



Original Articles

Combined inhibition of CDK4/6 and AKT is highly effective against the luminal androgen receptor (LAR) subtype of triple negative breast cancer

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ABSTRACT

Luminal Androgen Receptor (LAR) triple-negative breast cancers (TNBC) express androgen receptors (AR), exhibit high frequency of PIK3CA mutations and intact RB. Herein, we investigated combined blockade of the CDK4/6 and PI3K signaling with palbociclib, alpelisib, and capivasertib, which inhibit CDK4/6, PI3K α , and AKT1-3, respectively. The combination of palbociclib/capivasertib, but not palbociclib/alpelisib, synergistically inhibited proliferation of MDA-MB-453 and MFM-223 LAR cells [synergy score 7.34 ($p = 5.81 \times 10^{-11}$) and 4.78 ($p = 0.012$), respectively]. The AR antagonist enzalutamide was inactive against MDA-MB-453, MFM-223, and CAL148 cells and did not enhance the efficacy of either combination. Palbociclib/capivasertib inhibited growth of LAR patient-derived xenografts more potently than palbociclib/alpelisib. Treatment of LAR cells with palbociclib suppressed phosphorylated-RB and resulted in adaptive phosphorylation/activation of S473 pAKT and AKT substrates GSK3 β , PRAS40, and FoxO3a. Capivasertib blocked palbociclib-induced phosphorylation of AKT substrates more potently than alpelisib. Treatment with PI3K β inhibitors did not block phosphorylation of AKT substrates, suggesting that PI3K β did not mediate the adaptive response to CDK4/6 inhibition. Phosphokinase arrays of MDA-MB-453 cells treated with palbociclib showed time-dependent upregulation of PDGFR β , GSK3 β , STAT3, and STAT6. RNA silencing of PDGFR β in palbociclib-treated MDA-MB-453 and MFM-223 cells blocked the upregulation of S473 pAKT, suggesting that the adaptive response to CDK4/6 blockade involves PDGFR β signaling. Finally, treatment with palbociclib and the PDGFR inhibitor CP637451 arrested growth of MDA-MB-453 and MFM-223 cells to the same degree as palbociclib/capivasertib. These findings support testing the combination of CDK4/6 and AKT inhibitors in patients with LAR TNBC, and further investigation of PDGFR antagonists in this breast cancer subtype.

1. Introduction

Triple-negative breast cancer (TNBC) is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) amplification. This heterogeneous clinical subtype of breast cancer has been further stratified into four molecular subtypes based on gene expression profiles [1,2]. Among the TNBC subtypes, the Luminal Androgen Receptor (LAR) subtype is the least responsive to neoadjuvant chemotherapy, with a pathological complete response rate of ~10 % [3–6]. In addition to chemotherapy,

the treatment for TNBC patients includes immune checkpoint inhibitors, poly (ADP-ribose) polymerase (PARP) inhibitors, and the antibody drug conjugate sacituzumab govitecan [7]; however, their benefit in the LAR subtype is either limited or unclear [8].

LAR TNBCs are characterized by luminal gene expression and presence of androgen receptors (AR) [1,2]. They are predominantly *RB1* wild type (72 % in LAR vs. 42 % in non-LAR TNBC), are enriched in *CDKN2A* loss [9], and exhibit a high rate of somatic alterations in the PI3K/AKT pathway (*PIK3CA*, *AKT1*, *PTEN*) [10], all together suggesting these tumors might be sensitive to therapies targeting AR signaling,

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CDK4/6, and PI3K/AKT. Bicalutamide, a first generation AR antagonist with a partial agonist activity, and enzalutamide, a second generation AR full antagonist [11], showed modest clinical benefit rates (ranging between 19 % and 25 %) in patients with metastatic AR + TNBC [12, 13]. Coussy et al. reported that most LAR TNBC patient-derived xenografts (PDX) are resistant to enzalutamide [14], further suggesting AR signaling may not be the main driver of LAR TNBC progression. Sevitonol (Innocrin), a dual androgen biosynthesis inhibitor and AR antagonist, is currently in clinical trials for AR + TNBC. Treatment with the combination of sevitonol and the CDK4/6 inhibitor (CDK4/6i) abemaciclib resulted in modest growth inhibition of MDA-MB-453 LAR xenografts compared to the CDK4/6i alone [15]. Nevertheless, CDK4/6i have substantial activity against LAR TNBC cell lines, similar to that reported in estrogen receptor-positive (ER+) breast cancer cells [16]. However, results from a single-arm phase 2 study of the CDK4/6i abemaciclib in patients with RB-positive TNBC (NCT03130439) did not show clinical benefit [17], although the proportion of patients with the LAR subtype in this TNBC cohort was not reported. Combined treatment with CDK4/6 and PI3K inhibitors has demonstrated a synergistic anti-tumor effect in preclinical models of breast cancer, including LAR TNBC [16,18,19].

The CDK4/6 inhibitors palbociclib, ribociclib and abemaciclib, the PI3K α inhibitor alpelisib, and the pan-AKT ATP-competitive inhibitor capivasertib, in combination with antiestrogen therapies, are currently approved for the treatment of patients with advanced ER + breast cancer. More recently, the INAVO 120 trial showed safety and efficacy of the triple combination of fulvestrant, palbociclib, and the PI3Ki inavolisib in patients with ER + breast cancer [20]. Based on the above-mentioned molecular characteristics of LAR TNBC [1,2], and the feasibility of combining PI3K/AKT and CDK4/6 inhibitors, we sought to investigate the most effective combinations of AR, CDK4/6, and PI3K-AKT antagonists in preclinical models of LAR TNBC in order to inform future clinical trials in this breast cancer subtype.

2. Materials and Methods

Cell lines, Ligands, and Inhibitors: Molecular features of the breast cancer cell lines used in this study are detailed in [Supplementary Table 1](#). All cell lines (MDA-MB-453, MFM-223, CAL-148, SUM159PT, MCF7, BT20 and HCC38) were purchased from ATCC and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The 22Rv1 cell line was kindly provided by Dr. Suzanne Conzen (UT Southwestern Medical Center). Cell lines were tested for Mycoplasma contamination using the Lonza MycoAler Mycoplasma Detection Kit (catalog no. LT07418). MDA-MB-453 and MFM-223 cells were profiled with Cell Line Authentication kit purchased from ATCC (Lot:70044064). All cell lines were maintained at 37 °C and 5 % CO₂ in Dulbecco's Modified Eagle Medium (DMEM) (Genesee Scientific), except HCC38 that was maintained in RPMI-1640 supplemented with 10 % fetal bovine serum (FBS) and DHT 0.007 ng/mL (Omega Scientific, Inc.), 1 % penicillin-streptomycin (HyClone), and 1 % sodium pyruvate (Corning). Cell lines were tested routinely for Mycoplasma. For *in vitro* experiments, palbociclib, alpelisib, capivasertib, enzalutamide, ipatasertib, AZD8186, GSK2636771, TGX221, pictilisib and CP673451 were purchased from SelleckChem. For *in vivo* experiments, palbociclib was purchased from SelleckChem, and alpelisib and capivasertib were obtained from MedChemExpress. Recombinant human PDGF-BB and PDGF-DD proteins were purchased from R&D Systems (catalog numbers: 220-BB and 1159-SB, respectively).

Cell proliferation Assays: Cell proliferation after treatment with inhibitors was measured in clonogenic assays or using the Incucyte S3 Live-Cell Analysis system. For clonogenic assays, single-cell suspensions of 6000 cells were seeded into 60-mm culture dishes on day 0 and allowed to attach for 24 h at 37 °C in media containing 10 % FBS. Drugs or DMSO (for control cells) were added on day 1 and replenished with fresh media every 4 days. After 14 days, colonies were fixed in 33.3 %

acetic acid/66.7 % methanol for 15 min at room temperature and stained with 1.0 % crystal violet. Dishes were washed with water and colonies were digitally scanned. For Incucyte analysis, 1000 cells per well were seeded in 96-well plates (Corning) in triplicate. The next day, inhibitors or DMSO (control) were added to the monolayers. Cell proliferation was quantified after 6 days of treatment. For studies examining the synergistic anti-tumor effects of the inhibitors, we used the Loewe additivity model with the Synergy Finder tool (www.synergyfinder.org) [21].

