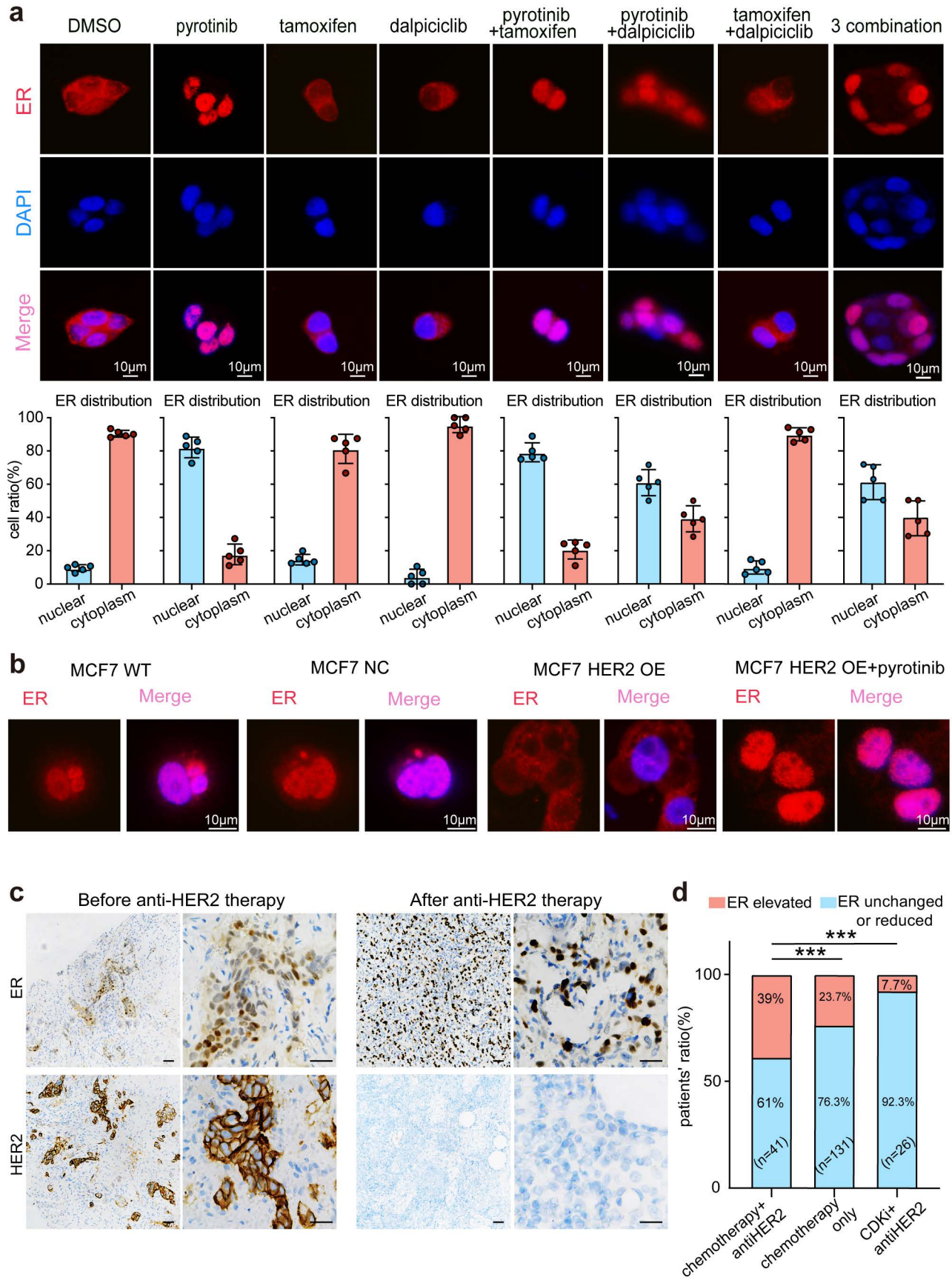


Fig 2



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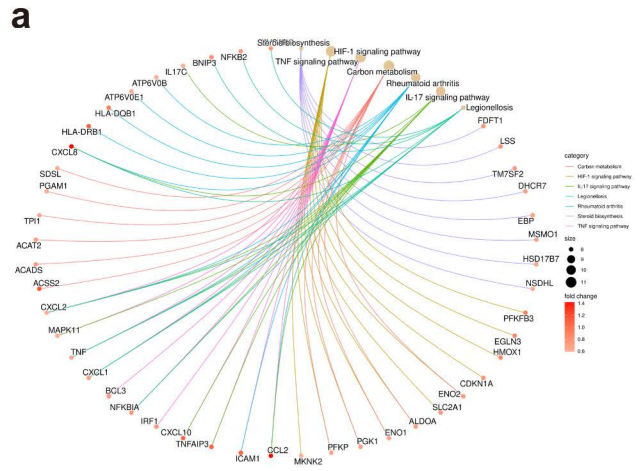

