# Functional analysis of gedatolisib combined with fulvestrant and/or palbociclib in breast cancer cell models adapted to estrogen receptor and/or CDK4/6 inhibitors

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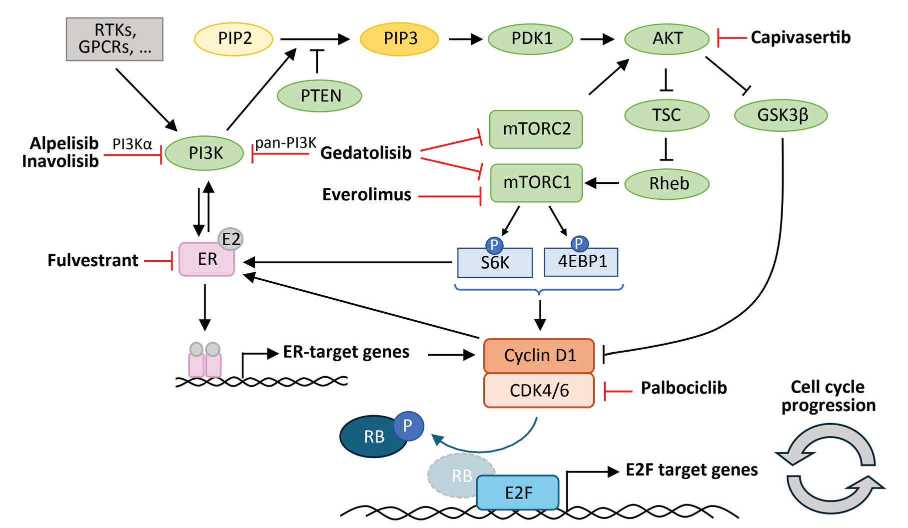
## BACKGROUND

- The PI3K, AKT, and mTOR (PAM) pathway is one of the most commonly activated oncogenic pathways in breast cancer (BC).[1]
- PAM pathway dysregulation has been linked to PAM pathway gene alterations (e.g., *PIK3CA*) but can also occur in their absence.[1,2]
- Adaptive activation of the PAM pathway contributes to resistance to endocrine therapy (ET) and CDK4/6 inhibitors, which are the first-line standard of care treatment for most patients with HR+/HER2- advanced breast cancer (ABC).[3,4]
- Due to the crosstalk between the PAM pathway and the estrogen receptor (ER) and CDK pathways (**Figure 1**), resistance can arise when only one of these pathways is inhibited.[3-6]
- Several FDA-approved inhibitors targeting individual PAM pathway components, such as everolimus (mTORC1), alpelisib (PI3Kα), capivasertib (AKT), and inavolisib (PI3Kα), are used to treat HR+/HER2- ABC in combination with ET or ET and CDK4/6 inhibitors.
- **The therapeutic efficacy of single-target inhibitors of the PAM pathway can be limited** by adaptive compensatory mechanisms and is often restricted to BCs with specific PAM pathway mutations (e.g., *PIK3CA*).[6,7]
- Inhibitors targeting multiple components of the PAM pathway are expected to be more efficacious than their single-target counterparts and potentially treat a broader range of BCs, including those without specific PAM pathway mutations.
- **Gedatolisib is a multi-target PAM inhibitor targeting all class I PI3K isoforms, mTORC1, and mTORC2** with potent growth inhibitory effects in vitro and in vivo.[8,9]
- Comparative studies have shown that gedatolisib inhibits the PAM pathway and PAM-controlled functions more effectively than single-target inhibitors of the PAM pathway, thus exerting greater anti-proliferative and cytotoxic effects.[10-12]
- Nonclinical studies have shown that the gedatolisib/fulvestrant/palbociclib triplet exerts greater anti-proliferative and cytotoxic effects than single drugs or double combinations in ER+ BC cell lines, regardless of *PIK3CA/PTEN* genetic alterations.[13]
- A phase 1b clinical trial has shown preliminary efficacy and tolerability for gedatolisib in combination with ET and a CDK4/6 inhibitor in HR+/HER2- ABC with or without *PIK3CA* mutations, both in treatment-naïve patients and those who received prior lines of therapy.[14]

**Study objective:** compare gedatolisib and single-target PAM pathway inhibitors in combination with fulvestrant and/or palbociclib in BC cells modeling mutant or wild-type *PIK3CA* BCs before and after progression on ET and/or CDK4/6 inhibitors.

