Pyrotinib and trastuzumab combination treatment synergistically overcomes HER2 dependency in HER2-positive breast cancer: insights from the PHILA trial

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eBioMedicine
2024;109: 105379
Published Online xxx
https://doi.org/10.1016/j.ebiom.2024.105379

Summary

Background The PHILA study suggests that pyrotinib, trastuzumab, and docetaxel significantly improved progression-free survival (PFS) compared with placebo, trastuzumab, and docetaxel in patients with untreated HER2-positive metastatic breast cancer. In this study, we aimed to investigate the synergistic mechanisms of pyrotinib plus trastuzumab and provide further insights for the PHILA trial.

Methods The *in vitro* activity of combination treatments was assessed through cell biological and biochemical experiments. The *in vivo* efficacy was evaluated in cell-derived xenografts, a TUBO tumour model, and one clinical case. Next-generation sequencing was performed on circulating tumour DNA (ctDNA) from patients in the PHILA trial.

Findings The combination of pyrotinib and trastuzumab more effectively inhibited cell growth than pyrotinib or trastuzumab alone in models of HER2-dependent breast cancer. It potentiated membrane HER2 ubiquitination and downregulation, which resulted in a comprehensive blockade of the HER2 signalling pathway. The pyrotinib-altered membrane HER2 levels had no significant effect on trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). We further validated the synergistic mechanisms in TUBO tumours and one clinical case, rather than models of HCC1954 cells harbouring the PIK3CA H1047R mutation. Similarly, in our centre cohort of the PHILA study, patients with genetic alterations in the HER2 signalling cascade had significantly shorter median PFS than individuals with the wild-type pathway.

Interpretation Our findings underscore the robust synergy between pyrotinib and trastuzumab in overcoming HER2 dependency and provide a rationale for pyrotinib, trastuzumab, and docetaxel as one of the optimal choices for patients with untreated HER2-positive metastatic breast cancer, who are dependent on the HER2 signalling cascade.

Funding This work was supported by the National Key Research and Development Program of China (2021YFF1201300), the National Natural Science Foundation of China (82172875), the CAMS Innovation Fund for Medical Sciences (CIFMS) (2022-I2M-2-001), and the Joint Innovative Fund of Beijing Natural Science Foundation and Changping District (L234004).

Keywords: PHILA study; Pyrotinib; Trastuzumab; HER2-positive breast cancer

Introduction

HER2-positive breast cancer accounts for approximately 20% of all breast cancers, which is characterised by its aggressive nature, high likelihood of early recurrence or metastasis, and rapid progression in advanced stages.[1] Moreover, a subset of HER2-positive breast cancer,

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Research in context

Evidence before this study

The results of the PHILA trial led by our group showed that the combination of pyrotinib, trastuzumab, and docetaxel significantly improved PFS (24.3 months versus 10.4 months, one sided $P < 0.001$) compared with placebo, trastuzumab, and docetaxel in patients with untreated HER2-positive metastatic breast cancer. Despite its remarkable efficacy, the synergistic mechanisms of pyrotinib plus trastuzumab and the optimal population benefiting from this regimen as a first-line treatment require further investigation.

Added value of this study

The combination of pyrotinib and trastuzumab demonstrated synergistic anti-tumour efficacy in models of HER2-dependent breast cancer. This combination reduced membrane HER2 levels by promoting receptor ubiquitination via HSP90 dissociation, and comprehensively inhibited the HER2 signalling pathway. We also detected that pyrotinib treatment had no significant effect on trastuzumab-mediated ADCC. Moreover, TUBO tumours and one clinical case were utilised to further validate the synergistic effects on overcoming HER2 dependency. In contrast, we did not observe a synergy between pyrotinib and trastuzumab in models of HCC1954 cells harbouring the PIK3CA H1047R mutation. Meanwhile, the PHILA regimen showed promising efficacy in our centre patients without genetic alterations in the HER2 signalling cascade.

Implications of all the available evidence

Our results further explain the substantial efficacy of pyrotinib and trastuzumab, and suggest that patients with HER2-dependent breast cancer may be the optimal beneficiaries of the PHILA regimen in advanced first-line treatment. This study may usher in a new era for the dual HER2 blockade with the use of a monoclonal antibody plus an irreversible tyrosine kinase inhibitor in the first-line setting of HER2-positive metastatic breast cancer in China.

defined as HER2-dependent breast cancer, exhibits high levels of HER2 protein and robust activation of the HER2 signalling cascade with no functional mutations in downstream pathways.[2–4] Concurrently, HER2-targeted drugs, including monoclonal antibodies (mAbs), tyrosine kinase inhibitors (TKIs), and antibody–drug conjugates (ADCs), have contributed to markedly improved survival outcomes in patients with HER2-positive breast cancer.[5]

Previous clinical trials have shown that the combination of trastuzumab and taxane, when used as a first-line treatment for patients with HER2-positive metastatic breast cancer, significantly prolongs PFS and overall survival (OS) compared with taxane alone.[6,7] Moreover, pyrotinib, which serves as an irreversible panHER receptor TKI that targets EGFR, HER2, and HER4, has favourable outcomes in patients with HER2-positive breast cancer.[8] In the PHOEBE study, pyrotinib plus capecitabine significantly improved PFS and OS compared with lapatinib plus capecitabine.[9–11] As a result, this treatment was approved as a second-line therapy for patients with advanced HER2-positive breast cancer in China. Notably, TKI and trastuzumab combination has shown encouraging anti-tumour activity in several trials, including NeoALTTO, PHEDRA, and HER2CLIMB, for patients with HER2-positive breast cancer receiving neoadjuvant or salvage therapy.[12–15]

We previously assessed the efficacy of pyrotinib plus trastuzumab as a first-line treatment for patients in advanced stage. In the PHILA study, the combination of pyrotinib, trastuzumab, and docetaxel significantly improved PFS (24.3 months versus 10.4 months, one sided $P < 0.001$) compared with placebo, trastuzumab, and docetaxel in patients with untreated HER2-positive metastatic breast cancer.[16] The results of the PHILA study suggest that pyrotinib, trastuzumab, and docetaxel could be recommended as a first-line treatment option for patients with advanced HER2-positive breast cancer. Despite its remarkable efficacy, the synergistic mechanisms between pyrotinib and trastuzumab require further investigation. In addition, the CLEOPATRA study demonstrated that the combination of pertuzumab and trastuzumab with docetaxel was superior to trastuzumab and docetaxel in terms of PFS and OS for patients with untreated HER2-positive metastatic breast cancer.[17,18] This pertuzumab-containing therapy is now the standard treatment for advanced HER2-positive breast cancer. However, to achieve the optimal clinical outcomes in treatment-naïve metastatic patients, further investigation is warranted to determine the selection between the two dual HER2 blockade regimens.

In light of the PHILA study, we aimed to explore the synergistic effects of pyrotinib and trastuzumab combination treatment. Our results have shown that pyrotinib plus trastuzumab induces membrane HER2 ubiquitin-mediated downregulation and exhibits superior inhibition in the HER2 signalling pathway to pyrotinib or trastuzumab alone, especially in the context of HER2-dependent breast cancer. Our findings corroborate the results of the PHILA study and offer compelling evidence for pyrotinib in combination with trastuzumab and docetaxel as one of the optimal choices for patients with untreated HER2-positive metastatic breast cancer.

Methods

Cells and cell culture

The human cell lines BT474 (CVCL_0179), SKBR3 (CVCL_0033), and K562 (CVCL_0004) were purchased

from Procell Life Science & Technology Co. (China). The human cell line HCC1954 (CVCL_1259) was purchased from BeNa Culture Collection Co. (China). The murine cell line TUBO (CVCL_2A33) was purchased from Fenghui Biotechnology Co. (China). SKBR3, HCC1954, and TUBO cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) supplemented with 10% foetal bovine serum (FBS, Gibco, USA) and 1% penicillin–streptomycin (HyClone). BT474 and K562 cells were maintained in cell-specific medium purchased from Procell Life Science & Technology Co. (China). All the cells were cultured in a humidified atmosphere with 5% CO2 at 37 °C and authenticated using short tandem repeat profiling.