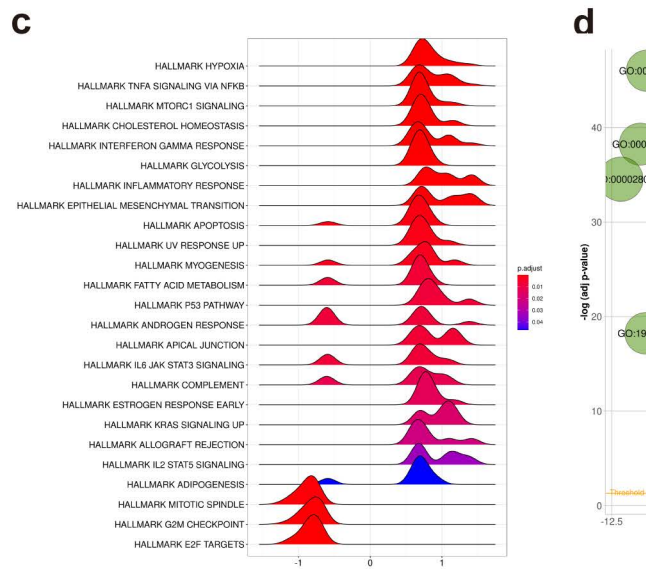

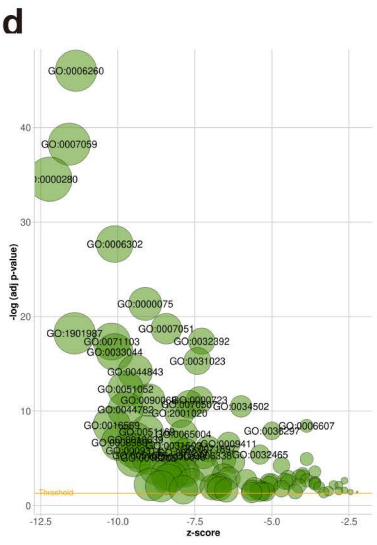

ID	Description
GO:0006260	DNA replication
GO:0007059	chromosome segregation
GO:0000280	nuclear division
GO:0006302	double-strand break repair
GO:0000075	cell cycle checkpoint
GO:0007051	spindle organization
GO:1901987	regulation of cell cycle phase transition
GO:0032392	DNA geometric change
GO:0071103	DNA conformation change
GO:0033044	regulation of chromosome organization
GO:0031023	microtubule organizing center organization
GO:0044843	cell cycle G1/S phase transition
GO:0051052	regulation of DNA metabolic process
GO:0000723	telomere maintenance
GO:0090068	positive regulation of cell cycle process
GO:0070507	regulation of microtubule cytoskeleton organization