## Figure 1. Crosstalk Between PAM, ER, and CDK Pathways




## METHODS

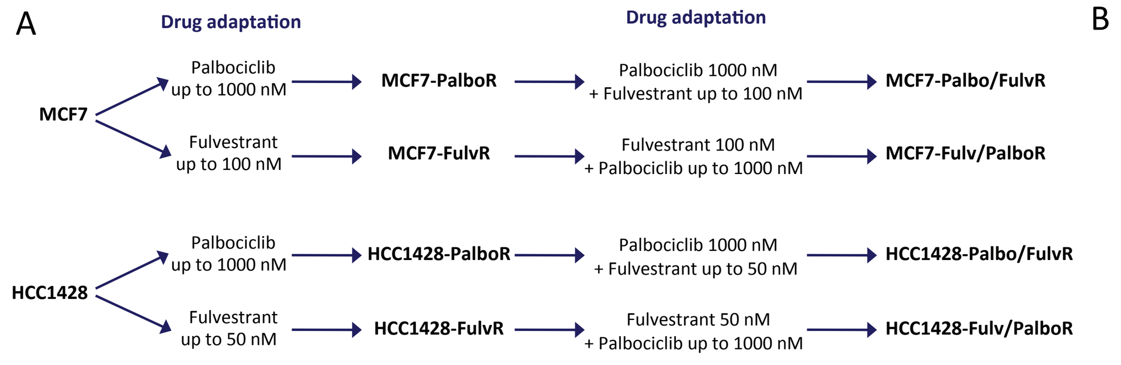
**Cell lines.** ER+ BC cell lines were obtained from ATCC and maintained based on the vendor's recommendations. Cells were authenticated by short tandem repeat profiling. Genetic alterations in PAM pathway genes were identified by cBioPortal (https://www.cbioportal.org) analysis of the Cancer Cell Line Encyclopedia. MCF7 and HCC1428 cells adapted to long-term treatment to fulvestrant and/or palbociclib were established as shown in **Figure 2**.[13]

**Growth rate (GR) assays.** Cells seeded in 96-well plates were analyzed for cell viability before and after drug treatment by SYTOX Green staining of dead and live cells before and after cell permeabilization as described.[11] Cell viability assessments were used to calculate GR values as described.[15] The GR approach was used to rule out confounding effects of traditional IC50 metrics, such as the number of cell divisions occurring during the assay. GR values between 0 and 1 indicate anti-proliferative effects, GR = 0 indicates fully cytostatic effects (no cell growth), and GR < 0 indicates cytotoxic effects (where -1 is complete cell killing).

**Analysis of cell cycle, cell death, and apoptosis by flow cytometry.** After treatment in 96-well plates, cells were harvested, stained with a viability dye (Zombie), fixed in 1.6% paraformaldehyde, permeabilized with methanol, stained with antibodies, and analyzed by flow cytometry on the Agilent NovoCyte 3005 as described.[13] Cell cycle and DNA replication were analyzed by combining DNA staining with FxCycle with 5-ethynyl-2'-deoxyuridine (EdU) incorporation Click-iT assay (Invitrogen). Cell death was analyzed by staining with Zombie dye, which identifies dead cells with compromised membrane permeability. Apoptosis in live cells (Zombie negative) was analyzed by immunostaining with anti-cleaved poly (ADP-ribose) polymerase (PARP) or anti-cleaved caspase 3.

