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LETTER TO THE EDITOR

Triple combination targeting PI3K, ER, and CDK4/6 inhibits growth of ER-positive breast cancer resistant to fulvestrant and CDK4/6 or PI3K inhibitor

Dear Editor,

Despite the improved outcome of advanced estrogen receptor-positive (ER^+) breast cancer patients treated with endocrine therapy in combination with either a cyclin-dependent kinase 4/6 inhibitor (CDK4/6i) or a phosphoinositide 3-kinase inhibitor (PI3Ki), the disease will eventually progress, and the optimal treatment strategy upon progression remains undefined [1–4]. To address this, we developed MCF-7- and T47D-derived *PIK3CA*-mutated breast cancer cell lines [5] resistant to combined CDK4/6i palbociclib and fulvestrant (MPF-R and TPF-R) or combined PI3Ki alpelisib and fulvestrant (MAF-R and TAF-R), respectively (Supplementary Materials and Methods). Drug-sensitive isogenic cells (M-S and T-S) grown in parallel with MPF-R and TPF-R cells and the original MCF-7/S0.5 and T47D cells were analyzed for comparison. To characterize the cell lines, we initially performed gene expression microarray analysis. The Gene Set Enrichment Analysis (GSEA) showed that alterations in the regulators of various pathways, including the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, apoptosis, cholesterol homeostasis, and interferon alpha response were significantly enriched gene datasets in MPF-R cells compared to M-S cells (Supplementary Table S1, Supplementary Figure S1A). Notably, alterations in the regulators of the cell cycle and the PI3K/AKT/mammalian target of the rapamycin (mTOR) signaling pathway were identified as significantly enriched in MAF-R cells compared to MCF-7/S0.5 cells (Supplementary Table S2, Supplementary Figure S1B). Conversely, the estrogen-dependent gene expression gene set was found to be significantly enriched in M-S and MCF-7/S0.5 cells compared to MPF-R and MAF-R cells, respectively (Supplementary Figure S1C-D), which suggests that the resistant cells are less dependent on the ER pathway than the parental cell lines.

Although most of the genomic alterations identified, such as *PIK3CA* and *ERBB2* mutations, *MYC* amplification, and *ESR1* fusion, were common to both sensitive and resistant cells, a unique *RB1* mutation (c.2125T>G, p.Tyr709Asp) was found in MPF-R cells, which likely is causally linked to the CDK4/6i-resistant phenotype in these cells (Supplementary Tables S3-S5). Based on these data, we firstly evaluated whether addition of an inhibitor of the PI3K/AKT/mTOR or the cell cycle pathway would overcome resistance to combined fulvestrant and palbociclib or alpelisib, respectively. The combination of alpelisib and fulvestrant was recently approved by the U.S. Food and Drug Administration for the treatment of ER^+ *PIK3CA*-mutated metastatic breast cancer following progression on endocrine therapy [6]. However, whether this drug combination would suppress the growth of tumors resistant to combined CDK4/6i and endocrine therapy remains to be determined, although the clinical trial BYLieve showed some response in patients who progressed on combined CDK4/6i and endocrine therapy [2]. In addition, it is unknown whether the clinical combination of CDK4/6i and fulvestrant therapy is efficacious following progression on combined PI3Ki and fulvestrant. Therefore, we tested the effects of the PI3Ki alpelisib, CDK4/6i palbociclib, and ER degrader fulvestrant as single drugs and in

List of abbreviations: CDK4/6, Cyclin-dependent kinase 4/6; CDK4/6i, CDK4/6-inhibitor; ER, Estrogen receptor; ERBB2, Erb-B2 receptor tyrosine kinase 2; ES, Enrichment score; ESR1, Estrogen receptor 1; FDR, False discovery rate; GSEA, Gene Set Enrichment Analysis; mTOR, Mammalian target of rapamycin; mTORC1, Mammalian target of rapamycin complex 1; NES, Normalized enrichment score; p-PRAS40, Phosphorylated proline-rich Akt substrate of 40 kDa; p-S6, Phosphorylated ribosomal protein S6; PARP, Poly (ADP-ribose) polymerase; PDX, Patient-derived xenograft; PFS, Progression-free survival; PI3K, Phosphoinositide 3-kinase; PI3Ki, PI3K-inhibitor; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PDK-1, 3-phosphoinositide dependent protein kinase-1; PTEN, Phosphatase and Tensin homolog; Rb, Retinoblastoma protein; RB1, Retinoblastoma 1.

various dual and triple combinations on growth and viability of breast cancer cells resistant to the combination of palbociclib or alpelisib and fulvestrant. We found that all the combinations were quite effective in the sensitive cell lines, whereas only the triple combination significantly reduced cell growth and viability of all resistant cells compared to the standard dual combinations (Supplementary Figures S2-S3).