Reagents and antibodies

Pyrotinib was obtained from Hengrui Medicine Co. (China). Trastuzumab and pertuzumab were obtained from the Cancer Hospital, Chinese Academy of Medical Sciences. Rituximab was purchased from MedChemExpress (USA). Anti-neu mAb 7.16.4 was purchased from BioXcell (BE0277, AB_2687800). The antibodies were purchased from commercial companies: pHER2 (CST, 2243S, AB_490899), HER2 (CST, 2165S, AB_10692490), HER2 (Abcam, ab237715, AB_3291601), HER2 (Abcam, ab214275, AB_3291603), pEGFR (CST, 3777S, AB_2096270), EGFR (CST, 4267S, AB_2246311), pHER3 (CST, 4791S, AB_2099709), HER3 (CST, 12708S, AB_2721919), pAKT (CST, 4060S, AB_2315049), AKT (CST, 4691S, AB_915783), pERK (CST, 4370S, AB_2315112), ERK (CST, 4695S, AB_390779), GAPDH (CST, 5174S, AB_10622025), Survivin (CST, 2808S, AB_2063948), cPARP (CST, 9541S, AB_331426), Ki67 (CST, 9129S, AB_2687446), and Na–K ATPase (Abcam, ab76020, AB_1310695).

Cell viability assay

Given the specific growth of each cell line, the cells were seeded in 6-well plates at 10,000 to 20,000 cells/well and cultured with either vehicle, 10 nmol/L pyrotinib, 10 μg/mL trastuzumab, 10 nmol/L pyrotinib plus 10 μg/mL trastuzumab, or 10 μg/mL trastuzumab plus 10 μg/mL pertuzumab for 1–3 weeks. The cells were then stained with crystal violet, and images of each plate were quantified using ImageJ software. Meanwhile, the cells were seeded in 96-well plates at 8000 cells/well and treated with increasing concentrations of pyrotinib with or without 10 μg/mL trastuzumab for 72 h. Cell viability was then determined by the CCK8 assay kit manual (Dojindo, Japan). The absorbance at 450 nm was measured by a microplate photometer (Thermo Scientific, USA).

Animal experiments

Five-week-old female BALB/c-nu (n = 50) and BALB/c mice (n = 28) were purchased from HFK Bioscience (China) and housed in a specific pathogen-free facility with optimal temperature and humidity, regular light/dark cycles, and high air quality. They also had free access to water and food. Each mouse served as an independent experimental unit. All procedures involving the mice were conducted in accordance with the approved protocols and guidelines from the Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences.

To investigate the HER2 signalling pathway *in vivo*, 1×10^7 BT474 cells or 2×10^6 HCC1954 cells were suspended in diluted Matrigel (354248, Corning, USA) and injected subcutaneously into BALB/c-nu mice (n = 25 for each xenograft model). Oestrogen pellets (0.36 mg 60-day-release, Innovative Research of America, USA) were specifically used to promote the growth of BT474 tumours. Once the tumours reached 150–200 mm^3, the mice were randomly allocated to treatment with either saline, pyrotinib (10 mg/kg, once daily oral gavage, 5 days/week), trastuzumab (10 mg/kg, IP, twice a week), pyrotinib plus trastuzumab, or trastuzumab plus pertuzumab (10 mg/kg, IP, twice a week). Each group included five mice. After short-term treatment for 7 days, the mice were euthanised, and the tumours were collected for IHC staining of pHER2, HER2, pAKT, pERK and Ki67 (n = 5 for each group).

To generate the TUBO tumour model, 5×10^5 TUBO cells were injected subcutaneously into BALB/c mice (n = 28). Once the tumours reached 50 mm^3, the mice were randomly allocated to treatment with either saline, pyrotinib (10 mg/kg, once daily oral gavage, 5 days/week), 7.16.4 (10 mg/kg, IP, twice a week), or pyrotinib plus 7.16.4. Each group included seven mice. When the maximum tumour length diameter reached 15 mm, the mice were euthanised, and the tumours were collected for Western blotting (pHER2, HER2, pAKT, AKT, pERK, ERK, Survivin, cPARP, and GAPDH, n = 3 for each group) and IHC staining (HER2 and Ki67, n = 7 for each group). Tumour volume and body weight were recorded every four days. Tumour volume was calculated using the following equation: tumour volume = length × width × width/2.

Protein extraction and Western blotting

BT474, SKBR3, and HCC1954 cells were treated with either vehicle, 10 nmol/L pyrotinib, 10 μg/mL trastuzumab, 10 nmol/L pyrotinib plus 10 μg/mL trastuzumab, or 10 μg/mL trastuzumab plus 10 μg/mL pertuzumab for 48 h, and TUBO tumour tissues were minced. The lysis buffer (P0013, Beyotime, China) supplemented with protease and phosphatase inhibitors (P1049, Beyotime, China) was used to extract total proteins from the samples. BT474 and SKBR3 cells were treated with increasing concentrations of pyrotinib with or without 10 μg/mL trastuzumab for 24 h. The Mem-PER™ Plus Membrane Protein Extraction Kit (89842, Thermo Fisher, USA) was used for the isolation of membrane proteins. The lysates were separated by 10%

SDS-PAGE gel and transferred onto PVDF membranes (Millipore, USA). The samples were blocked with 5% non-fat milk in TBST for 1 h at room temperature and incubated with the appropriate primary antibodies overnight at 4 °C. The membranes were further incubated with HRP-conjugated secondary antibodies at room temperature for 2 h and visualised using enhanced chemiluminescence substrate (32106, Thermo Fisher, USA). Images were quantified using ImageJ software.

IHC staining

The tumours were fixed overnight and processed for paraffin embedded tissues. Five-μm sections were subjected to dewaxing, rehydration, and heat-induced antigen retrieval. The slides were incubated with primary antibodies overnight at 4 °C, followed by HRP-conjugated secondary antibodies at room temperature for 1 h. Visualization was performed using DAB, and the slides were counterstained with haematoxylin. Protein expression for each marker was evaluated by measuring the average intensity and proportion of positive cells using QuPath (version 0.5.1).

Immunofluorescence

The cells were cultured on sterilised coverslips with either vehicle, 100 nmol/L pyrotinib, 10 μg/mL trastuzumab, or 100 nmol/L pyrotinib plus 10 μg/mL trastuzumab for 6 h. Then, the cells were fixed with 4% paraformaldehyde, rinsed with PBS, permeabilised with 0.2% Triton X-100, and blocked with goat serum working solution. The cells were subsequently incubated with anti-HER2 primary antibody (ab214275, Abcam, UK) overnight at 4 °C and fluorescein secondary antibody at room temperature for 1 h. The coverslips with cells were finally mounted with mounting medium containing DAPI. Images were acquired using confocal laser scanning microscopy.

HER2 immunoprecipitation

The cells were cultured in 5% FBS medium supplemented with the proteasome inhibitor MG-132 (S1748, Beyotime, China) and treated with or without the IgG control (CST, 2729S, AB_1031062), 100 nmol/L pyrotinib, or 10 μg/mL trastuzumab at 37 °C for 6 h. Total protein lysates were extracted from the cells and incubated with 10 μg/mL trastuzumab on a rotating shaker at 4 °C for 4 h. Protein A/G magnetic beads (HY–K0202, MedChemExpress, USA) were then added, and the samples were rotated at 4 °C overnight. The immunoprecipitants were washed with the lysis buffer, boiled in 1 × loading buffer for 10 min, and subjected to Western blotting.