In vivo Experiments: Animal experiments were approved by the UTSW Institutional Animal Care and Use Committee. The LAR TNBC HCI-009 (PIK3CA mutant, RB wild type) PDX was kindly provided by Dr. Alana Welm (Huntsman Institute) [22]. PDX fragments were implanted s.c. into 6-week-old female NOD scid gamma (NSG) mice. Once tumors reached approximately 200 mm³, mice were randomized to receive treatment with vehicle (5 % DMSO, 40 % PEG-300, and 5 % Tween-80 in sterilized water), palbociclib (50 mg/mouse/day, via orogastric gavage), alpelisib (25 mg/kg/day, via orogastric gavage), capivasertib (100 mg/kg/bid 4 days on-3 days off, via orogastric gavage), or combination of these drugs. For a second *in vivo* study, 10 million MDA-MB-453 cells were inoculated s.c. into 6-week-old female nude mice. Once tumors reached approximately 100 mm³, mice were randomized to receive treatment with vehicle (5 % DMSO, 40 % PEG-300, and 5 % Tween-80 in sterilized water), palbociclib (50 mg/mouse/day, via orogastric gavage), alpelisib (30 mg/kg/day, via orogastric gavage), capivasertib (150 mg/mouse/day, via orogastric gavage), or combination of these drugs. Tumor size was serially measured with calipers every three days and tumor volume was calculated using the formula: volume = width² × length/2. At the end of the treatment, tumors were harvested and snap-frozen in liquid nitrogen or fixed in 10 % neutral-buffered formalin.

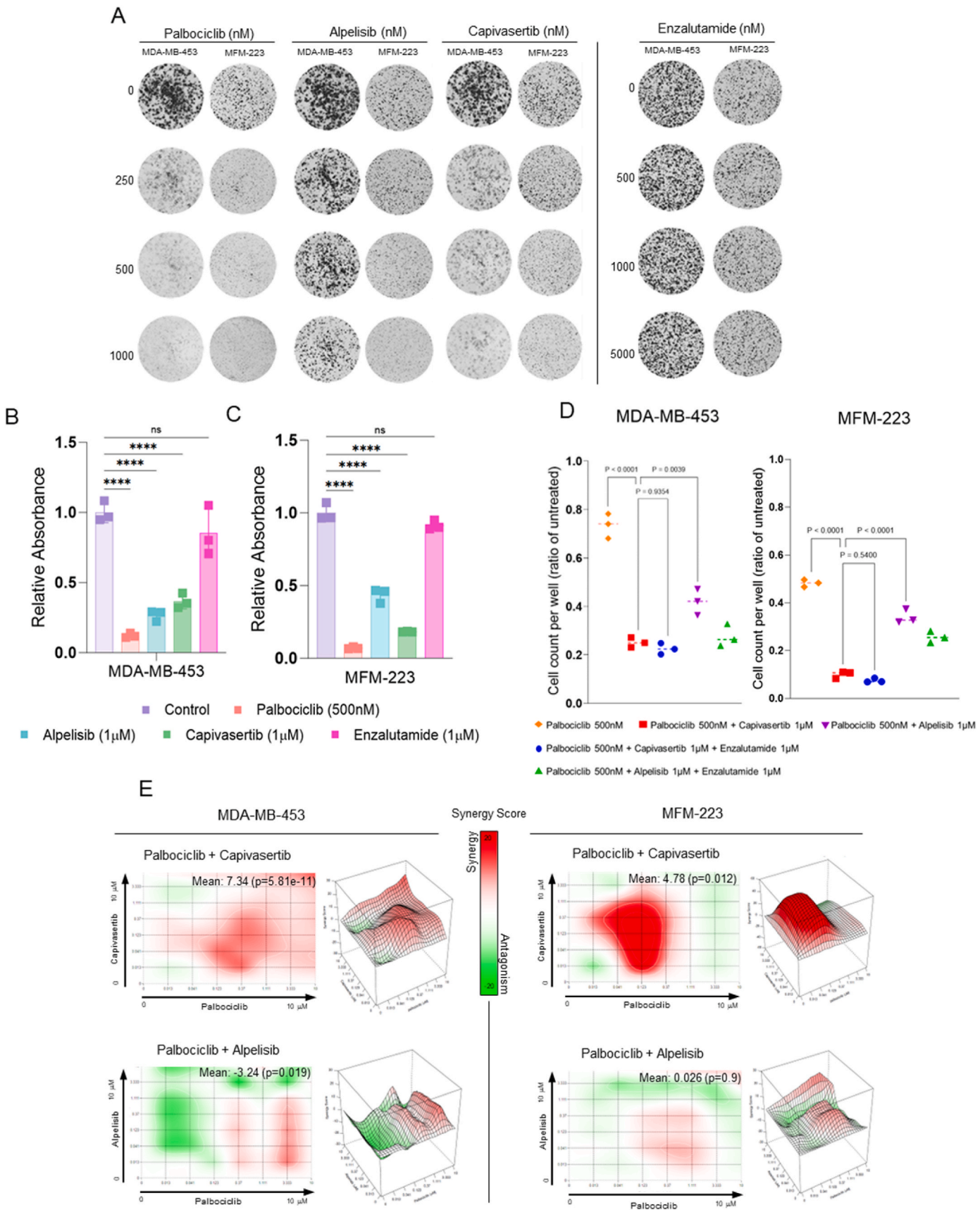
Immunohistochemistry (IHC): Formalin-fixed tumors were embedded in paraffin; 5- μ m tumor sections were stained with the antibodies listed in [Supplementary Table 2](#). The percentage of Ki67, pRB-S807, and Cleaved caspase-3 positive cells was quantified by an expert breast pathologist blinded to treatment arms and using standard CAP breast tumor biomarkers scoring guidelines.

AR transcriptional activity assay: A human ARE reporter plasmid was kindly provided by Dr. Ganesh Raj (UT Southwestern). MDA-MB-453 cells were grown in white-walled, flat-surface 96-well plates and transfected an ARE reporter plasmid using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). After 24 h, cells were treated with DMSO, palbociclib (500 nM), alpelisib (1 μ M), capivasertib (1 μ M) or DHT (1 pM). Cell lysates were prepared 24 h later and subjected to a dual luciferase enzyme assay system (Promega Corp., Madison, WI, USA). Each experiment was performed in triplicate on at least three separate occasions.

Immunoblot analysis: Cell lysates were prepared in RIPA buffer (ThermoFisher Scientific) containing 1X protease inhibitor (Protease Inhibitor Cocktail, Roche) and phosphatase inhibitor for 30 min on ice and then subjected to centrifugation at 15,000 rpm for 15 min. Protein concentration in the supernatants was measured with the Pierce BCA Protein Assay kit (ThermoFisher Scientific). Thirty μ g of protein was subjected to SDS-PAGE followed by transfer to nitrocellulose membranes. Membranes were incubated with primary antibodies (listed in [Supplementary Table 2](#)) followed by rabbit or mouse antibodies conjugated with HRP or fluorescence dyes. Immunoblot images were taken using the Chemidoc MP imaging system (BIO-RAD).

siRNA transfection: Silencer Select siRNAs targeting RICTOR (ID: s226000 and s226001) and PDGFR β (ID: s10242) were purchased from Invitrogen. siRNA transfection was conducted using Lipofectamine RNAiMAX (Invitrogen).

Phospho-Kinase Arrays: The proteome profiler human phospho-kinase array kit was purchased from R&D Systems (catalog number ARY003C). The experiment was performed according to the manufacturer's protocol.