Next, we assessed the long-term effect of the different treatments and confirmed that all combinations were comparably effective in the sensitive cell lines (Figure 1A-C). In resistant MPF-R and TPF-R cells, combined alpelisib and fulvestrant was more effective than combined palbociclib and fulvestrant, but failed to maintain growth inhibition over the 8 weeks of treatment (Figure 1D-E). In contrast, the triple combination was highly effective in both resistant cell lines, preventing the outgrowth of resistant colonies in the 8-week period in MPF-R, while few resistant colonies of TPF-R cells were observed at weeks 7 and 8 (Figure 1D-E). Similarly, only the triple combination completely arrested the growth of the resistant MAF-R cells over the entire treatment period, while combined fulvestrant and palbociclib or alpelisib failed to maintain growth inhibition as cell confluency was observed as early as week 2 (Figure 1F).

By studying the underlying mechanisms of the inhibitory effect caused by the triple combination, we observed a marked increase in apoptosis and cleaved poly(ADP-ribose) polymerase (PARP) levels in both MPF-R and M-S cells treated with the triple combination compared to the palbociclib and fulvestrant combination (Supplementary Figure S4A-B), but these changes were not observed in TPF-R and T-S cells (Supplementary Figure S4C). A slight increase in apoptosis was also observed in the MAF-R cells, but not in TAF-R cells (Supplementary Figure S4D-E). Furthermore, triple combination efficiently inhibited cell proliferation (Supplementary Figure S4F-I), and reduced the expression of key proteins of the ER, PI3K/AKT/mTOR and cyclin D-CDK4/6-Rb pathways in both MPF-R and TPR-R cells (Supplementary Figure S5A-D), and induced cell cycle arrest in MPF-R cells (Supplementary Figure S5E-F).

Next, we evaluated the antitumor activity of the triple combination and the clinically used dual combinations in MPF-R and MAF-R cell line xenografts. The triple combination significantly inhibited growth of both MPF-R and MAF-R tumors compared to the clinically used dual combinations (Figure 1G-H). In addition, we evaluated the efficacy of the triple combination in a palbociclib- and fulvestrant-resistant patient-derived xenograft (PDX) model (Gar15-13-FPR) and found that the triple combination significantly reduced tumor growth compared with combined palbociclib and fulvestrant ($P < 0.0001$) (Figure 1I, Supplementary Figure S6). The triple combination-treated tumors demonstrated a greater decrease in Ki67 expression compared to combined palbociclib and fulvestrant-treated tumors (Figure 1J-K).

To investigate the clinical relevance of our findings, we next assessed whether p-S6 and p-PRAS40 levels, found to be upregulated in the combined palbociclib- and fulvestrant-resistant cell lines compared to sensitive cells (Supplementary Figure S5A-B), correlated with the clinical outcome in ER^+ advanced breast cancer patients treated with combined CDK4/6i and endocrine therapy. Survival analysis did not show a significant correlation between the expression level of either p-S6 or p-PRAS40 and progression-free survival (PFS, Supplementary Figure S7). High p-AKT levels have been previously found in the combined palbociclib- and fulvestrant-resistant cells and were correlated with shorter PFS in the advanced ER^+ breast cancer patients treated with combined CDK4/6i and fulvestrant [7]. Therefore, we next evaluated the clinical relevance of other markers of the PI3K/AKT/mTOR pathway, including 3-phosphoinositide dependent protein kinase-1 (PDK-1) and phosphatase and tensin homolog (PTEN), which are key regulators of the pathway and have previously been shown to play a key role in the resistance mechanisms to CDK4/6i [8, 9]. Moreover, *PDK-1* was found to be among the genes significantly enriched in the palbociclib- and fulvestrant-resistant cell line in the GSEA analysis (Supplementary Table S1). Although PDK-1 and p-PDK-1 protein expression were not increased in cells resistant to combined CDK4/6i and endocrine therapy compared to the sensitive cells, we observed induction of phosphorylated and non-phosphorylated PDK-1 expression upon treatment with combined fulvestrant and CDK4/6i (Supplementary Figure S8), which suggests involvement of this regulator in the response to the combined therapy. The expression levels of PDK-1 and PTEN were evaluated in a cohort of advanced ER^+ patients treated with combined CDK4/6i and endocrine therapy ($n = 80$). The survival analysis indicated strong associations between PDK-1 and PTEN levels and PFS. High levels (intensity ≥ 2) of PDK-1 were significantly associated with shorter PFS compared to low levels of PDK-1 ($P = 0.019$) (Figure 1L). PTEN showed, as expected, the opposite association, as tumors exhibiting low (intensity < 2) nuclear and cytoplasmic PTEN expression were associated with poor outcome compared to tumors with high nuclear and cytoplasmic PTEN expression (Figure 1M). Univariate Cox regression analysis showed that PDK-1 status ($P = 0.022$), PTEN status ($P = 0.030$), endocrine status ($P = 0.007$), and line of therapy ($P = 0.006$) were all significantly associated with PFS for patients treated with the combination of the endocrine therapy and