ADCC reporter bioassay

The target cells and the engineered Jurkat effector cells (GS-J2C/CD16A 158 V, GenScript ProBio, China) were co-incubated for 6 h in 10% FBS medium with or without increasing concentrations of pyrotinib or 10 μg/mL trastuzumab. The effector-to-target cell ratio was 3:1. Bio-Lite™ Luciferase Assay System working solution was added, and the luminescence signal was recorded with a microplate reader. Relative EC50 values were obtained using a four-parameter function as follows, characterizing a sigmoid curve where the relative luminescence unit was plotted against the concentration of pyrotinib: $Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{[(\text{LogEC50} - X) \times \text{Hill slope}]})$, X represents the concentration of pyrotinib, Y represents the relative luminescence unit. Rituximab was used as the negative control for the HER2-targeted ADCC response.

PBMC isolation and LDH assay

Whole blood was collected from three healthy female donors with written informed consent. Human PBMCs were isolated by Ficoll–Paque (GE Healthcare), resuspended in cell-specific medium purchased from Procell Life Science & Technology Co. (CM-H158, China), and cultured overnight with 50 IU/mL IL-2. The target cells were seeded in 96-well plates at 5000 cells/well for 24 h and pre-treated with 100 nmol/L pyrotinib for 1 h. The target cells and various ratios of PBMCs were then co-incubated for 6 h in 5% FBS medium with or without 10 μg/mL trastuzumab or 10 μg/mL pertuzumab. LDH release was detected following the manufacturer's instructions (C0017, Beyotime, China). The percentage of specific lysis was calculated as described in a previous study.[19] Rituximab was used as the negative control for the HER2-targeted ADCC response, and K562 cells were used as the positive control target cells for direct cytotoxicity.

Clinical specimens

Blood samples and clinical data were collected from the Cancer Hospital, Chinese Academy of Medical Sciences, with appropriate written informed consent. Tumour response was evaluated according to the Response Evaluation Criteria in Solid Tumours (RECIST, version 1.1). PFS was assessed by a physician based on radiological data and defined as the period between the start of treatment and the occurrence of disease progression.

Clinical case study

A 42-year-old patient with HER2-positive accessory breast cancer and liver metastasis received pyrotinib, trastuzumab and taxane in the Cancer Hospital, Chinese Academy of Medical Sciences. We collected paired biopsy samples from this patient's accessory breast tumour before and 3 weeks after treatment initiation. Written informed consent was obtained from the patient.

Targeted next generation sequencing and bioinformatics analysis

Plasma DNA from 18 patients in the PHILA trial was subjected to analysis with a customised panel of 1021

genes. The samples were processed following the standard laboratory protocols and manufacturer's guidelines. DNA extraction, library preparation, hybrid capture, and sequencing were carried out as previously described (GenePlus, Suzhou, China).[20] Single nucleotide variants were called using MuTect and NChot. Small insertions and deletions were identified by GATK. Somatic copy-number alterations were identified with CONTRA. Structural variations were identified using NoahCare structural variations detection. Gene Ontology (GO) annotation analysis was performed using the cluster Profiler package.[21]

Ethics

This study was conducted in compliance with the declaration of Helsinki and received informed consent from all human subjects. The Ethics Committee of Cancer Hospital, Chinese Academy of Medical Sciences provided the ethics approval statements for human subjects (NCC2021C-122) and all the animal experiments (NCC2021A155).

Statistical analysis

All animal experiments were performed adhered to the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines. Sample size was determined based on the resource equation method and the requirements of various biochemical experiments. E = N − T, where E is the error degrees of freedom, N is the total number of animals, and T is the number of groups. The appropriate acceptable range for E is 10–20. For BT474 and HCC1954 xenografts, BALB/c-nu mice (n = 25) were divided into five groups, and each experimental group included five mice. For the TUBO tumour model, BALB/c mice (n = 28) were divided into four groups, and each experimental group included seven mice. The quantitative data were shown as mean ± standard errors (SEM). We verified the normality distribution of continuous variables by Shapiro–Wilk test, and the differences between groups were examined by Student's *t* test. We determined combination index (CI) using Bliss combination model. $CI = ((E_A + E_B) - (E_A{*}E_B))/E_{AB}$, where E_A, E_B, and E_{AB} are the inhibition percentages of drug A, drug B, and combination AB, respectively (CI < 1.0 synergistic, CI = 1.0 additive, CI > 1.0 antagonistic). We used the log-rank test for univariate survival analyses and the Kaplan–Meier plot for presentation. Each experiment was performed with at least three independent rounds. All the statistical analyses were performed using GraphPad Prism (version 9.0) and R (version 4.2.0). All the statistical tests were two-tailed, and $P < 0.05$ was considered significant.

Role of the funding source

The funding sources were not involved in the study design; data collection, analysis, and interpretation of data; or decision to publish.

Results

Pyrotinib plus trastuzumab shows superior inhibition of cell growth than pyrotinib or trastuzumab alone in HER2-dependent breast cancer

First, the efficacy of pyrotinib plus trastuzumab was evaluated and compared with pyrotinib or trastuzumab alone in BT474 (HER2+, hormone receptor positive, HR+) and SKBR3 (HER2+, hormone receptor negative, HR–) cells, which are breast cancer cells highly dependent on HER2.[22,23] We then treated these cells with either vehicle, pyrotinib, trastuzumab, pyrotinib plus trastuzumab, or trastuzumab plus pertuzumab and examined their effects on cell viability. In BT474 and SKBR3 cells, pyrotinib plus trastuzumab caused a significant decrease in cell growth compared with pyrotinib, trastuzumab, or trastuzumab plus pertuzumab (Fig. 1a–c, $P < 0.05$ each treatment condition versus its respective control, and $P < 0.001$ pyrotinib plus trastuzumab versus other treatment groups by Student's *t* test). The bliss analyses also suggest a synergy between pyrotinib and trastuzumab. In parallel, pyrotinib plus trastuzumab substantially reduced cell viability in a dose-dependent manner (Fig. 1d and e). Taken together, these results indicate that pyrotinib plus trastuzumab more effectively inhibits cell growth than pyrotinib or trastuzumab alone, as well as trastuzumab plus pertuzumab, in HER2-dependent breast cancer.

Pyrotinib plus trastuzumab shows superior inhibition of HER2 signalling pathway than pyrotinib or trastuzumab alone in HER2-dependent breast cancer

To examine the impacts of these HER2-directed agents on intracellular signalling, we treated BT474 and SKBR3 cells with previous regimens for 48 h and performed Western blotting (WB). Compared with pyrotinib or trastuzumab alone, pyrotinib plus trastuzumab substantially reduced pHER2, pEGFR, pHER3, pAKT, and pERK protein levels (Fig. 1f, Supplementary Fig. S1). Moreover, trastuzumab plus pertuzumab also exhibited suboptimal inhibition of HER family phosphorylation and downstream signalling pathway. It is noteworthy that the WB analyses aligned with previous cell proliferation results. We also observed that pyrotinib plus trastuzumab induced a marked decrease in total HER2 levels compared with pyrotinib, trastuzumab, or trastuzumab plus pertuzumab. Subsequently, we established BT474 xenografts in athymic nude mice to confirm these molecular changes. After short-term treatment with previous regimens for 7 days, we obtained tumour tissues and performed IHC staining. Consistent with the WB results, the IHC analyses further verified the effects of pyrotinib plus trastuzumab on the HER2 signalling cascade (Fig. 2, Supplementary Fig. S2). In addition, we detected the substantial inhibition of tumour cell proliferation, as indicated by Ki67