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Fig. 1. LAR TNBC cells are sensitive to CDK4/6 and PI3K/AKT inhibitors but not to an AR antagonist. A. Clonogenic assay to measure cell viability of MDA-MB-453 and MFM-223 after 14 days of treatment with the indicated nM concentrations of palbociclib, alpelisib, capivasertib, or enzalutamide. DMSO was used as control. B, C. One-way ANOVA analysis of relative absorbance from the clonogenic assays of MDA-MB-453 and MFM-223 cells, respectively, as in (A); ns, p not significant; ****, $p < 0.0001$. D. Cell viability assay measured by CellTiter-Glo in MDA-MB-453 and MFM-223 cells treated during 6 days with palbociclib (500 nM) alone or in combination with capivasertib (1 μ M) or alpelisib (1 μ M); enzalutamide (1 μ M) was added to each combination. Data were analyzed with one-way ANOVA multiple comparisons test. E. MDA-MB-453 and MFM-223 cells were treated with the indicated concentrations of palbociclib $\pm$ alpelisib or palbociclib $\pm$ capivasertib. The average excess response due to drug interactions was measured by a synergy score with SynergyFinder 2.0. We employed the Loewe additive model to assess the combined effects of the drugs.

Statistical Analysis: Analysis for T-tests (nonparametric tests), Pearson correlation coefficients, log-rank hazard ratio, and one-way ANOVA simple or multiple comparisons were performed with GraphPad Prism version 10.2. Data were represented as mean $\pm$ standard deviation. All in vitro experiments were conducted at least three times. A p-value < 0.05 was considered statistically significant.

3. Results

LAR TNBC cells are sensitive to CDK4/6 and PI3K/AKT inhibitors but not to enzalutamide. We first tested the CDK4/6 inhibitor palbociclib, the PI3K α inhibitor alpelisib, the AKT inhibitor capivasertib, and the AR antagonist enzalutamide as single agents against PIK3CA-mut/RB-intact LAR TNBC MDA-MB-453 and MFM-223 cells. RB-null TNBC CAL148 cells and ER + MCF7 breast cancer cells were used as negative and positive controls, respectively, for palbociclib. In clonogenic assays, treatment with sub- μ M concentrations of palbociclib, capivasertib or alpelisib for 14 days resulted in a dose-dependent inhibition of proliferation of both MDA-MB-453 and MFM-223 cells, whereas enzalutamide was completely ineffective even at high concentrations (5 μ M) (Fig. 1A–C, Suppl. Fig. 1A). RB-deficient CAL148 cells were resistant to palbociclib, whereas MCF7 cells were highly sensitive. The IC₅₀ of capivasertib in LAR cells was lower than that of alpelisib (Suppl. Fig. 1A). For both drugs, 1 μ M was chosen as a fixed concentration since it blocked PI3K/AKT signaling and downstream transducers, p-GSK3 β , p-PRAS40 and p-S6 (Suppl. Fig. 1B). For palbociclib, 500 nM was chosen as that concentration completely suppressed RB phosphorylation (Suppl. Fig. 1C). Treatment with alpelisib reduced p-AKT^{S473}, whereas treatment with capivasertib markedly elevated p-AKT^{S473} levels (Suppl. Fig. 1B). This effect on p-AKT is attributed to the binding of the small molecule to the ATP pocket in AKT, which maintains the protein in its hyperphosphorylated but kinase inactive state [23,24]. It has been previously reported that inhibition of the PI3K pathway in PTEN-null prostate cancer cells results in feedback reactivation of HER2/HER3, subsequently leading to activation of AR transcription [25]. In LAR TNBC cells, treatment with palbociclib, alpelisib, or capivasertib did not affect AR protein levels (Suppl. Fig. 1D) or AR transcriptional activity, as measured with an ARE-based luciferase reporter (Suppl. Fig. 1E), suggesting that LAR TNBC cells lack PI3K to AR feedback signaling. Since the AR transcript AR-V7 has been linked to resistance to antiandrogen therapy [26], we next examined its presence in MDA-MB-453 and MFM-223 cells. By immunoblot analysis, the AR-V7 variant was undetectable in LAR TNBC cells but present in 22Rv1 antiandrogen-resistant prostate cancer cells (Suppl. Fig. 1F). Finally, siRNA-mediated silencing of AKT1 or AKT2 inhibited MDA-MB-453 cell growth, whereas silencing AR did not (Suppl. Fig. 1G). This suggests that LAR TNBC is dependent on PI3K/AKT but not AR signaling.

CDK4/6 and PI3K/AKT inhibitors synergize in LAR TNBC cells and tumors. We next examined the effect of CDK4/6 and PI3K/AKT inhibitors in combination. The combination of fixed doses of palbociclib (500 nM) plus capivasertib (1 μ M) was more effective than palbociclib plus alpelisib (1 μ M) against MDA-MB-453 and MFM-223 cells ($p = 0.0039$ and $p < 0.0001$, respectively). Addition of enzalutamide did not increase the efficacy of either combination (Fig. 1D). Next, LAR cells were treated with increasing concentrations of palbociclib $\pm$ alpelisib vs. palbociclib $\pm$ capivasertib for 6 days and performed a synergy analysis using the Loewe additivity equation calculated by SynergyFinder + [21]. In both

MDA-MB-453 and MFM-223 cells, palbociclib/capivasertib exerted a synergistic anti-proliferative effect (7.34, $p = 5.81\text{--}11$; 4.78, $p = 0.012$, respectively), whereas the combination of palbociclib and alpelisib was only additive (-3.24 , $p = 0.019$; 0.026, $p = 0.9$, respectively; Fig. 1E). Similar results were observed with the ATP-competitive pan-AKT inhibitor ipatasertib (Suppl. Fig. 1H). We next examined the antitumor activity of CDK4/6i and PI3K/AKT pathway antagonists in LAR TNBC xenografts *in vivo*. Mice, with established PIK3CA-mutant, AR + RB + PDXs (shown in Suppl. Fig. 2A and B), were treated with palbociclib, alpelisib, capivasertib, or combinations of these. The combination of palbociclib/capivasertib inhibited tumor growth *in vivo* more potently than palbociclib/alpelisib ($p = 0.042$), and induced regression in of 10/10 tumors (Fig. 2A and B). Capivasertib monotherapy was effective (Fig. 2A and B), although in combination with palbociclib, tumor regression was more pronounced (Fig. 2C). IHC of tumors harvested on day 28 of treatment showed consistent inhibition of phosphorylated-RB (p-RB) in all xenografts treated with palbociclib, and evidence of cell death as measured by cleaved caspase-3 staining only in tumors treated with palbociclib/capivasertib (Fig. 2D–G), suggesting a cytotoxic effect. Immunoblot analyses of tumor lysates showed p-RB levels were markedly reduced in all xenografts treated with palbociclib, alone or in combination, whereas the AKT substrate p-PRAS40 was reduced only in xenografts treated with palbociclib/capivasertib. Of note, in tumors treated with palbociclib alone there was upregulation of p-AKT, and in those treated with palbociclib alone and palbociclib/alpelisib, there was an increase in p-PRAS40 compared to untreated tumors (Fig. 2H). Similar results were observed in mice bearing MDA-MB-453 xenografts. Both combinations of palbociclib/capivasertib and palbociclib/alpelisib exerted a more potent growth inhibitory effect compared to palbociclib monotherapy ($p < 0.0001$, Suppl. Fig. 2C and D). In all *in vivo* experiments, the combination of capivasertib and palbociclib appeared to be safe, with no mice experiencing weight loss (Fig. 2I, Suppl. Fig. 2E).