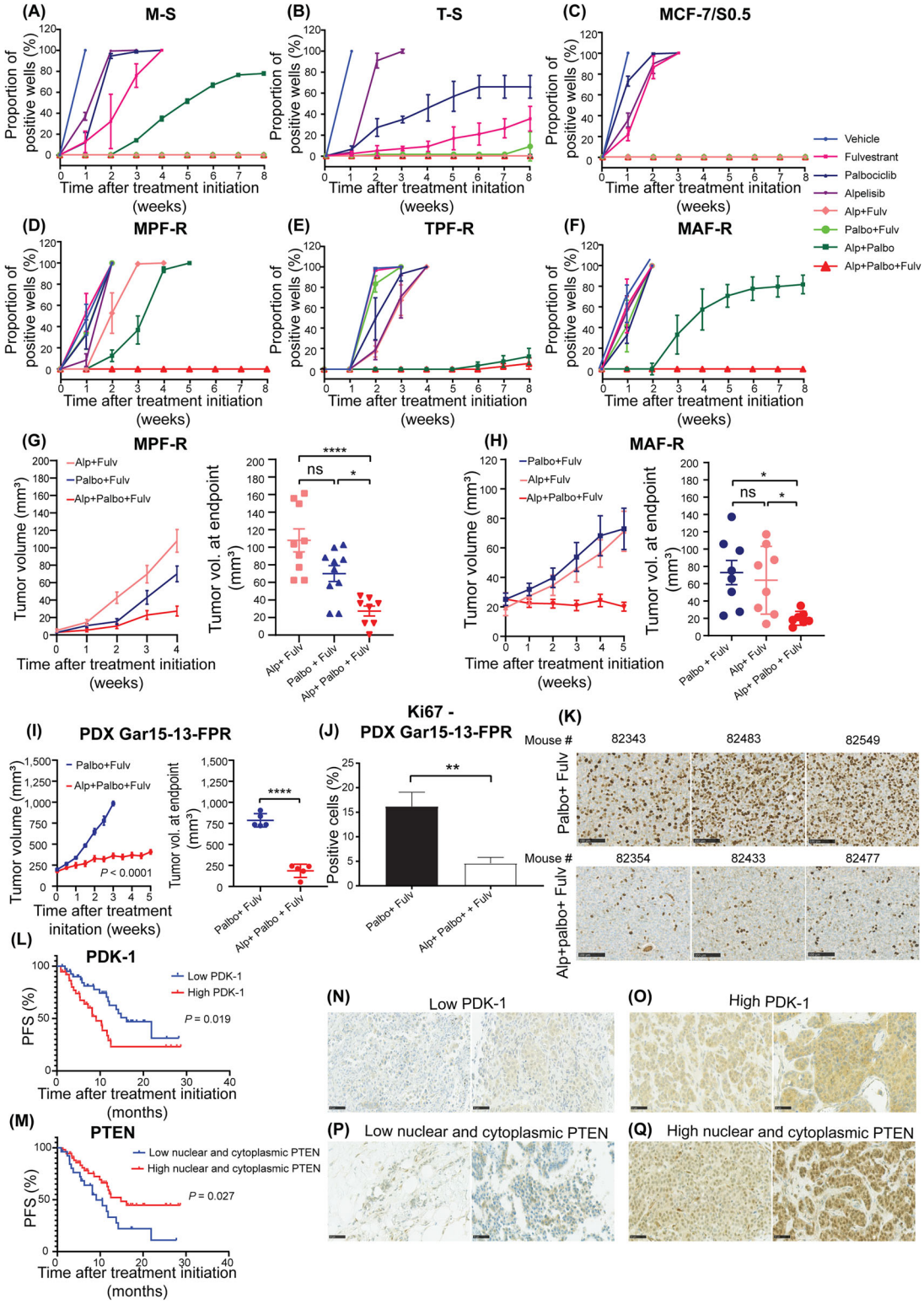

FIGURE 1 Triple combination with alpelisib, palbociclib, and fulvestrant prevents or significantly delays the emergence of resistance and significantly inhibits the growth of cell line-based xenograft and PDX tumors resistant to combined fulvestrant and palbociclib or alpelisib. (A-F) MCF-7- and T47D-derived cells resistant to combined palbociclib and fulvestrant (MPF-R and TPF-R) or to combined alpelisib and fulvestrant (MAF-R) and corresponding sensitive cell lines (M-S, T-S, MCF-7/S0.5) were treated weekly with vehicle, fulvestrant alone (Fulv, 100 nmol/L), palbociclib alone (Palbo, 200 nmol/L) and alpelisib alone (Alp, 1 μmol/L for M-S/MPF-R, 500 nmol/L for T-S/TPF-R, and 5 μmol/L for MCF7/S0.5/MAF-R) or different combinations of these for 8 weeks. Medium with the treatments was changed once a week. The percentage of wells ($n = 48$) exhibiting 50 % or greater confluence (defined as positive) was assessed weekly. The experiment was performed in

CDK4/6i (Supplementary Table S6). Multivariate Cox regression analysis of these parameters revealed that both PTEN ($P = 0.006$) and PDK-1 expression ($P = 0.031$) were independent prognostic factors for PFS (Supplementary Table S6). Description of patient clinicopathological characteristics in the early and advanced settings are shown in Supplementary Tables S7-S8, respectively. Although χ^2 test identified a significant difference in line of therapy for combined CDK4/6 inhibitor and endocrine therapy between low and high PDK-1 groups (Supplementary Table S8), multivariate analysis showed that PDK-1, but not line of therapy, was an independent prognostic factor for PFS in this patient cohort (Supplementary Table S6). No significant association between PDK-1 or PTEN expression and other clinical parameters at the metastatic disease was identified (Supplementary Table S8).