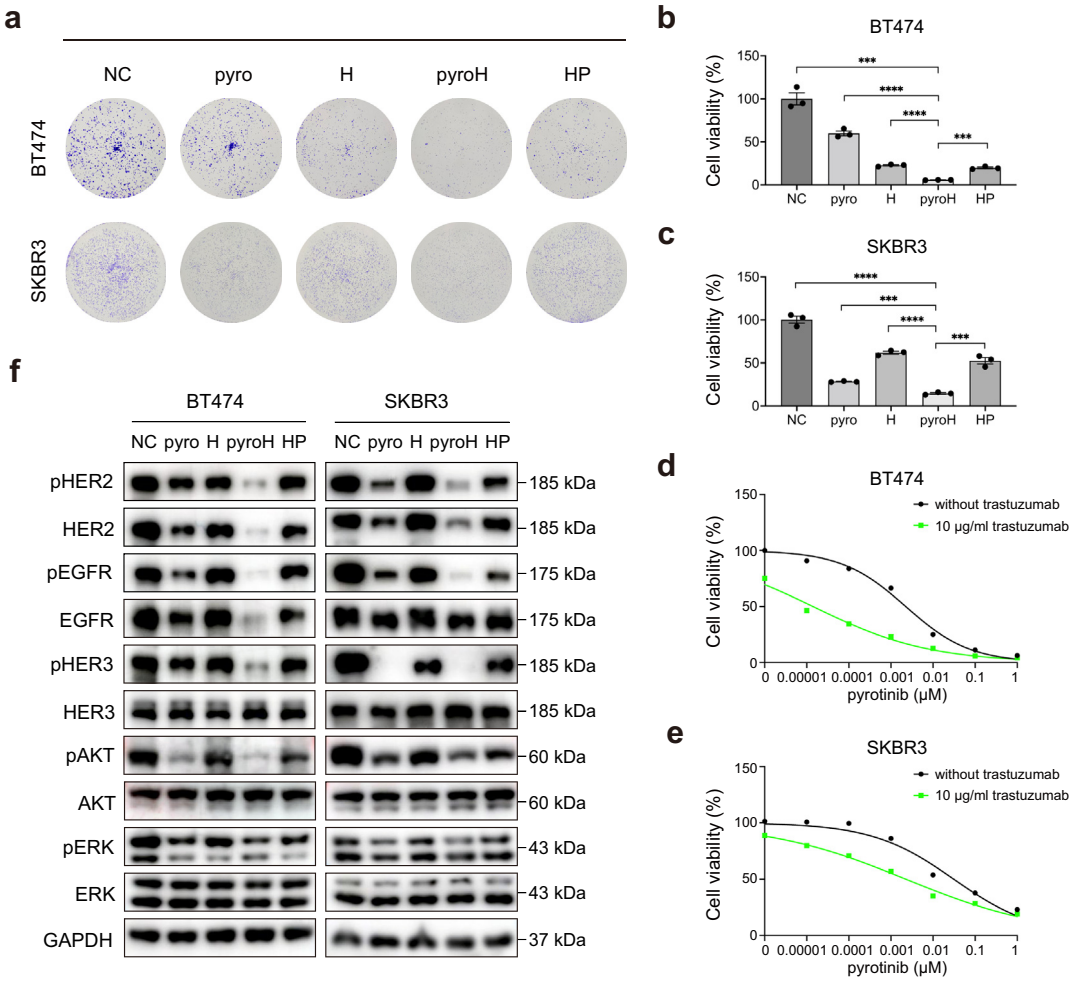

Fig. 1: Pyrotinib plus trastuzumab shows superior inhibition of cell growth than pyrotinib or trastuzumab alone in HER2-dependent breast cancer. **a–c**, BT474 and SKBR3 cells were fixed and stained with 0.06% crystal violet. Representative images of breast cancer cells after various treatments are shown in **a**. Cell viability was quantified using ImageJ and normalised with vehicle controls (**b** and **c**). **d** and **e**, Cell viability of BT474 and SKBR3 cells upon treatment with increasing concentrations of pyrotinib as monotherapy or in combination with trastuzumab for 72 h. **f**, BT474 and SKBR3 cells were cultured with various anti-HER2 agents for 48 h. Phosphorylation and total levels of HER2, EGFR, HER3, AKT, and ERK were detected by Western blotting. Three independent experiments were performed. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, and ns not significant by Student's t test. Data are represented as mean ± SEM. NC vehicle, pyro pyrotinib, H trastuzumab, pyroH pyrotinib plus trastuzumab, HP trastuzumab plus pertuzumab.

expression, after pyrotinib plus trastuzumab combination treatment. Collectively, these results suggest that pyrotinib plus trastuzumab more effectively inhibits the HER2 signalling pathway than pyrotinib or trastuzumab alone, as well as trastuzumab plus pertuzumab, in HER2-dependent breast cancer.

The combination of pyrotinib and trastuzumab potentiates membrane HER2 ubiquitination and downregulation

Our results revealed a marked decrease in total HER2 levels upon pyrotinib and trastuzumab combination treatment, leading us to further assess membrane HER2 levels, which serve as a crucial factor in HER2-targeted therapy. Subsequently, we isolated membrane proteins from BT474 and SKBR3 cells, and detected that pyrotinib plus trastuzumab more effectively reduced membrane HER2 expression than pyrotinib or trastuzumab alone (Fig. 3a and b). We also observed the substantial downregulation of HER2 surface expression following the combination treatment by immunofluorescence (Fig. 3c). Previous studies have suggested that irreversible HER2 inhibitors induce receptor ubiquitination and downregulation via HSP90 dissociation.[24,25] We thus evaluated whether trastuzumab could enhance this process. Cotreatment of these breast cancer cells with pyrotinib and trastuzumab resulted in stronger ubiquitination of immunoprecipitated HER2 than pyrotinib or