## Figure 2. Development of Cell Lines Adapted to Fulvestrant and/or Palbociclib

A




B

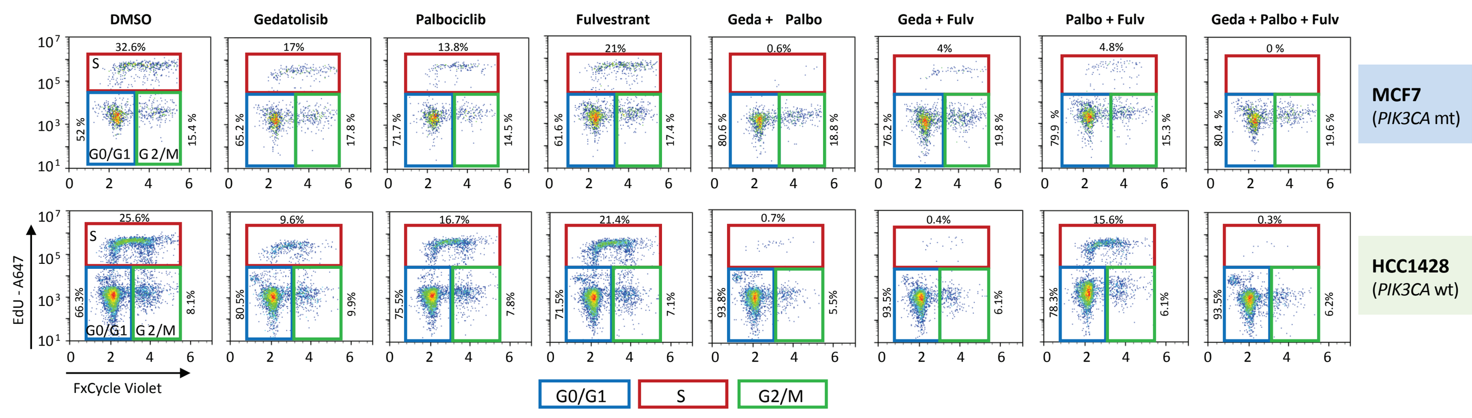
| Cell line | EdU IC50 (nM) Palbo | EdU IC50 (nM) Fulv |
|---|---|---|
| MCF7 | 56.3 | <1.4 |
| MCF7-PalboR | 530.5 | 72.5 |
| MCF7-FulvR | 45.6 | >1000 |
| MCF7-Palbo/FulvR | 415.4 | >1000 |
| MCF7-Fulv/PalboR | 861.8 | >1000 |
| HCC1428 | 222.6 | 7.3 |
| HCC1428-PalboR | 2164 | 4.0 |
| HCC1428-FulvR | 104.5 | >1000 |
| HCC1428-Palbo/FulvR | >9000 | >1000 |
| HCC1428-Fulv/PalboR | 1761 | >1000 |

**A.** MCF7 and HCC1428 cells adapted to fulvestrant and/or palbociclib were established by continuous treatment with the two drugs as indicated.
**B.** Analysis of DNA replication by EdU incorporation showed that the adapted cell lines had decreased sensitivity (higher IC50) to the selection drug.