GO:0034502	protein localization to chromosome
GO:0044782	cilium organization
GO:2001020	regulation of response to DNA damage stimulus
GO:0016569	covalent chromatin modification
GO:0006607	NLS-bearing protein import into nucleus
GO:0036297	interstrand cross-link repair
GO:0051169	nuclear transport
GO:0065004	protein-DNA complex assembly
GO:0010639	negative regulation of organelle organization
GO:0008380	RNA splicing
GO:0009411	response to UV
GO:0031503	protein-containing complex localization
GO:0071897	DNA biosynthetic process
GO:0009314	response to radiation
GO:0072331	signal transduction by p53 class mediator
GO:0032465	regulation of cytokinesis
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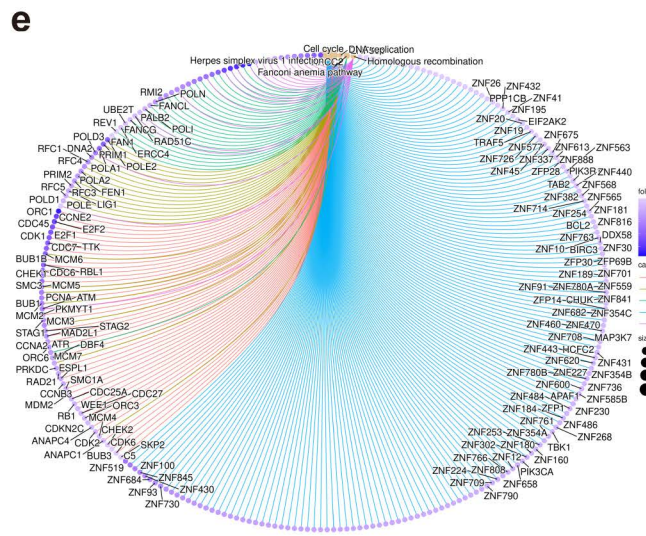
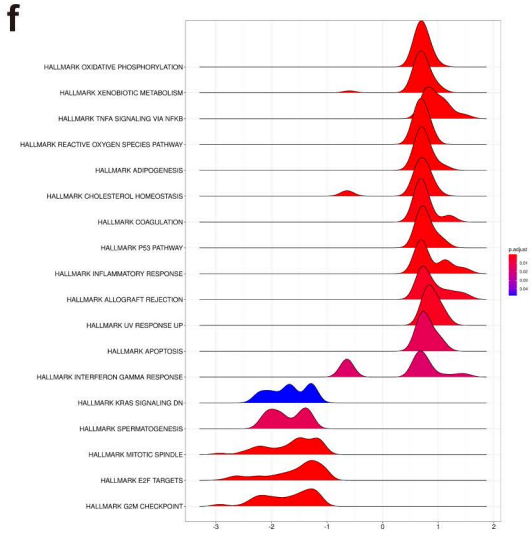