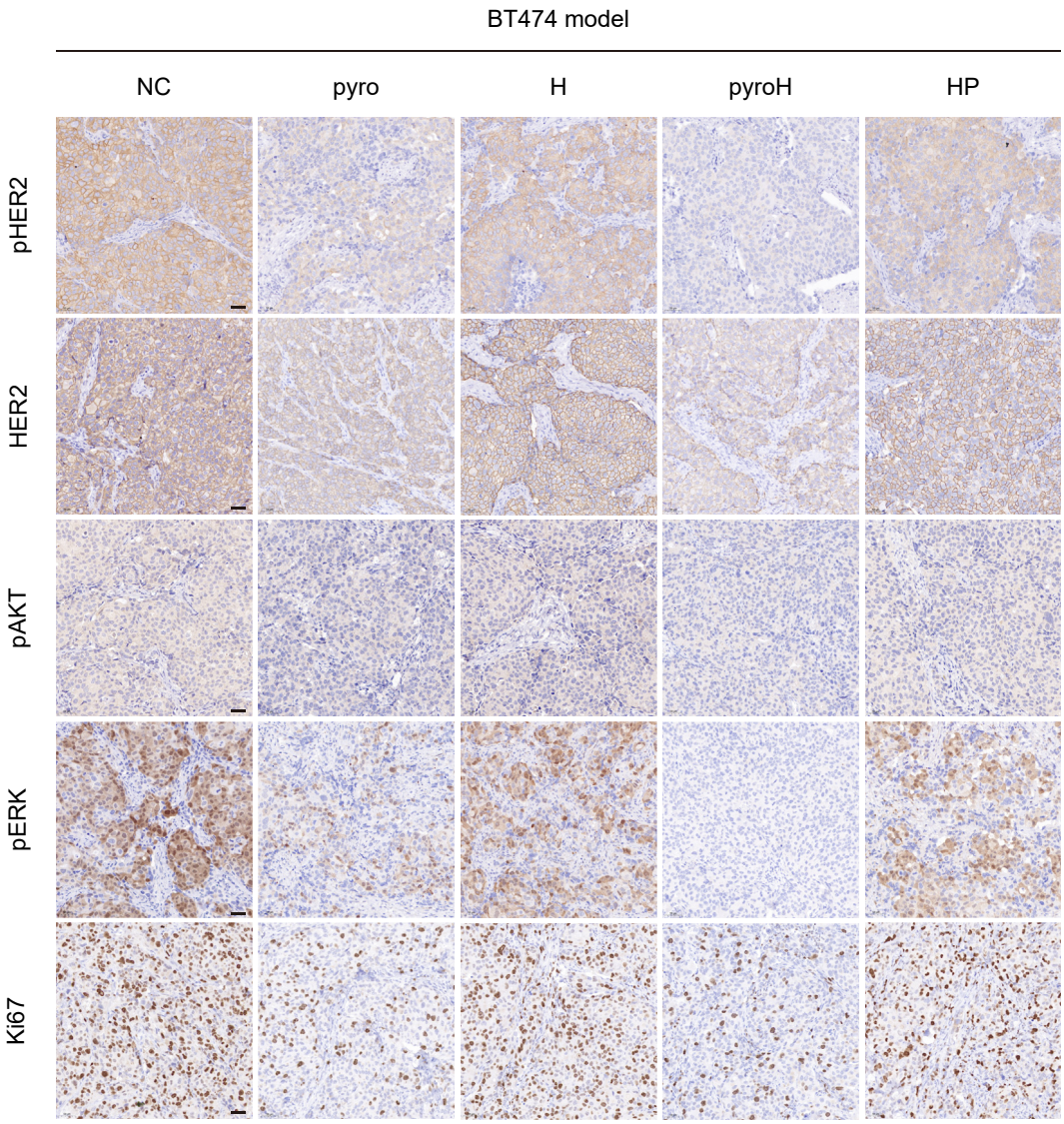

Fig. 2: Pyrotinib plus trastuzumab shows superior inhibition of HER2 signalling pathway than pyrotinib or trastuzumab alone in HER2-dependent breast cancer. The mice bearing BT474 xenografts were treated with various anti-HER2 agents for 7 days, and the tumours were collected. Protein levels of pHER2, HER2, pAKT, pERK, and Ki67 were detected by IHC. Representative images are shown. Scale bar, 50 μm. n = 5 animals per group. NC vehicle, pyro pyrotinib, H trastuzumab, pyroH pyrotinib plus trastuzumab, HP trastuzumab plus pertuzumab.

trastuzumab alone (Fig. 3d and e). Consistently, a weakened interaction between HER2 and HSP90 was identified upon the combination treatment. Taken together, these results indicate that pyrotinib plus trastuzumab promotes membrane HER2 ubiquitination and downregulation in HER2-dependent breast cancer.

We then evaluated the clinical efficacy of this combination in a 42-year-old patient with HER2-positive accessory breast cancer and liver metastasis. Immediately upon diagnosis, this patient received pyrotinib in combination with trastuzumab and taxane. The patient achieved a partial response after 9 weeks of therapy (Fig. 3f) and has continued with this treatment regimen as of now. In addition, we collected paired biopsy samples from this patient before and 3 weeks after treatment initiation and found a substantial decrease in membrane HER2 expression (Fig. 3g). This result further confirms that pyrotinib plus trastuzumab potentiates membrane HER2 downregulation and is consistent with the findings of PHILA study showing the promising efficacy of pyrotinib, trastuzumab, and docetaxel as a first-line treatment for patients with untreated HER2-positive metastatic breast cancer.

To find out whether pyrotinib-altered membrane HER2 levels determine the ADCC response, we employed a reporter cell line overexpressing human Fc

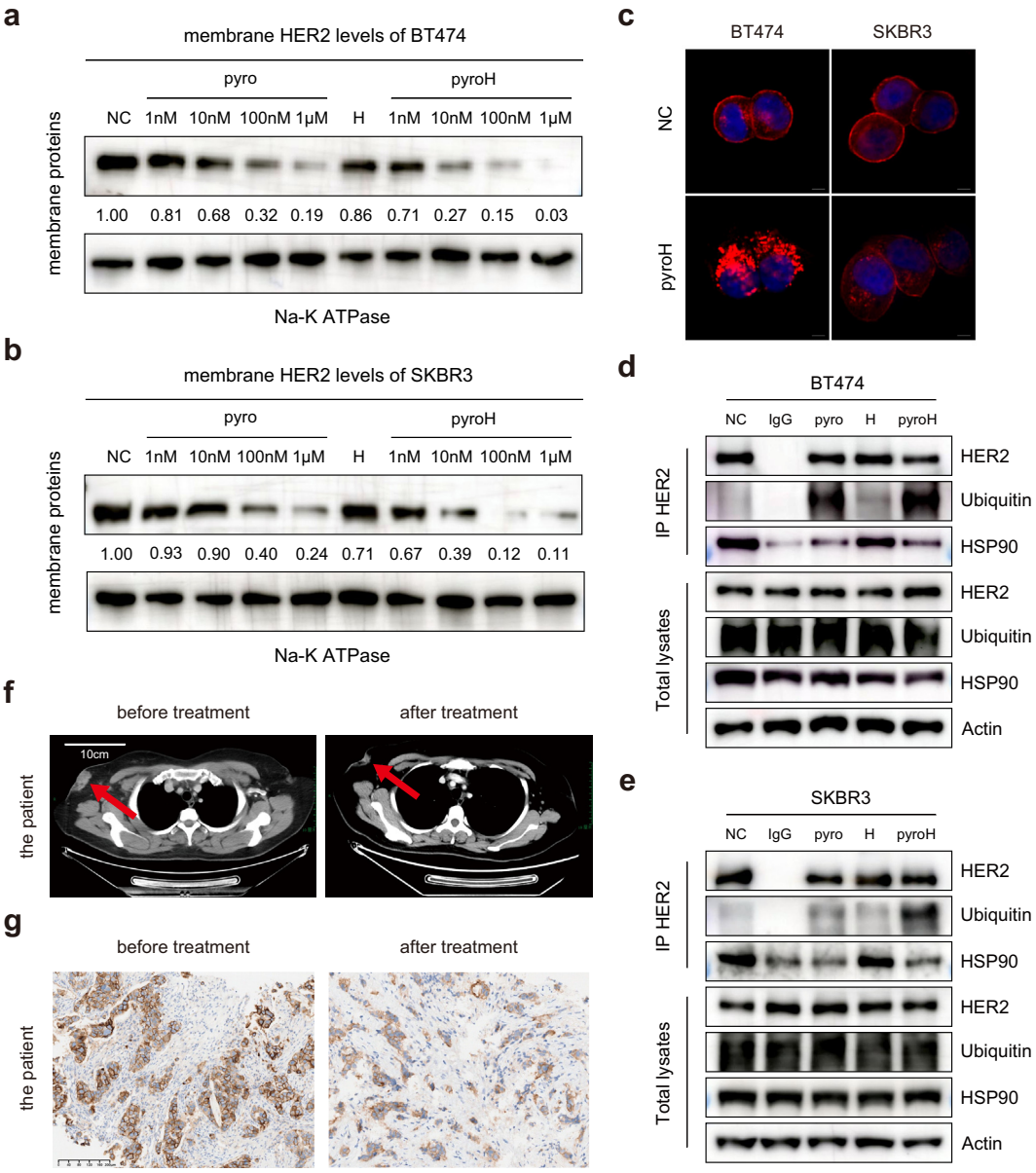

Fig. 3: The combination of pyrotinib and trastuzumab potentiates HER2 ubiquitination and downregulation. **a** and **b**, BT474 and SKBR3 cells were treated with increasing concentrations of pyrotinib with or without trastuzumab for 24 h. Membrane-bound HER2 was detected by Western blotting. **c–e**, BT474 and SKBR3 cells were treated with various anti-HER2 agents for 6 h. HER2 protein expression was examined by immunofluorescence. Representative images are shown in **c**. Scale bar, 5 μm. HER2 immunoprecipitations were performed using trastuzumab itself (or IgG control) as primary antibody in BT474 and SKBR3 cells (**d** and **e**). **f** and **g**, Computed tomography and HER2 IHC staining of a patient with untreated HER2-positive metastatic breast cancer who responded to pyrotinib, trastuzumab and taxane. Scale bar, 200 μm. Three independent experiments were performed. Data are represented as mean ± SEM. NC vehicle, pyro pyrotinib, H trastuzumab, pyroH pyrotinib plus trastuzumab.