## RESULTS

## Figure 3. Effects of the Gedatolisib Triplet in Treatment-Naïve Cells

A



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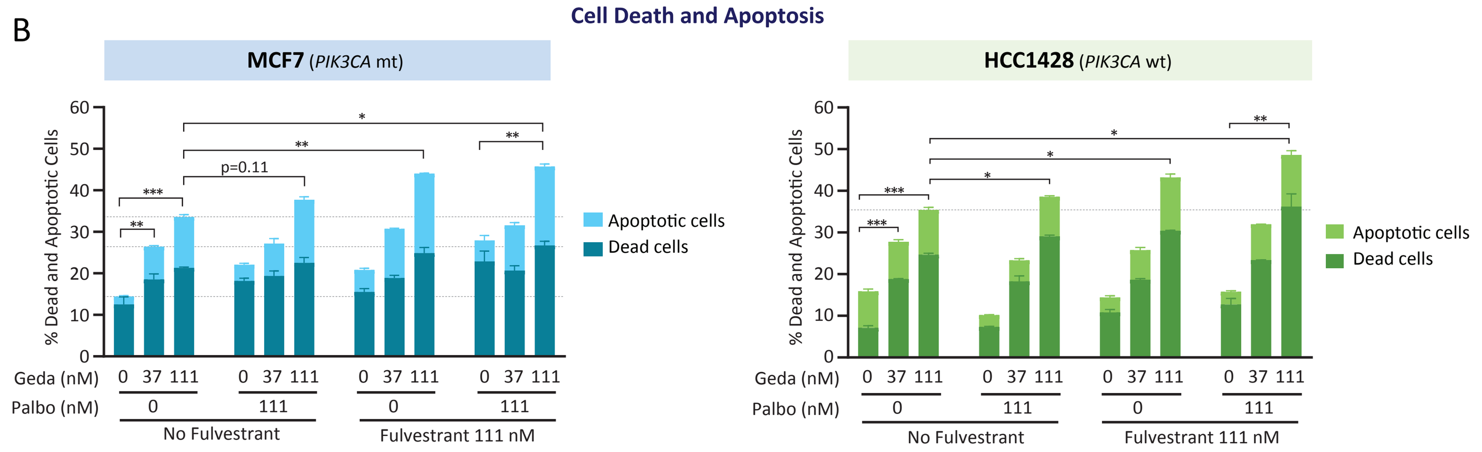


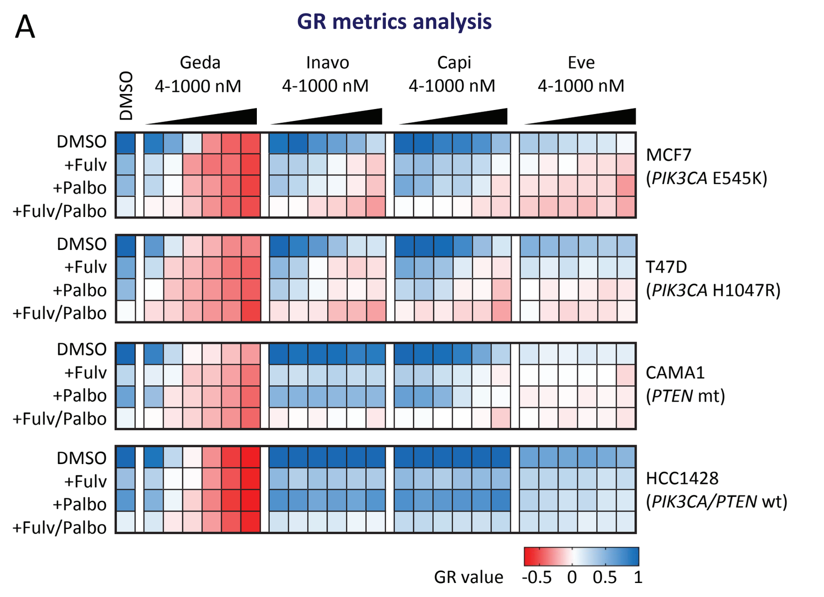
**A.** MCF7 and HCC1428 cells were treated with various combinations of gedatolisib (37 nM), fulvestrant (1.4 nM for MCF7 and 12 nM for HCC1428), and palbociclib (111 nM) for 72 hours and analyzed for cell cycle phases and DNA replication by flow cytometry analysis of DNA content (assessed by FxCycle staining) and incorporation of the nucleoside analog EdU into newly synthesized DNA. The percentage of cells in S, G0/G1, and G2/M phases was calculated by FxCycle/EdU gating of live cells. **B.** MCF7 and HCC1428 were also analyzed by flow cytometry for cell death (assessed by Zombie staining) and apoptosis (assessed by immunodetection of cleaved PARP or cleaved caspase 3) after 72 hours of treatment with the indicated combinations of gedatolisib, fulvestrant, and/or palbociclib. The graphs show the quantification of dead cells (% parent) and live apoptotic cells (% grandparents) in response to the various drug treatments. Data represent mean +/- standard deviation (n=2). * p<0.05, ** p<0.01, *** p<0.001 by unpaired, two-sided t-test. Statistical analysis refers to the sum of dead cells plus live apoptotic cells.