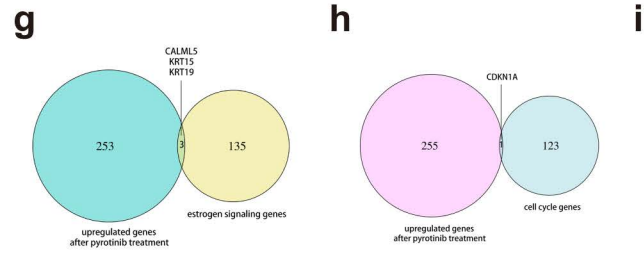
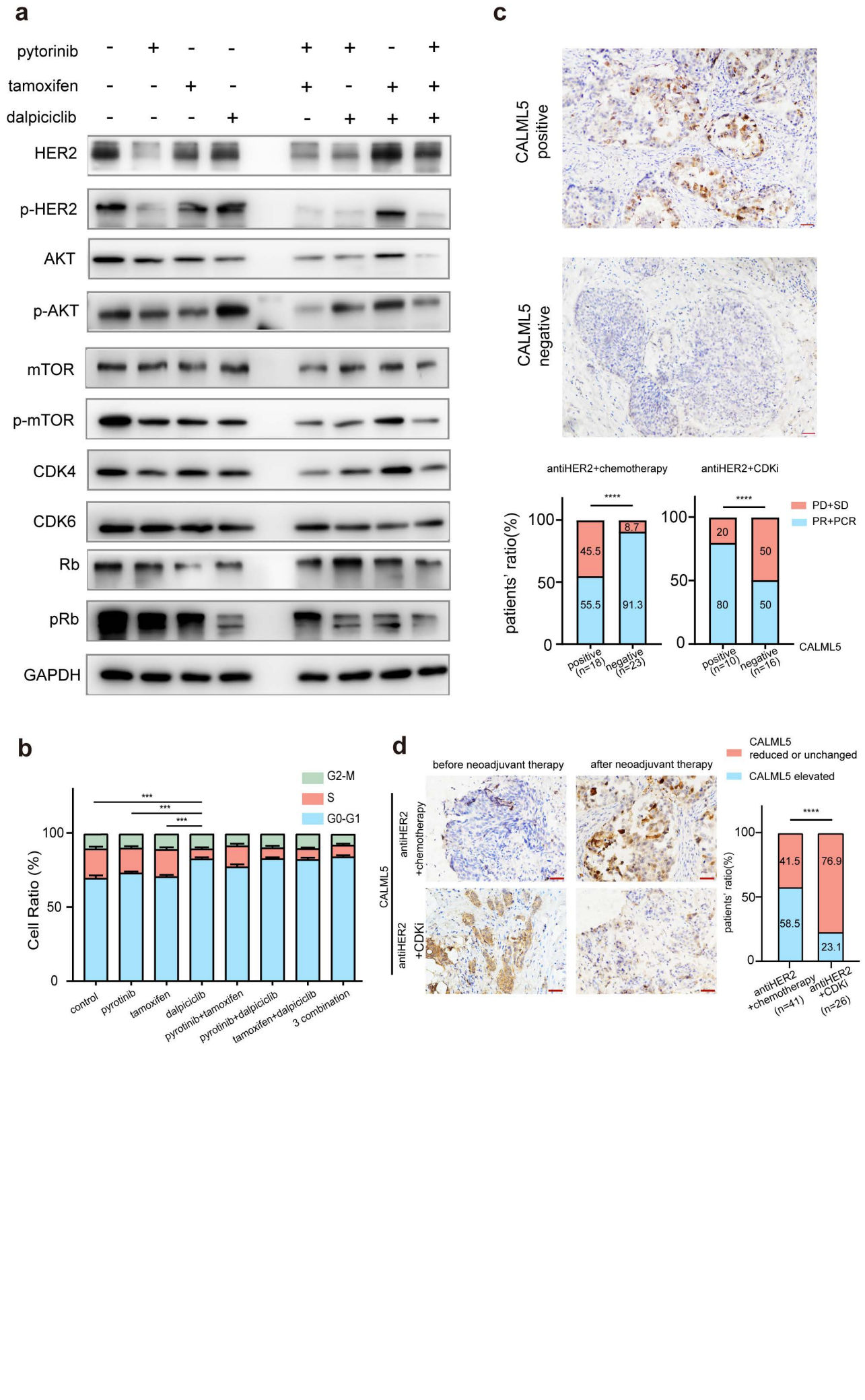