Palbociclib was tested as single agent or in combination with either alpelisib or capivasertib in other RB-intact TNBC subtypes. The Mesenchymal Stem-Like (MSL) subtype SUM159PT (PIK3CA, TP53, and HRAS mutated), basal-like 1 (BL1) subtype HCC38 (TP53, CDKN2A mutated), and unclassified subtype BT20 (PIK3CA, TP53, CDKN2A mutated) cell lines were used for this purpose (Suppl. Table 1). SUM159PT, HCC38, and BT20 cells were resistant to palbociclib (Suppl. Fig. 3A), even though p-RB was decreased (Suppl. Fig. 3D). Palbociclib/capivasertib reduced SUM159PT and BT20 cell proliferation more potently palbociclib/alpelisib (Suppl. Fig. 3B), but the effect of the combination was less than in LAR TNBC cells (Suppl. Fig. 3C).

Overall, these data suggest a potent effect of the combination of palbociclib/capivasertib against LAR TNBC cells and PDXs, and a potential compensatory activation of AKT when CDK4/6 is inhibited, which is not abrogated by PI3K α blockade with alpelisib.

Activation of AKT after CDK4/6 blockade is better suppressed by AKT than by PI3K α inhibitors. To further investigate the upregulation of activated AKT observed in palbociclib-treated PDXs (Fig. 2H), MDA-MB-453 and MFM-223 cells were treated with CDK4/6i over a 24-h time course. Concurrent with palbociclib-induced downregulation of p-RB, there was a time-dependent increase in p-AKT^{S473} and the AKT substrates p-PRAS40 and p-GSK3 β in both cell lines (Fig. 3A and B). In LAR RB-null CAL-148 cells, and non-LAR RB-intact SUM159PT, HCC38, and BT20 cells, the compensatory activation of AKT following treatment with the CDK4/6i was not observed (Suppl. Fig. 4A, Suppl. Fig. 3D).

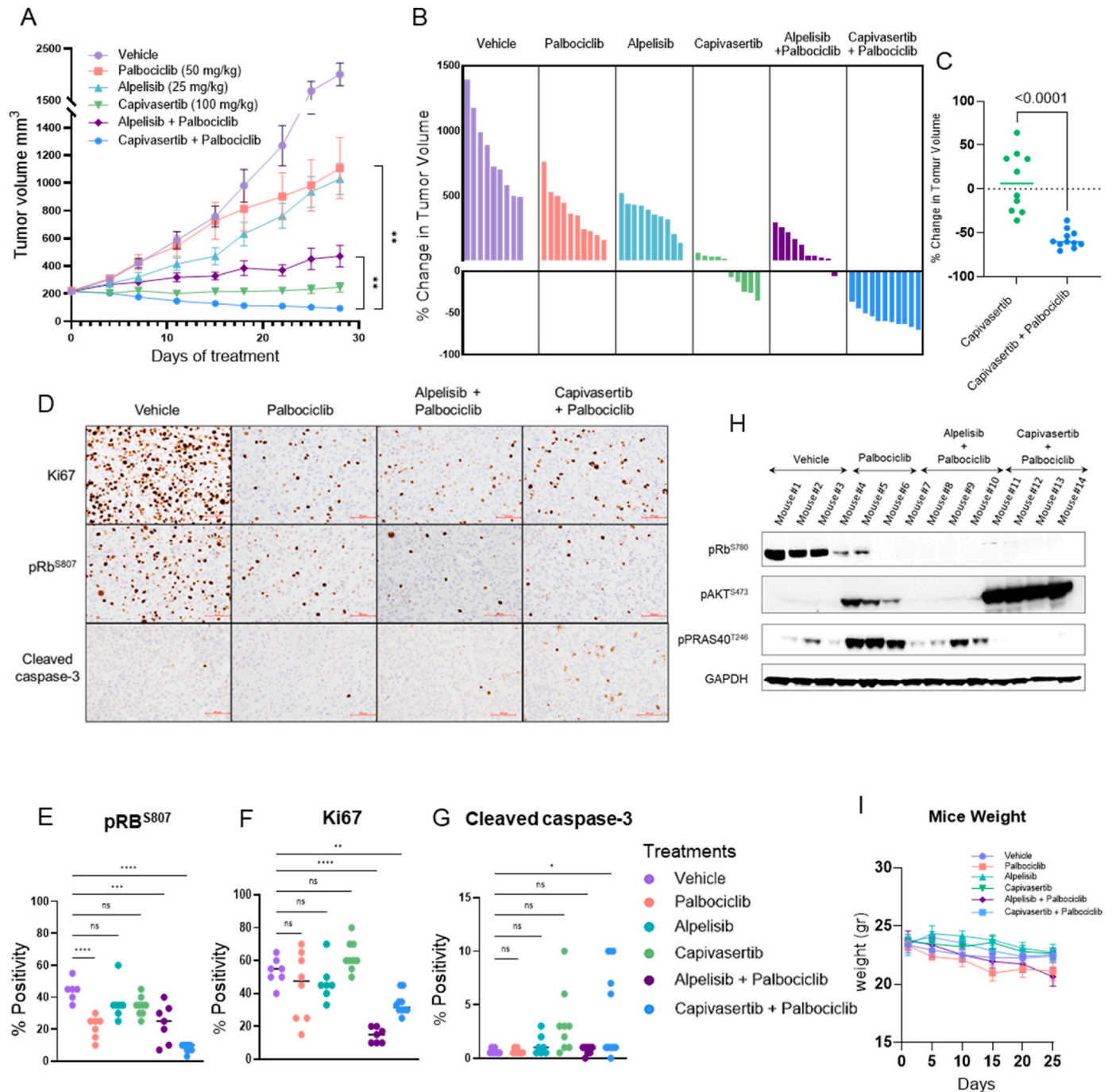


Fig. 2. The combination of CDK4/6 and AKT inhibitors is highly effective *in vivo* against LAR TNBC. **A.** HCl-009 PDX fragments were transplanted into female NSG mice. When tumors reached an average of $\sim 200 \text{ mm}^3$, mice were randomized and treated daily with vehicle ($n = 8$), 50 mg/kg palbociclib ($n = 9$), 25 mg/kg alpelisib ($n = 9$), 100 mg/kg BID 4 days on/3 days off of capivasertib ($n = 9$), palbociclib + alpelisib ($n = 9$), or palbociclib + capivasertib ($n = 10$). Each data point represents the mean tumor volume in $\text{mm}^3 \pm \text{SD}$. Data was analyzed with one-way ANOVA multiple comparison test (**, $p < 0.01$). **B.** Percent change in individual tumor volumes between day 0 and day 28 of treatment. All tumors treated with palbociclib/capivasertib exhibited an objective response. **C.** Cells were treated with capivasertib $\pm$ palbociclib. Percent change in individual tumor volumes between day 0 and day 28 of treatment. Each dot represents an individual xenograft. Data was analyzed with *t*-test. **D.** Representative Ki67, p-RB^{S807}, and cleaved caspase-3 IHC of tumors treated in A, B. **E-G.** Percent IHC positive tumor cells for p-RB^{S807}, Ki67, and cleaved caspase-3, respectively. Each dot represents an individual xenograft. Data was analyzed with one-way ANOVA multiple comparisons test (ns, *p* not significant; *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$). **H.** Immunoblot analysis of mouse tumors harvested after 28 days of treatment with palbociclib $\pm$ alpelisib or $\pm$ capivasertib as indicated. Whole tumor lysates were subjected to immunoblot analysis with the indicated antibodies. **I.** Mouse weight during experiment with HCl009 PDXs. Each color represents a treatment arm.