DMSO, dimethylsulfoxide; EdU, 5-Ethynyl-2'-deoxyuridine; fulv, fulvestrant; geda, gedatolisib; mt, mutant; palbo, palbociclib; wt, wild-type.

- **Gedatolisib combined with fulvestrant and palbociclib inhibited cell cycle progression by arresting cells in G0/G1 and induced apoptotic cell death more effectively than each single agent or the fulvestrant/palbociclib doublet in treatment-naïve BC cell lines**
- **The triplet benefits were observed in both *PIK3CA* mutated (MCF7) and *PIK3CA* wild-type (HCC1428) BC cells**

## Figure 4. Gedatolisib Versus Single-Target Inhibitors in Combination With Fulvestrant and/or Palbociclib in Treatment-Naïve BC Cells

A



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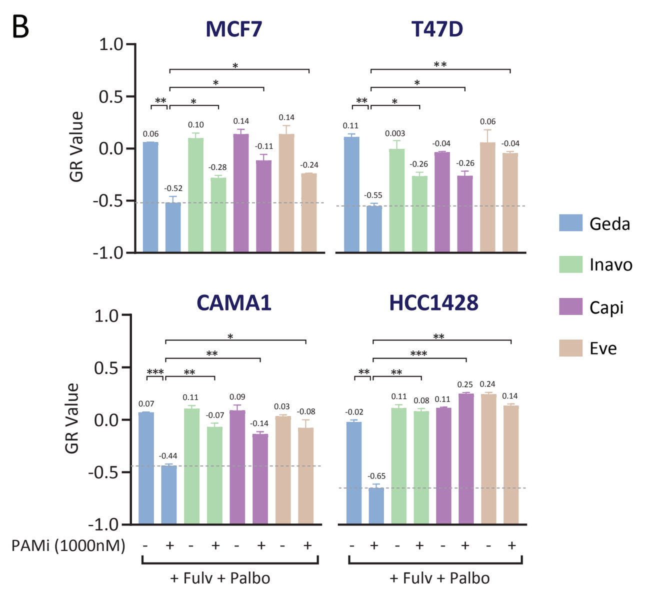