gamma receptor IIIA (FcγRIIIA) to measure the biological activity of therapeutic antibodies in an ADCC mechanism of action assay. In BT474 and SKBR3 cells, the biological activity of trastuzumab (Supplementary Fig. S3a) remained stable at concentrations of pyrotinib up to 100 nmol/L; however, a marked decrease was observed upon treatment with high doses of pyrotinib (1 μmol/L or 10 μmol/L). In addition, we performed a LDH assay to evaluate the impact of pyrotinib on trastuzumab-mediated ADCC at different effector-to-target cell ratios (Supplementary Fig. S3b).[19] Breast cancer cells were pre-treated with pyrotinib (100 nmol/L) for

1 h. In BT474 and SKBR3 cells, pyrotinib treatment had no significant effect on trastuzumab-mediated ADCC. Moreover, we detected comparable ADCC levels between pyrotinib plus trastuzumab and trastuzumab plus pertuzumab.

Pyrotinib synergises with anti-neu antibody in the TUBO tumour model

TUBO tumours, derived from spontaneous carcinoma, have been established as a HER2-driven model in neu-transgenic mice.[26,27] We utilised this model to assess the anti-tumour efficacy of pyrotinib and the anti-neu antibody 7.16.4, which mimics trastuzumab. BALB/c mice bearing TUBO tumours were then treated with vehicle, pyrotinib, 7.16.4, or pyrotinib plus 7.16.4 (Fig. 4a). We detected that pyrotinib plus anti-neu antibody synergistically delayed tumour growth with a CI value of 0.75 (Fig. 4b–d, Supplementary Fig. S4a and Table S1, for tumour volume and tumour weight, $P < 0.05$ each treatment condition versus its respective control, and $P < 0.001$ pyrotinib plus 7.16.4 versus other treatment groups by Student's t test, for body weight, no significant differences between the groups by Student's t test). HER2 surface expression was decreased in TUBO tumours following the combination treatment (Fig. 4f, Supplementary Fig. S4c). We also observed the potent inhibition of tumour cell proliferation, as measured by Ki67 expression, in TUBO tumours treated with pyrotinib plus anti-neu antibody (Supplementary Fig. S4b and d). Moreover, it strongly reduced the protein levels of pHER2, HER2, pAKT, pERK, and Survivin with a marginal increase in cleaved PARP (Fig. 4e, Supplementary Fig. S4e). Taken together, these results strengthen the argument that pyrotinib and anti-neu antibody can significantly reduce membrane HER2 levels and completely inhibit the HER2 signalling cascade (Fig. 4g).

Pyrotinib and trastuzumab combination treatment shows marginal efficacy in HER2-positive breast cancer with genetic alterations in HER2 signalling cascade

To confirm the significant efficacy of pyrotinib plus trastuzumab especially in HER2-dependent breast cancer, we performed reverse validation using models of HCC1954 breast cancer cells harbouring the PIK3CA H1047R mutation. Subsequently, pyrotinib plus trastuzumab modestly decreased the cell viability (no significant differences between the groups by Student's t test) and had no inhibitory effect on the HER2 signalling cascade both *in vitro* and *in vivo* (Fig. 5a–d, Supplementary Figs. S5–S7). We did not detect a synergy between pyrotinib and trastuzumab using the Bliss model. We further substantiated the marginal efficacy of these anti-HER2 regimens in the PHILA trial. Plasma samples were collected from our centre cohort of 18 patients prior to the start of pyrotinib or placebo in combination with trastuzumab and docetaxel (Supplementary Table S2). We obtained ctDNA from the samples and performed targeted next generation sequencing. The detected molecular alterations in ctDNA are presented in Fig. 5e and Supplementary Table S3. GO annotation analysis revealed that the mutated genes were significantly enriched in the PI3K-Akt signalling pathway, Ras signalling pathway, MAPK signalling pathway, and mTOR signalling pathway (Fig. 5f). Patients with genetic alterations in the HER2 signalling cascade had markedly shorter median PFS than individuals with wild-type pathway among the 18-patient cohort (10.4 months versus 24.8 months, $P = 0.039$) (Fig. 5g). A similar trend was also observed in terms of median PFS within the group receiving pyrotinib, trastuzumab, and docetaxel combination (10.4 months versus 27.5 months, $P = 0.39$) (Supplementary Fig. S8). Collectively, these results suggest that pyrotinib and trastuzumab combination treatment exhibits limited efficacy in HER2-positive breast cancer with genetic alterations in the HER2 signalling cascade.

Discussion

We have revealed that pyrotinib plus trastuzumab displays superior anti-tumour efficacy compared to pyrotinib or trastuzumab alone, especially in models of HER2-dependent breast cancer. This study demonstrates that pyrotinib plus trastuzumab can induce membrane HER2 receptor ubiquitin-mediated downregulation, block the entire HER2 signalling pathway, and completely overcome HER2 dependency in HER2-positive breast cancer. Thus, patients who are dependent on the HER2 signalling cascade may benefit more from the combination of pyrotinib, trastuzumab, and docetaxel.

Previous studies have demonstrated that trastuzumab binding to subdomain IV of HER2 disrupts HER2 homodimers and ligand-independent HER2/HER3/PI3K complex.[28,29] However, this monoclonal antibody cannot effectively hinder the dimerization of HER2 with ligand-activated EGFR or HER3. Notably, TKIs, a class of small-molecule inhibitors, target the intracellular domain of HER2, compete with ATP, and block tyrosine kinase enzymes. Dual HER2 blockade with TKI and trastuzumab was reported to exhibit more potent inhibition of the HER2 signalling pathway than TKI or trastuzumab alone.[3,30,31] Irreversible inhibitors, when combined with trastuzumab, achieve more effective blockade of HER receptors compared to reversible inhibitors. Consistent with prior reports, we have demonstrated that pyrotinib, an irreversible pan-HER TKI,[32] in combination with trastuzumab, results in a profound inhibition of HER family phosphorylation and downstream signalling, significantly surpassing the efficacy of pyrotinib or trastuzumab alone. We have further elucidated the robust inhibitory effects of