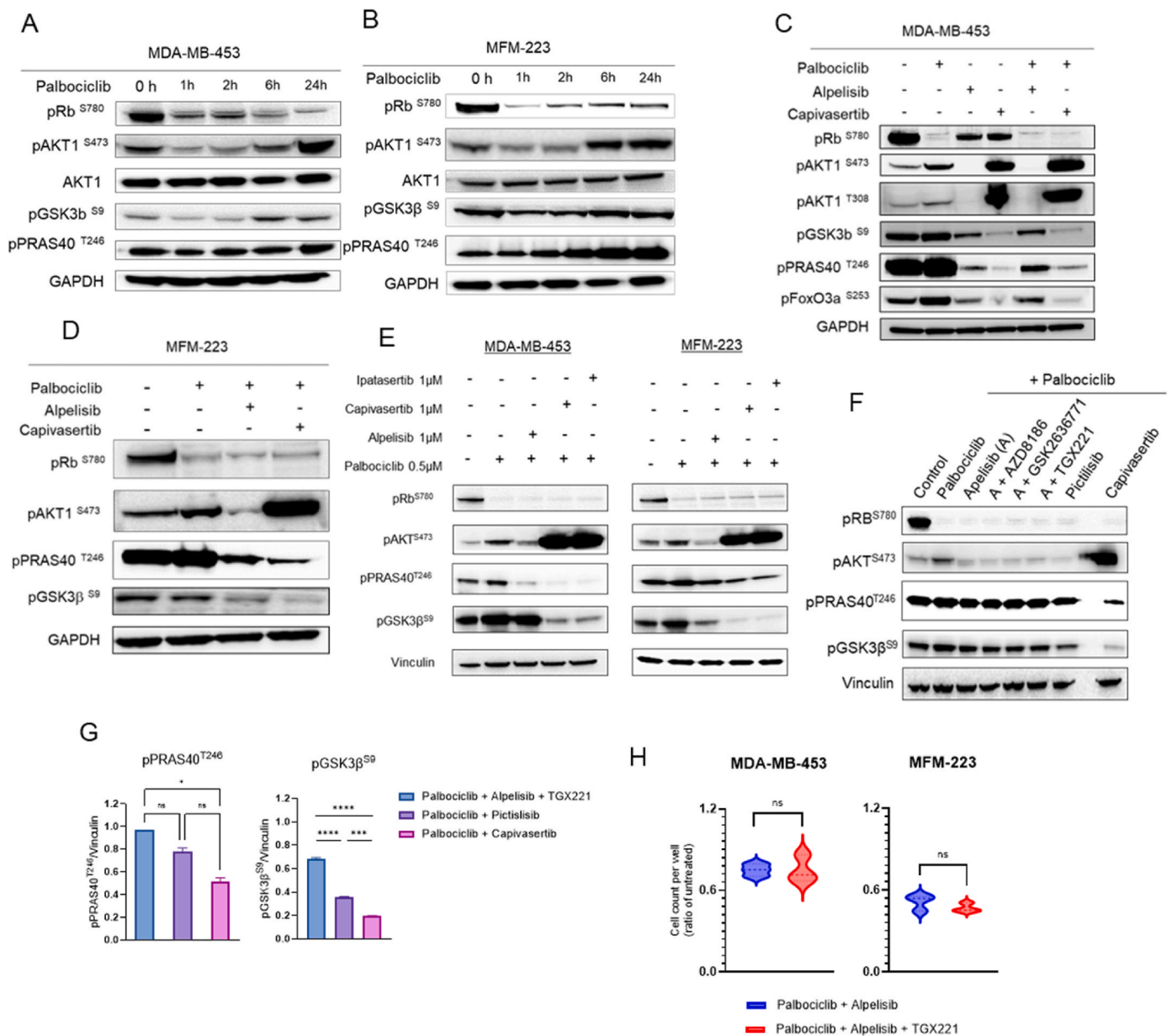


Fig. 3. CDK4/6i-induced activation of the PI3K/AKT pathway is more completely blocked by an AKT inhibitor. **A, B.** Immunoblot analysis of lysates from MDA-MB-453 (**A**) and MFM-223 cells (**B**) treated with 500 nM palbociclib over a time course. **C, D.** Immunoblot analysis of MDA-MB-453 (**C**) and MFM-223 (**D**) cells treated with palbociclib 500 nM, alpelisib 1 μ M, capivasertib 1 μ M, or the indicated combinations for 24 h. **E.** MDA-MB-453 and MFM-223 cells treated with palbociclib 500 nM alone or in combination with capivasertib 1 μ M, alpelisib 1 μ M, or ipatasertib 1 μ M. **F.** MDA-MB-453 cells treated with palbociclib 500 nM alone or in combination with alpelisib 1 μ M, pictilisib 1 μ M or capivasertib 1 μ M (lanes 2,3,7,9). Lanes 4–6 show immunoblots of lysates from cells treated with palbociclib/alpelisib plus AZD8186 1 μ M, GSK2636771 1 μ M, or TGX221 1 μ M for 24 h. Antibodies used for immunoblots in A–E are shown to the left of each panel. **G.** Quantitative immunoblot analysis of pPRAS40^{T246} and pGSK3 β ^{S9} protein expression relative to Vinculin. **H.** Cell viability assay for MDA-MB-453 and MFM-223 cells treated with palbociclib 500 nM, alpelisib 1 μ M $\pm$ the PI3K β inhibitor TGX221 1 μ M for 24 h. Results were analyzed with *t*-test analysis, ns, *p* not significant.

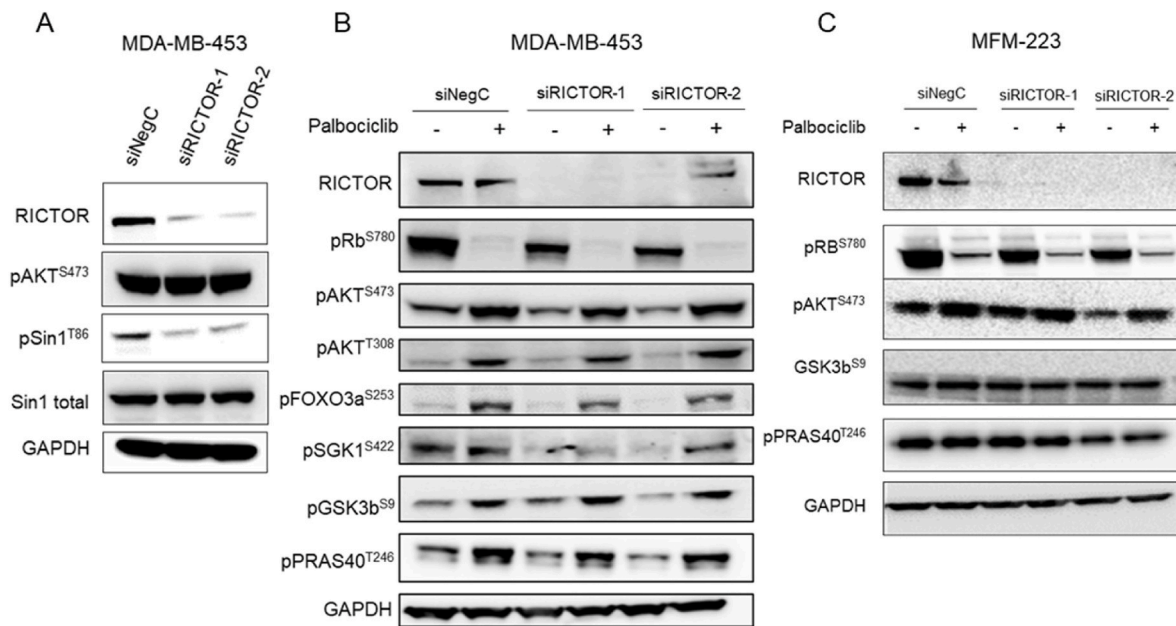


Fig. 4. mTORC2 is dispensable for CDK4/6i-induced pAKT^{S473}. **A.** Immunoblot of MDA-MB-453 cells were transfected with Rictor siRNAs as described in Methods. Immunoblots were prepared and with the indicated antibodies. **B.** **C.** Immunoblot analysis of MDA-MB-453 (**B**) and MFM-223 cells (**C**) transfected with a negative control siRNA (siNegC) or two independent RICTOR siRNAs (siRCTOR) and then treated with palbociclib 500 nM for 24 h. Antibodies used in A-B are shown to the left of each panel.