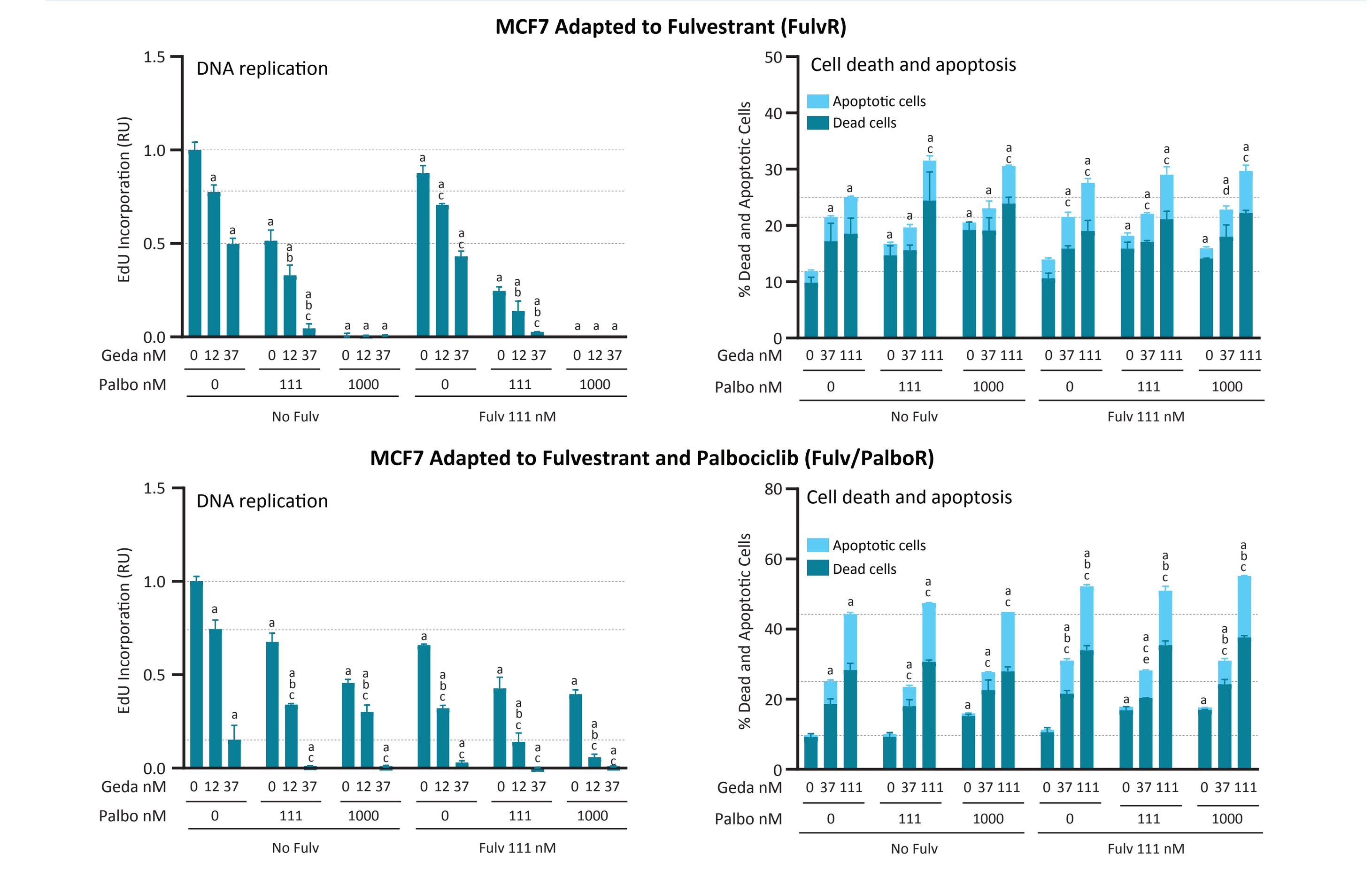
GR metrics analysis of BC cell lines treated with increasing concentration of gedatolisib (geda), inavolisib (inavo), capivasertib (capi), or everolimus (eve), alone or in combination with fulvestrant (100 nM) and/or palbociclib (100 nM for MCF7, T47D, CAMA1; 250 nM for HCC1428) for 6 days. A heatmap summarizing the GR values in all cell lines is shown in **A**; GR values in response to 1000 nM PAM pathway inhibitor (PAMi) in the presence of fulvestrant and palbociclib are shown in **B**. Data represent the mean +/- standard deviation (n=2) and are relative to DMSO-treated cells (set as 1). * p< 0.05, ** p<0.01, ***p<0.001 by two-sided, unpaired t-test.

BC, breast cancer; DMSO, dimethylsulfoxide; EdU, 5-Ethynyl-2'-deoxyuridine; fulv, fulvestrant; GR, growth rate; mt, mutant; palbo, palbociclib; wt, wild type.