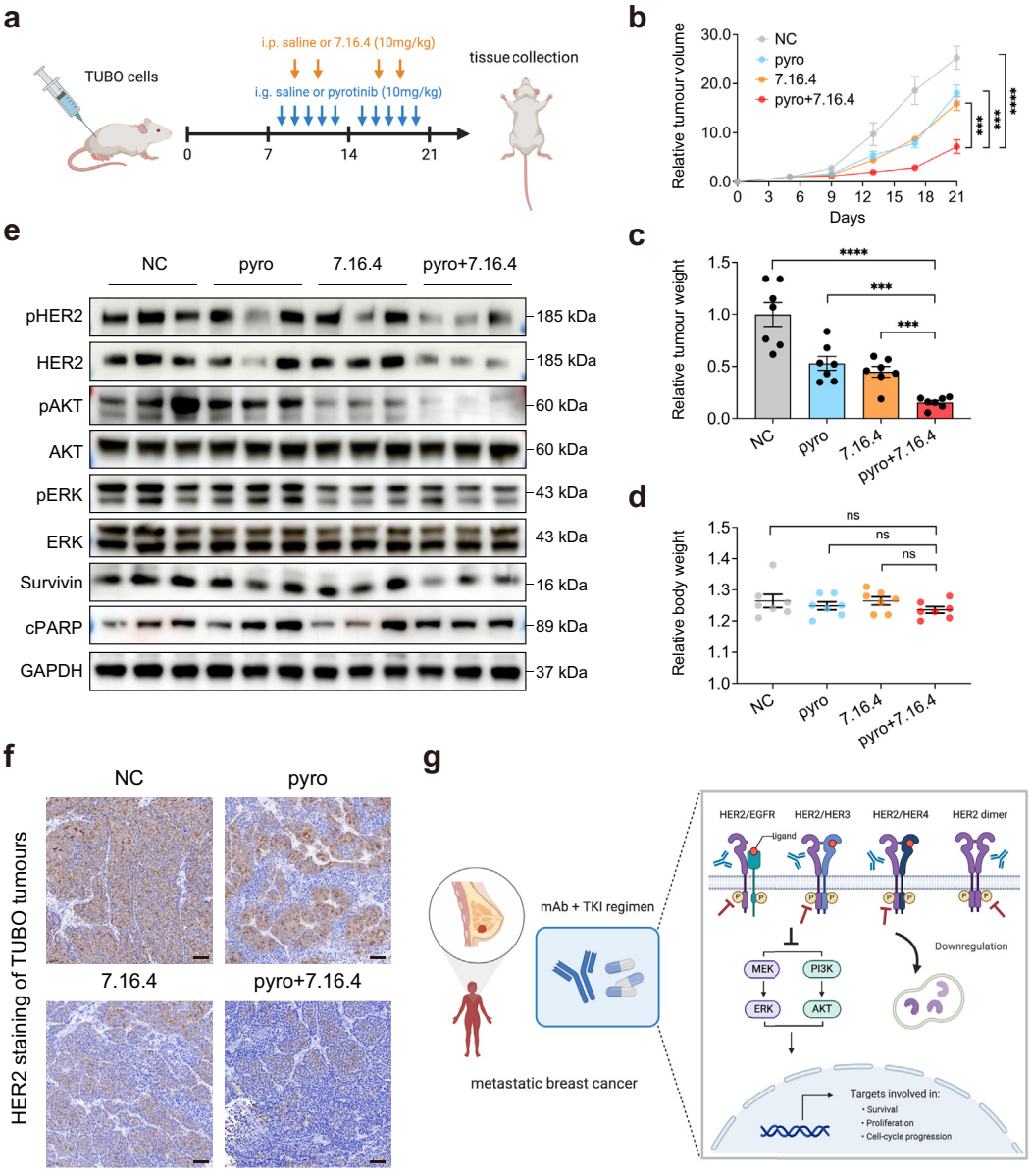

Fig. 4: Pyrotinib synergises with anti-neu antibody in the TUBO tumour model. **a**, Treatment scheme of BALB/c mice bearing TUBO tumours. **b–d**, Tumour volume (**b**), tumour weight (**c**), and body weight (**d**) changes of the mice in various treatment groups over 21 days. **e**, Western blotting for pHER2, HER2, pAKT, AKT, pERK, ERK, Survivin, and cPARP in TUBO tumours. **f**, HER2 IHC staining of TUBO tumours. Representative images are shown. Scale bar, 50 μm **g**, Schematic representation for the synergistic mechanisms of pyrotinib plus trastuzumab in HER2 positive breast cancer cells. n = 7 animals per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, and ns not significant by Student's t test. Data are represented as mean ± SEM. NC vehicle, pyro pyrotinib, pyro+7.16.4 pyrotinib plus 7.16.4.

pyrotinib and trastuzumab, especially in the context of HER2-dependent BT474 and SKBR3 models. We have also confirmed that the combination regimen exhibits high sensitivity in a HER2-driven TUBO model. Conversely, we have observed no synergy between pyrotinib and trastuzumab in models of HCC1954 cells, which display a functional mutation in PIK3CA. Thus, our results underscore the notable advantage of pyrotinib and trastuzumab combination treatment compared to pyrotinib or trastuzumab alone in HER2-dependent breast cancer. However, the underlying mechanisms of this robust synergy in overcoming HER2 dependency remain poorly understood.

Interactions with HSP90 play a crucial role in stabilizing HER2 at the cell membrane. Early studies have demonstrated that HSP90 inhibition induces receptor polyubiquitination, which results in either proteasomal degradation or the initiation of HER2 internalization.[33–36]