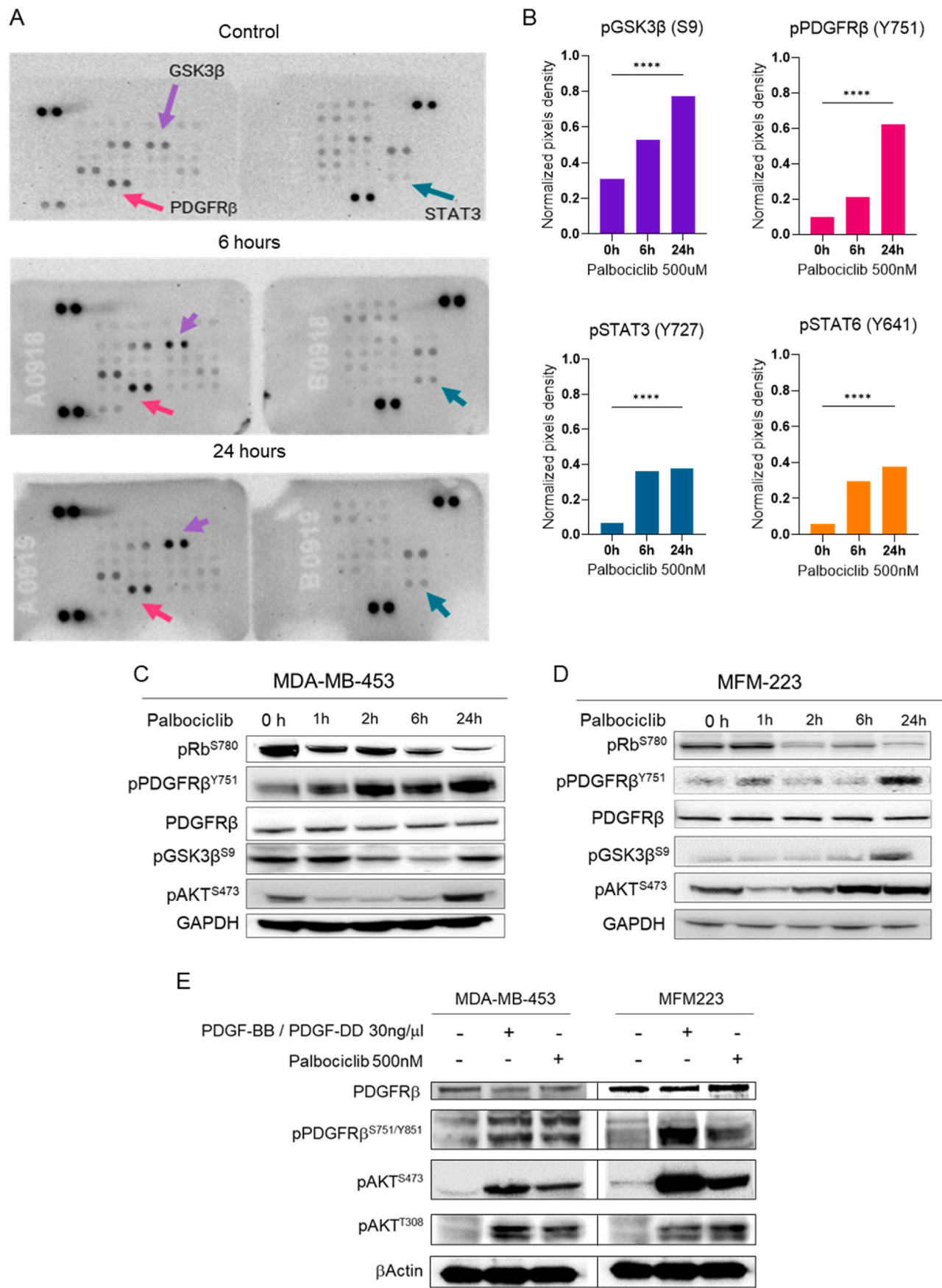
Next, we treated both MDA-MB-453 and MFM-223 cells with palbociclib $\pm$ alpelisib and palbociclib $\pm$ capivasertib for 24 h. In both cell lines, the increase in p-PRAS40 and p-GSK3 β upon treatment with CDK4/6i was more effectively suppressed by capivasertib than alpelisib, in line with the synergistic anti-proliferative effect observed with the palbociclib/capivasertib combination (Fig. 1E). Similar results were observed with the ATP-competitive pan-AKT inhibitor, ipatasertib (Fig. 3E). This result suggested the possibility that the compensatory activation of AKT was mediated by a PI3K isozyme other than PI3K α . MDA-MB-453 and MFM-223 cells express PI3K α and PI3K β , and all three AKT isoforms (Suppl. Fig. 4B). Thus, we next examined whether the small molecule PI3K β inhibitors, AZD8186, GSK26236771, or TGX221 could block the upregulation of PI3K/AKT signaling in LAR cells treated with CDK4/6i. Capivasertib effectively inhibited the phosphorylation of GSK3 β ^{S9} and PRAS40^{T246} in palbociclib-treated cells, but none of the PI3K β inhibitors did (Fig. 3E). The addition of TGX221 did not enhance the anti-proliferative effect of palbociclib/alpelisib in MDA-MB-453 and MFM-223 cells (Fig. 3H). Similarly, treatment with the pan-PI3K inhibitor pictilisib [27] in combination with palbociclib modestly reduced p-GSK3 β , but was not as effective as capivasertib (Fig. 3F and G). Addition of the pan-PI3Ki buparlisib or the dual PI3K/mTORi dactolisib to palbociclib did not prevent the increase in p-GSK3 β and p-PRAS40 either (data not shown). Altogether, these results suggest PI3K α or other PI3K isozymes may not mediate the adaptive response to CDK4/6 inhibition.

Phosphorylation of AKT in Ser473 by the mTORC2 complex is required for full activation of AKT [28]. Phosphorylated cytoplasmic RB has been shown to interact with Sin1, a component of the mTORC2 complex, and suppress the function of mTORC2, thus blocking mTORC2-mediated phosphorylation of AKT^{Ser473} [29]. Hence, we speculated that inhibition of p-RB by treatment with a CDK4/6i would release mTORC2 from RB-mediated suppression. In turn, this would activate mTORC2 and lead to mTORC2-mediated compensatory activation of AKT, thus explaining the synergistic effect of the combination of palbociclib and capivasertib that we had observed. To test this hypothesis, we knocked down RICTOR, a key component of mTORC2, required for its function [30], in MDA-MB-453 cells. Transduction of two independent RICTOR siRNAs led to a decrease in pSin1^{T86} [28], but did

not affect baseline pAKT^{S473} levels (Fig. 4A). As shown previously, treatment of MDA-MB-453 and MFM-223 cells with palbociclib for 24 h inhibited p-RB, but increased levels of p-AKT^{S473} and phosphorylated AKT substrates. This upregulation of PI3K/AKT signaling was not blocked by RICTOR silencing (Fig. 4B and C), suggesting activation of mTORC2 upon inhibition of p-RB did not explain the feedback activation of AKT in palbociclib-treated LAR cells.