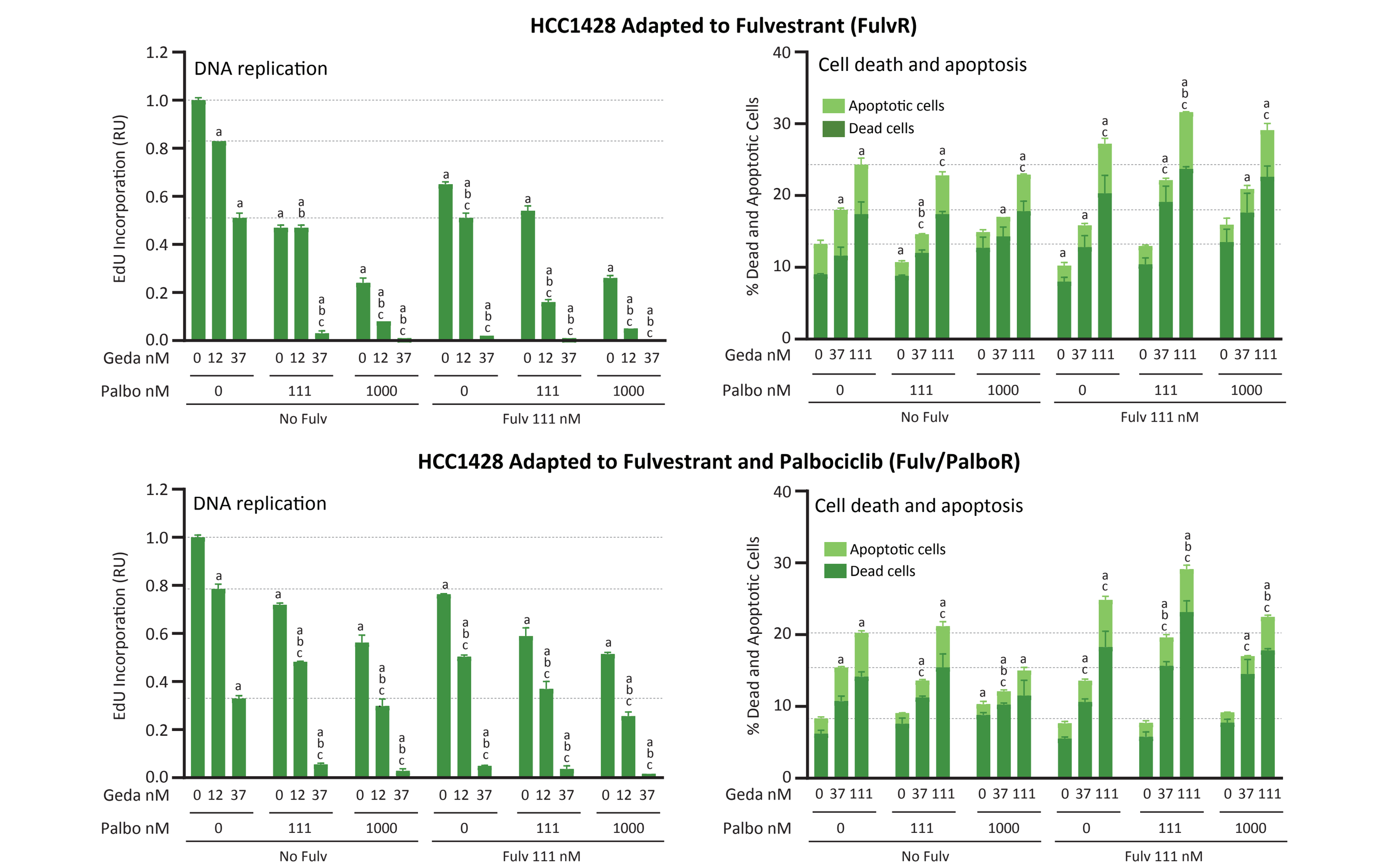
- **The gedatolisib/fulvestrant/palbociclib triplet exerted similar growth-inhibitory effects on all BC cell lines tested, while the single-target inhibitor triplets were less effective in *PIK3CA/PTEN* wild-type cells**
- **The gedatolisib triplet was more effective than the single-target inhibitor triplets in all cell lines, regardless of their *PIK3CA/PTEN* mutational status**