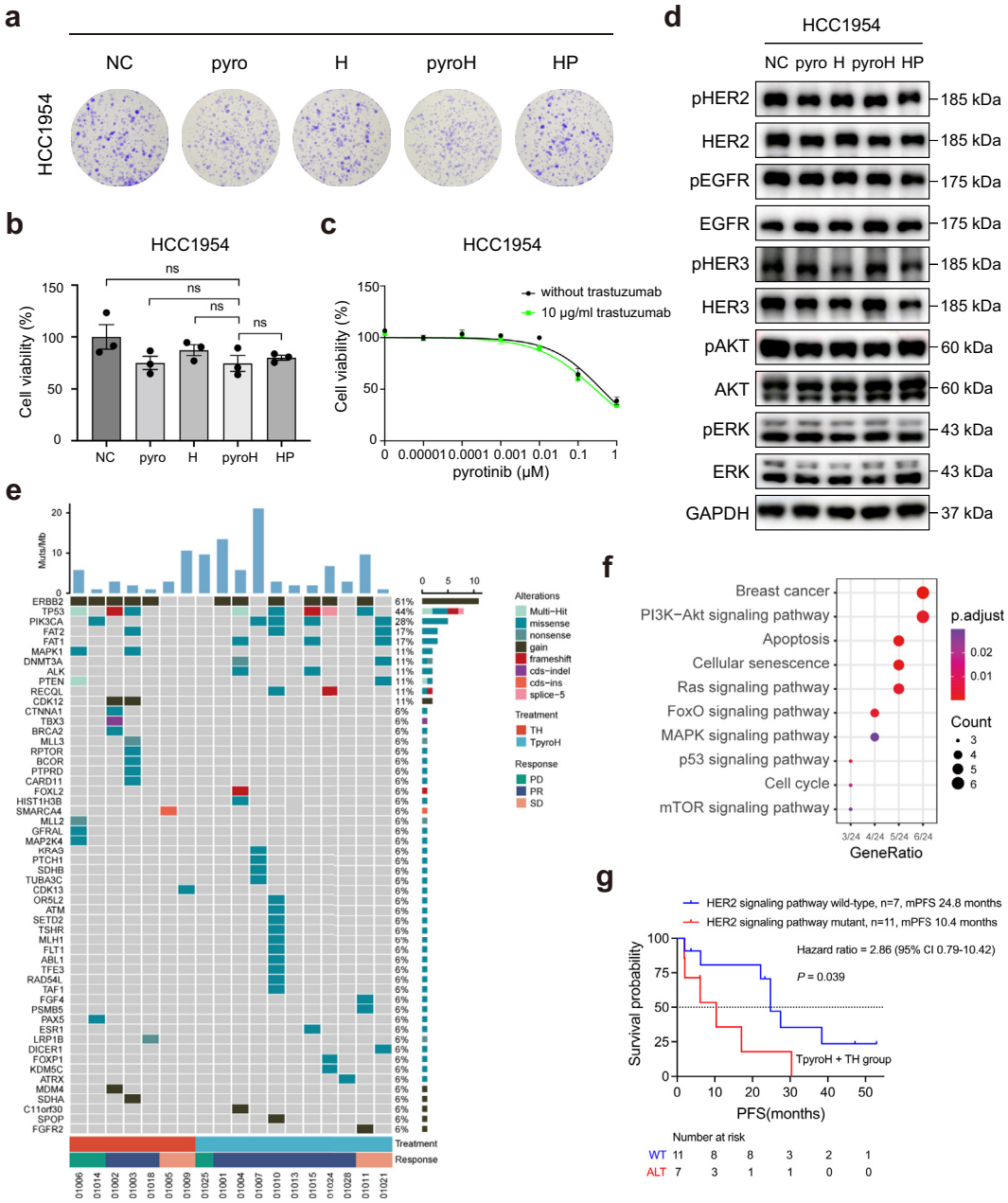

Fig. 5: Pyrotinib and trastuzumab combination treatment shows marginal efficacy in HER2-positive breast cancer with genetic alterations in HER2 signalling cascade. **a** and **b**, HCC1954 cells were fixed and stained with 0.06% crystal violet. Representative images are shown in **a**. Cell viability was quantified using ImageJ and normalised with vehicle controls (**b**). **c**, Cell viability of HCC1954 cells upon treatment with increasing concentrations of pyrotinib as monotherapy or in combination with trastuzumab for 72 h. **d**, HCC1954 cells were cultured with various anti-HER2 agents for 48 h. Phosphorylation and total levels of HER2, EGFR, HER3, AKT, and ERK were detected by Western blotting. **e**, The landscape of altered genes at baseline in a cohort of 18 patients at our centre from the PHILA trial. **f**, GO enrichment analysis of mutated genes in plasma from our centre cohort. **g**, The Kaplan–Meier curves of PFS in the 18-patient cohort by the alteration status of HER2 signalling pathway. Three independent experiments were performed. ns not significant by Student's *t* test. Data are represented as mean ± SEM. NC vehicle, pyro pyrotinib, H trastuzumab, pyroH pyrotinib plus trastuzumab, HP trastuzumab plus pertuzumab. TpyroH pyrotinib, trastuzumab, and docetaxel. TH placebo, trastuzumab, and docetaxel.

Moreover, when HSP90 dissociates from HER2, the E3 ubiquitin ligase CHIP or Cullin5 can efficiently downregulate HER2.[37,38] Disruption of HER2-HSP90 interactions has been suggested as a promising strategy for treating HER2-dependent breast cancer.[39,40] HSP90 inhibitors were reported to overcome trastuzumab resistance induced by full-length HER2 or p95 HER2.[41,42] Given the potential toxicity of HSP90 inhibitors, irreversible pan-HER TKIs are considered to be alternatives for blocking HER2-HSP90 interactions. Consistent with prior reports, we have demonstrated that pyrotinib, which acts as a HER2-specific HSP90 inhibitor, enhances the downregulation of membrane-bound HER2. We have revealed that pyrotinib and trastuzumab combination treatment can potently reduce HER2-HSP90 interactions and further promote the HER2 ubiquitination in a synergistic manner. Furthermore, breast cancer cells and a TUBO model were utilised to confirm the substantial decrease in membrane HER2 levels. We have also demonstrated such a decrease in one patient with HER2-positive metastatic breast cancer who could benefit from pyrotinib in combination with trastuzumab and taxane as a first-line treatment. In our study, we observed that trastuzumab alone induced HER2 ubiquitination with no significant change in the interaction between HER2 and HSP90. This finding is consistent with a previous study, and we postulate that other proteins involved in HER2 stabilization may be crucial for the trastuzumab-induced ubiquitination.[38] Further investigation is necessary to elucidate the alternative synergistic mechanisms induced by pyrotinib and trastuzumab combination treatment.

Moreover, accumulating evidence suggests that TKIs could alter the trastuzumab-mediated ADCC response.[43–45] For example, lapatinib is thought to induce membrane HER2 accumulation and potentiate trastuzumab-mediated ADCC in HER2-overexpressing cancer cells.[46–48] However, the impact of TKIs on the ADCC response remains controversial. Recent studies have indicated that ADCC activity is not directly correlated with membrane HER2 levels, and that lapatinib does not increase the trastuzumab-mediated ADCC response.[45,49] Consistent with prior reports, we have demonstrated that 100 nmol/L pyrotinib, which significantly reduces HER2 surface expression in combination with trastuzumab, exhibits no substantial effects on ADCC response induced by trastuzumab alone. We have also confirmed the synergistic efficacy of pyrotinib plus trastuzumab in the TUBO tumour model with an intact immune system. However, the exact impact of pyrotinib and trastuzumab combination treatment on anti-tumour immunity warrants further study.

In phase I trials of pyrotinib or pyrotinib plus capecitabine, concurrent mutations in the HER2-related signalling network were associated with limited efficacy.[50–52] A prospective neoadjuvant study revealed that patients with HER2-positive breast cancer harbouring PIK3CA-activating mutations exhibited a suboptimal response to pyrotinib and trastuzumab plus chemotherapy.[53] In the 18-patient cohort of the PHILA trial, patients with genetic alterations in the HER2 signalling cascade have also demonstrated a shorter median PFS than individuals with the wild-type pathway. Thus, we propose that patients who do not depend on HER2 may exhibit an unsatisfactory response to the PHILA combination regimen and potentially benefit more from anti-HER2 ADCs.[54,55]

Since pertuzumab, trastuzumab, and docetaxel combination treatment is the standard regimen, we have also assessed the efficacy of pyrotinib plus trastuzumab and trastuzumab plus pertuzumab in models of HER2-positive breast cancer. Consistent with previous studies,[3,56–58] pyrotinib plus trastuzumab superiorly inhibited the HER2 signalling pathway compared to trastuzumab plus pertuzumab. Moreover, we detected comparable ADCC levels between pyrotinib plus trastuzumab and trastuzumab plus pertuzumab. However, there is currently a lack of head-to-head clinical trials between the two dual HER2 blockade strategies. Meanwhile, this study has some limitations. First, pyrotinib plus trastuzumab may involve other key synergistic mechanisms that we have not explored. Second, we utilised a small number of clinical specimens to demonstrate the HER2 dependency, and the sample size should be increased for further validation. Third, we have not yet identified specific biomarkers to screen for patients with HER2-dependent breast cancer, which would allow for the prioritization of the PHILA regimen. Therefore, further investigations are needed to determine which comprehensive biomarkers can be used to accurately predict HER2 dependency in clinical patients with breast cancer.

In summary, pyrotinib and trastuzumab combination treatment showed synergistic anti-tumour efficacy and comprehensively inhibited the HER2 signalling pathway, especially in models of HER2-dependent breast cancer. Moreover, pyrotinib plus trastuzumab potentiated membrane HER2 ubiquitination and downregulation, thus significantly contributed to overcoming HER2 dependency in HER2-positive breast cancer. In addition, pyrotinib-altered membrane HER2 levels had no significant effect on trastuzumab-mediated ADCC. Based on these results, we propose that pyrotinib, trastuzumab, and docetaxel might be one of the optimal choices for patients with untreated HER2-positive metastatic breast cancer that is dependent on HER2 signalling cascade.

Contributors

F.M., H.Q., and B.X. conceptualised and supervised this study. S.L. and B.L. contributed to the study design and conducted the experiments. Y.W. and T.Y. collected the samples and processed the data. L.L., H.G., and C.Z. contributed to the statistical analysis. S.L. and B.L. wrote the manuscript. S.L., B.L., and F.M. revised the manuscript and verified the

underlying data. All authors have read and approved the article. S.L. and B.L. contributed equally to this work.

Data sharing statement

The data generated and analysed in the present study are included in this manuscript and the supplementary files. Additional information is available from the corresponding author upon reasonable request.

Declaration of interests

The authors declare that they have no competing interests.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2021YFF1201300), the National Natural Science Foundation of China (82172875), the CAMS Innovation Fund for Medical Sciences (CIFMS) (2022-I2M-2-001), and the Joint Innovative Fund of Beijing Natural Science Foundation and Changping District (L234004). We would like to thank Geneplus Inc. for the production and analysis of ctDNA data, and BioRender.com for the creation of schematic diagrams.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105379.

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