PDGFR β signaling is activated following inhibition of CDK4/6. Next, to identify kinases that could potentially induce the compensatory activation of AKT, we treated MDA-MB-453 cells with 500 nM palbociclib over a 24-h time-course and added the cell lysates to a human phospho-kinase array containing 43 phospho-antibodies. We observed a time-dependent increase in the phosphorylation of the receptor tyrosine kinase PDGFR β (p < 0.0001), GSK3 β ^{S9} (p < 0.0001), STAT3^{Y750} (p < 0.0001), STAT3^{Y727} (p < 0.0001), STAT6^{Y641} (p < 0.0001) (Fig. 5A and B). Although p-STAT6^{Y641} was not as apparent in the array images in Fig. 5A, it was clearly identified as an upregulated phosphoprotein by the software analysis of the arrays in Fig. 5B. We confirmed results of this unbiased screen by treating MDA-MB-453 and MFM-223 cells with palbociclib over the same time-course and subjecting the cell lysates to immunoblot analyses. In both cell lines treated with the CDK4/6i we observed a time-dependent increase p-PDGFR β ^{Y751}, p-AKT^{S473}, and p-GSK3 β ^{S9} (Fig. 5C and D). To confirm that PDGFR β signaling can activate the PI3K/AKT pathway in LAR cells, we treated MDA-MB-453 and MFM-223 cells with the receptor ligands PDGF-BB and PDGF-DD and compared the results of PDGFR β pathway activation with palbociclib. Both treatment with PDGF ligands and with palbociclib induced p-PDGFR β ^{Y751} and p-PDGFR β ^{Y849} as well as p-AKT^{S473} and p-AKT^{T308}, indicating full AKT activation (Fig. 5E).

Finally, we investigated the effects of PDGFR β inhibition, both pharmacologically and genetically. First, using the Incucyte® Live-Cell Analysis System, we examined the cell proliferation of the LAR cell lines treated with PDGFR α/β inhibitor, CP-673451, $\pm$ palbociclib for 6 days. CP-673451 was inactive as a single agent, while in combination with palbociclib exerted an identical anti-proliferative effect as palbociclib/capivasertib (Fig. 6A–D). Further, similar to palbociclib/capivasertib, the combination of palbociclib/CP-673451 inhibited growth of MFM-223 cells more potently than palbociclib/alpelisib (Fig. 6E).



(caption on next page)

Fig. 5. PDGFR β signaling is activated following inhibition of CDK4/6. A. MDA-MB-453 cells were treated with 500 nM palbociclib for 0, 6 and 24 h; lysates were prepared and incubated in a Human Phospho-Kinase/RTK Array with 43 phospho-antibodies spotted onto nitrocellulose membranes. Arrows denote signaling molecules upregulated over the time course (p-GSK3 β , p-PDGFR β , p-STAT3). B. Plot of normalized pixel density of phosphoproteins that were induced upon treatment with palbociclib. Data was analyzed using one-way ANOVA multiple comparisons test; ****, $p < 0.0001$. C, D. Immunoblot analysis of MDA-MB-453 (C) and MFM-223 (D) cells treated with 500 nM palbociclib for the indicated time points. Lysates were probed with the indicated antibodies. E. MDA-MB-453 and MFM-223 cells were treated with PDGFR β ligands PDGF-BB and PDGF-DD (30 ng/ μ L) or palbociclib 500 nM for 24 h. Protein lysates were incubated with the indicated antibodies.

Finally, transduction of a PDGFR β siRNA into MDA-MB-453 and MFM-223 cells reduced PDGFR β levels and dampened the increase in p-AKT, p-GSK3 β , and p-PRAS40 that followed treatment with palbociclib (Fig. 6F). Taken together, these data suggest a novel adaptive response to CDK4/6 blockade that involves PDGFR β signaling.

4. Discussion

Approximately 10 % of TNBC are of the luminal androgen receptor (LAR) subtype, which is enriched in hormonally regulated pathways, including androgen and estrogen metabolism and luminal gene expression, similar to ER + breast cancer [1,2]. Approximately one-third of patients with TNBC benefit from neoadjuvant/adjuvant chemotherapy, yet patients with LAR TNBC are less likely to benefit. Further, clinical trials with AR antagonists, like bicalutamide and enzalutamide, and the androgen biosynthesis inhibitor abiraterone have shown only modest efficacy in patients with AR + metastatic TNBC [12,13,31], suggesting AR signaling is not a major pathogenic driver in these cancers. Activating PIK3CA mutations are enriched in LAR TNBC [10]. Based on these data, Lehmann et al. conducted a phase Ib/II trial testing enzalutamide in combination with the pan-PI3K inhibitor, taselisib, in patients with AR + TNBC. Clinical benefit was quite modest, and AR expression in the tumors was insufficient to identify patients who more likely to respond to the combination [32]. Currently, the PI3K α inhibitor alpelisib and the AKT inhibitor capivasertib are approved for treatment of ER + breast cancers with PIK3CA activation mutations and PIK3CA/AKT1/PTEN alterations, respectively. However, these agents remain relatively understudied in TNBC.

CDK4/6 inhibitors are highly active and widely used for the treatment of ER + breast cancer. These drugs require presence of the Retinoblastoma protein (RB) to exert their antitumor effect, as they primarily inhibit the phosphorylation of RB, thereby activating it into coupling to E2F transcription factors, thus preventing cells from entering into S phase. Most TNBC are RB-deficient as a result of RB genomic loss, inactivating somatic mutations, small gene deletions, methylation, and LOH [33,34], explaining why CDK4/6i have also been understudied in TNBC. However, the LAR subtype is predominantly RB-intact compared to the other subtypes of TNBC [9]. Few preclinical studies have investigated the role of CDK4/6i in TNBC. Ashgar et al. reported that palbociclib-treated MDA-453 LAR cells exit mitosis with a low level of CDK2 activity and enter a quiescent state that requires CDK4/6 resulting in sensitivity to the CDK4/6i [16]. Teo et al. tested the combination of alpelisib (BYL719) and ribociclib (LEE0110) in TNBC cells and xenografts. Combined inhibition resulted in increased cell cycle arrest, apoptosis, and tumor immunogenicity, particularly in combination with immune checkpoint inhibitors [19]. Their study, however, did not focus on LAR TNBC. Therefore, with the limited clinical activity of anti-androgens and the increasing use and safety of PI3K/AKT pathway and CDK4/6 inhibitors in ER + breast cancer, we interrogated rational combinations with these agents in LAR TNBC.

In this study, using LAR TNBC cells and xenografts, the combination of palbociclib/capivasertib emerged as the most active. Unexpectedly, we noticed that upon treatment with a CDK4/6i, LAR cells and tumors showed upregulated PI3K/AKT signaling. This adaptive response was

blocked more potently by capivasertib than alpelisib or pan-PI3K inhibitors, suggesting PI3K isozymes other than PI3K α were not involved. At this point, we cannot rule out the possibility of an off-target effect of capivasertib that may explain its superior efficacy in blocking AKT reactivation. Of note, in addition to AKT1-3, capivasertib has been reported to also directly inhibit ROCK1/2, PKA, p70S6K, pGSK3 β , pPRAS40, and pFOXO3a [23]. These actions could potentially explain the superior efficacy of capivasertib over alpelisib in reducing pathway flux as a consequence of AKT inhibition.

In order to explore the mechanism(s) driving AKT activation upon CDK4/6 blockade, we first investigated whether mTORC2 mediates this compensatory response after RB dephosphorylation [29]. Our results showed that knockdown of RICTOR, an essential component of the mTORC2 [30,35,36], did not block AKT activation following CDK4/6 blockade, arguing against de-repressed mTORC2 function mediating AKT feedback reactivation. To gain further insight, we used an unbiased phosphokinase array in LAR cells treated with palbociclib and found a time-dependent increase in phosphorylated PDGFR β , a member of the PDGFR family of RTKs that regulates cell proliferation, differentiation, growth, and development. Ligand-induced homodimerization or heterodimerization of PDGFR β with other PDGFR subunits activates the receptor kinase activity and induces MAPK, PI3K/AKT, PLC γ , and STAT3 signaling [37]. In breast cancer, PDGFR β expression has mainly been reported in stroma, and high expression has been associated with HER2 positivity, high histopathologic tumor grade, ER negativity, and shorter survival [38]. PDGFR β is expressed in TNBC cells, with higher mRNA levels in the basal-like and mesenchymal subtypes [39]. Genetically engineered mice with deletion of *Brca1* in p18^{INK4C} or p16^{INK4A}-deficient mice develop basal-like mammary cancers that exhibit activated PDGFR β signaling evidence of epithelial-to-mesenchymal transition. In the same study, targeted deletion of *Pdgfr β* in *Brca1*-deficient mammary cells resulted in mesenchymal-to-epithelial transition, tumor cell death, and suppression of tumorigenesis [40]. In a preclinical study, Van Swearingen et al. showed that treatment with the combination of the MEK inhibitor selumetinib and the PDGFR inhibitor pazopanib improved the survival of mice with intracranial SUM149 and MDA-MB-231 TNBC grafts [41]. Activation of PDGF signaling has also been associated with aromatase inhibitors resistance in ER + breast cancer cells through enhanced proliferation and angiogenesis [42], suggesting that luminal breast cancers can be targeted by PDGFR inhibition [43,44]. Despite this suggestive preclinical evidence, PDGFR inhibitors have not been formally tested in patients with TNBC. The mechanism by which CDK4/6i activate AKT through PDGFR β requires further elucidation. Taken together, these data suggest the combination of PDGFR β and CDK4/6i merits further investigation in LAR TNBC.