Collectively, we found that the triple combination with fulvestrant, palbociclib and alpelisib is superior in abolishing the growth of breast cancer resistant to combined fulvestrant and palbociclib or alpelisib. Moreover, our data suggested that the benefit of treating tumors resistant to combined palbociclib and fulvestrant with the alternative approved combination of alpelisib and fulvestrant, or vice versa, was very limited. Our data supported the clinical development of triple combination therapy targeting PI3K, CDK4/6, and ER in advanced ER$^+$ breast cancer following progression on the combined CDK4/6i or PI3Ki and endocrine therapy.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The immunohistochemical study was approved by the Ethics committee of the Region of Southern Denmark (approval no S-2008-0115) and the Danish Data Protection Agency. All patient samples were collected in compliance with informed consent policy and coded to maintain patient confidentiality. Procedures and endpoints involving laboratory animals were approved by the Experimental Animal Committee of The Danish Ministry of Justice (2021-15-0201-0084) and the Garvan Institute of Medical Research Animal Ethics Committee (protocol 15/25, 18/20, and 18/26).

CONSENT FOR PUBLICATION

Not applicable.

three biological replicates and data are presented as mean ± SEMs. The triple combination therapy durably impairs growth of resistant breast cancer cells and prevents the emergence of resistance.

(G-H) MPF-R and MAF-R cells were transplanted into the mammary fat pad of NOG CIEA mice. Mice were then treated with fulvestrant (Fulv, 100 mg/kg, subcutaneously, weekly) combined with either palbociclib (Palbo, 25 mg/kg, oral gavage, 5 days per week, $n = 8$-10) or alpelisib (Alp, 25 mg/kg, oral gavage, 5 days per week, $n = 8$-9) or a triple combination of alpelisib, palbociclib, and fulvestrant ($n = 8$). Tumor size was measured weekly for 4-5 weeks. For each subfigure, tumor growth curves are shown to the left and endpoint bar graphs of the same values are shown to the right. Data are presented as mean ± SEMs. Significant differences are calculated by one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, **** $P < 0.0001$; ns, non-significant) at endpoint (week 4 or 5). Triple combination of alpelisib, palbociclib and fulvestrant significantly inhibited growth of MPF-R and MFA-R tumor xenografts.