Fig 4



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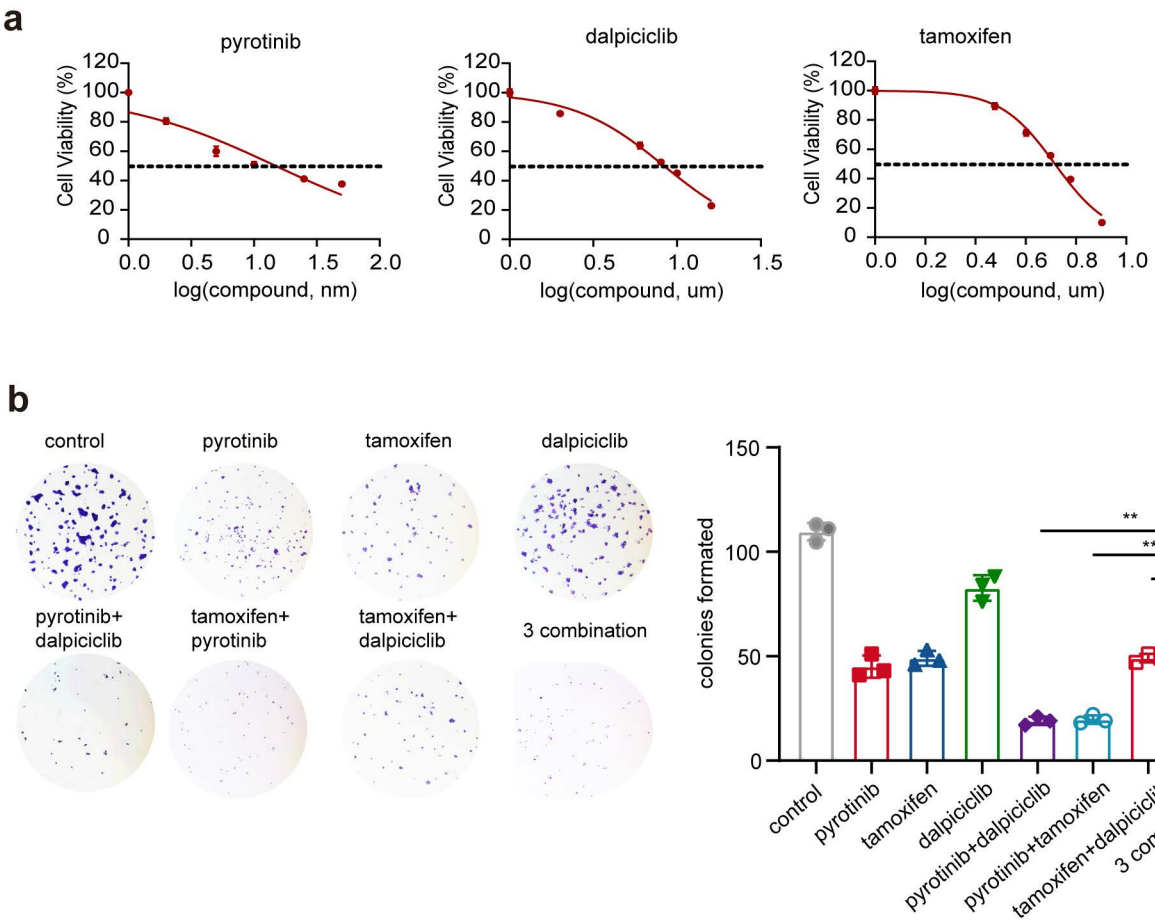

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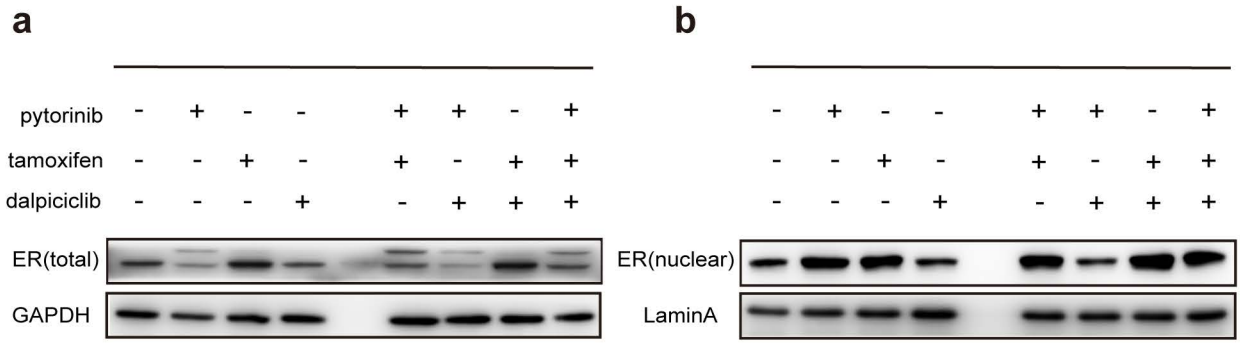