In summary, herein we showed that CDK4/6 blockade resulted in adaptive compensatory activation of the PI3K/AKT pathway. The addition of capivasertib was superior to alpelisib in blocking that compensation, and synergizing with palbociclib against LAR TNBC. In parallel, we uncovered, for the first time, that upregulation of PDGFR β is a novel adaptive mechanism triggered by CDK4/6 inhibition. These findings strongly encourage clinical trials of CDK4/6 and AKT inhibitors in patients with LAR TNBC and warrant further investigation of PDGFR antagonists for this breast cancer subtype.

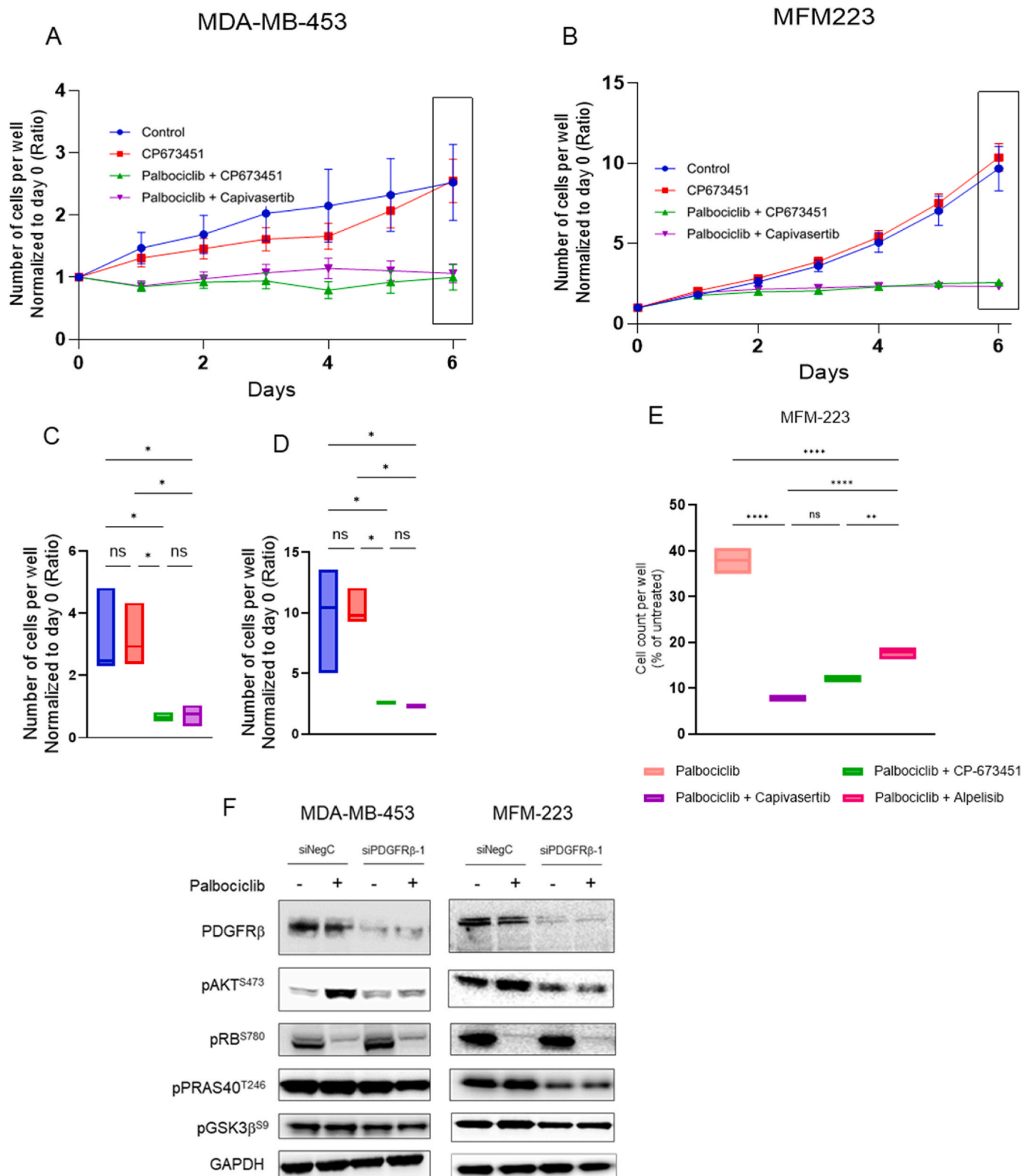


Fig. 6. PDGFR β inhibition in combination with palbociclib inhibits LAR TNBC cells. A, B. Cell viability assay using Incucyte S3 live cell of MDA-MB-453 (A) and MFM-223 (B) treated for 6 days with the PDGFR small molecule inhibitor CP673451 1 μ M $\pm$ palbociclib 500 nM or palbociclib plus capivasertib 1 μ M. C, D. Cell numbers on day 6 of treatment (A and B). Each data point represents the mean cell number $\pm$ SD ($n = 3$). One-way ANOVA multiple comparisons were used to analyze the data; ns, p not significant; *, $p < 0.05$. E. Cell viability assay in MFM-223 cells with palbociclib 500 nM alone or in combination with capivasertib 1 μ M, CP-673451 1 μ M or alpelisib 1 μ M treated for 6 days. Data were analyzed with one-way ANOVA multiple comparisons test; ns, p value not significant; **, $p < 0.01$, ****, $p < 0.0001$. F. MDA-MB-453 and MFM-223 cells were transfected with negative control siRNA (siNegC) or a PDGFR β siRNA (siPDGFR β) and treated $\pm$ palbociclib 500 nM. Lysates were probed for the indicated antibodies.

CRediT authorship contribution statement

María Rosario Chica-Parrado: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gun Min Kim:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Yasuaki Uemoto:** Methodology, Investigation. **Fabiana Napolitano:** Data curation, Conceptualization. **Chang-Ching Lin:** Methodology, Investigation. **Dan Ye:** Methodology. **Emmanuel Bikorimana:** Methodology, Investigation. **Yisheng Fang:** Investigation. **Kyung-min Lee:** Conceptualization. **Saurabh Mendiratta:** Resources. **Ariella B. Hanker:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Carlos L. Arteaga:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ariella B. Hanker received or has received research grants from Takeda and Lilly and nonfinancial support from Puma Biotechnology, Daiichi Sankyo, and Tempus. Carlos L. Arteaga receives or has received research grants from Pfizer, Lilly, and Takeda; holds minor stock options in Provista; serves or has served in an advisory role to Novartis, Merck, Lilly, Daiichi Sankyo, Taiho Oncology, Origimed, Puma Biotechnology, Immunomedics, AstraZeneca, Arvinas, and Sanofi; and reports scientific advisory board remuneration from the Susan G. Komen Foundation.

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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.2024.217219>.

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