(I) Left panel: Average tumor growth curves from mice bearing the Gar15-13-FPR patient-derived xenograft (PDX) model resistant to combined palbociclib and fulvestrant treated with palbociclib (25 mg/kg, 5 days per week, oral gavage) and fulvestrant (2 mg/body, subcutaneously weekly, $n = 5$), or a triple combination of alpelisib (50 mg/kg, 5 days per week, oral gavage), palbociclib, and fulvestrant ($n = 5$). The addition of alpelisib resulted in a clear inhibition of tumor growth. Growth rate of palbociclib- and fulvestrant-resistant PDX was similar to that of the untreated parental PDX (Supplementary Figure S6). Right panel: Tumor volumes at endpoint (3 weeks for palbociclib and fulvestrant; and 5 weeks for triple combination).

(J) Quantification of Ki67 expression in PDX-Gar15-13FPR tumor sections treated with double or triple drug combinations, performed by ImageJ. (K) Representative images of Ki67 expression in 6 PDX-Gar15-13-FPR tumors treated with double or triple combinations (Scale bar 100 μm). (L-M) Kaplan-Meier curves evaluating PFS according to the levels of PDK-1 and PTEN in metastatic lesions ($n = 80$) from patients with advanced ER$^+$ breast cancer treated with combined CDK4/6i (palbociclib or ribociclib) and endocrine therapy (fulvestrant or letrozole). A two-sided P-value was calculated using log-rank testing.

(N-Q) Representative images of breast cancer metastasis sections showing low PDK-1 expression (intensity < 2, N), High PDK-1 expression (intensity ≥ 2, O), low nuclear and cytoplasmic PTEN expression (intensity < 2, P) and high nuclear and cytoplasmic PTEN expression (intensity ≥ 2, Q) (Scale bar 50 μm).

Abbreviations: Alp, alpelisib; Fulv, fulvestrant; Palbo, palbociclib; PDX, patient-derived xenografts; PDK-1, phosphoinositide-dependent kinase-1; PTEN, phosphatase and tensin homolog; SEM, standard error of the mean.

AVAILABILITY OF DATA AND MATERIALS

The gene expression data generated during the study are publicly available in the gene expression omnibus (GEO) database under the accession number GSE210400. Survival analyses and immunohistochemistry data are not publicly available to protect patient privacy but will be made available to authorized researchers who have an approved Institutional Review Board application and have obtained approval from the Regional Committees on Health Research Ethics for Southern Denmark. Please contact the corresponding author with data access requests. All other datasets generated during the study will be made available upon reasonable request to the corresponding author. Uncropped Western blots are part of the supplementary information.

AUTHOR CONTRIBUTIONS

Conceptualization: Leena Karimi, Carla L. Alves, and Henrik J. Ditzel. Methodology: Leena Karimi, Carla L. Alves, Mikkel G. Terp, Martina Tuttolomondo, Neil Portman, Sidse Ehmse, Lene E. Johansen, Martin Bak, Elgene Lim, and Henrik J. Ditzel. Investigation: Leena Karimi, Carla L. Alves, Mikkel G. Terp, Martina Tuttolomondo, Neil Portman, Sidse Ehmsen, Lene E. Johansen, Martina Bak, Elgene Lim, and Henrik J. Ditzel. Writing the original draft: Leena Karimi, Carla L. Alves, Henrik J. Ditzel. Writing and Review & Editing: all authors. Funding acquisition, Leena Karimi, Carla L. Alves, and Henrik J. Ditzel. Resources: Henrik J. Ditzel. Supervision: Carla L. Alves and Henrik J. Ditzel.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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Leena Karimi[1]
Carla L. Alves[1]
Mikkel G. Terp[1]
Martina Tuttolomondo[1]
Neil Portman[2,3]
Sidse Ehmsen[1,4]
Lene E. Johansen[1]
Martin Bak[5]
Elgene Lim[2,3]
Henrik J. Ditzel[1,4,6]

[1]*Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark*
[2]*Garvan Institute of Medical Research, Darlinghurst, Sydney, New South Wales, Australia*
[3]*St. Vincent's Clinical School, Faculty of Medicine, University of New South Wales Sydney, Sydney, New South Wales, Australia*
[4]*Department of Oncology, Institute of Clinical Research, Odense University Hospital, Odense, Denmark*
[5]*Department of Pathology, Sydvestjysk Sygehus, Esbjerg, Denmark*
[6]*Academy of Geriatric Cancer Research (AgeCare), Odense University Hospital, Odense, Denmark*

Correspondence
Henrik J. Ditzel, Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, Odense 5000, Denmark.
Emai: hditzel@health.sdu.dk

ORCID

Henrik J. Ditzel https://orcid.org/0000-0003-3927-5135

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.