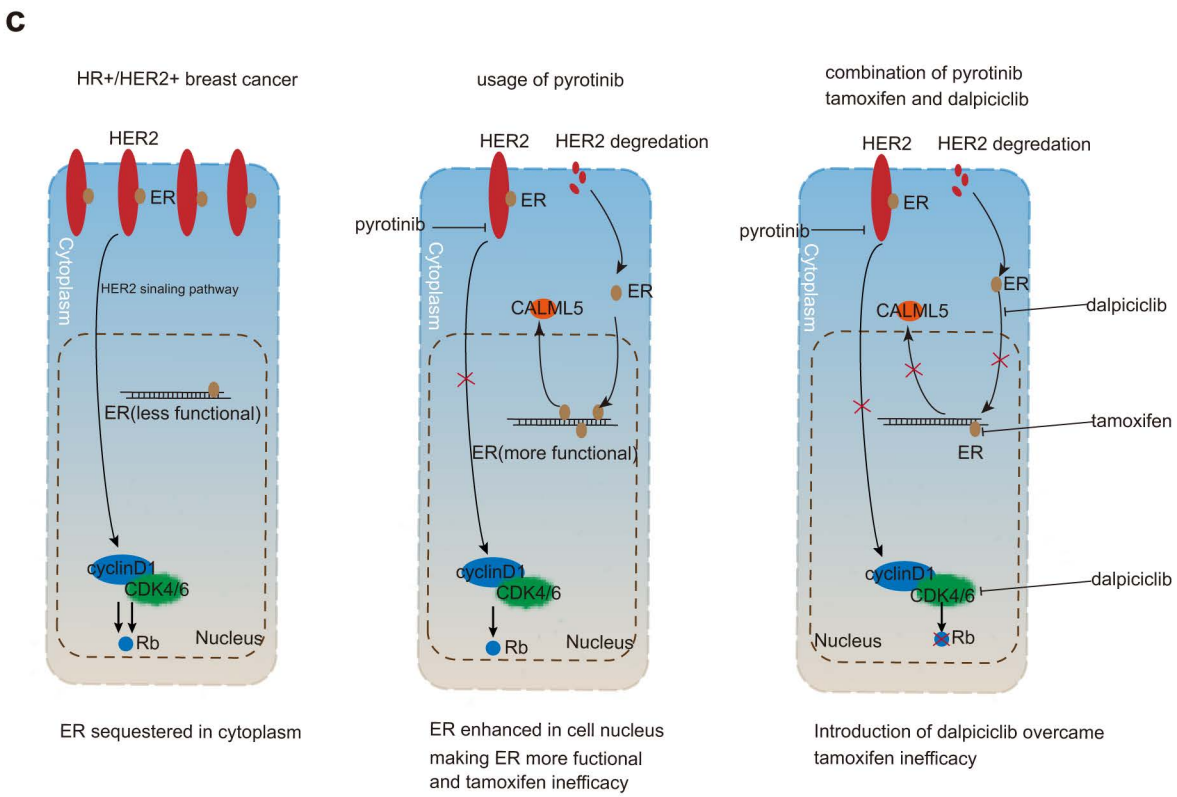

ER sequestered in cytoplasm

ER enhanced in cell nucleus making ER more fuctional and tamoxifen inefficacy

Introduction of dalpiciclib overcame tamoxifen inefficacy