## Figure 5. Effects of the Gedatolisib Triplet in BC Cells Adapted to Fulvestrant and/or Palbociclib

A **MCF7 adapted cell lines** (*PIK3CA* mutant)



B **HCC1428 adapted cell lines** (*PIK3CA* wild-type)



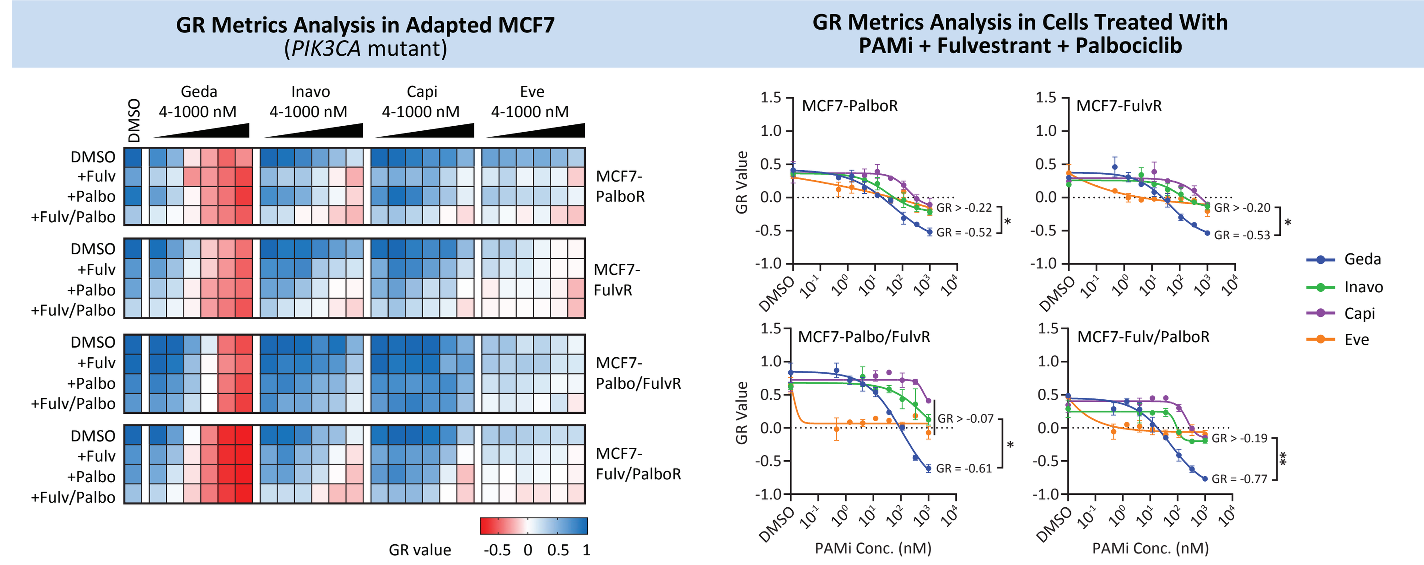
MCF7 **(A)** and HCC1438 **(B)** cells adapted to fulvestrant (FulvR) or fulvestrant + palbociclib (Fulv/PalboR) were treated with various combinations of gedatolisib, fulvestrant, and palbociclib for 72 hours and analyzed by flow cytometry for DNA replication (assessed by EdU incorporation), cell death (assessed by Zombie dye staining), and apoptosis (assessed by cleaved PARP or cleaved caspase 3). Data represent mean +/- standard deviation (n=2). a, p<0.05 vs DMSO; b, p<0.05 vs gedatolisib only; c, p<0.05 vs no gedatolisib within group; d, p=0.07 vs no gedatolisib within group; e, p=0.05 vs gedatolisib only by unpaired, two-sided t-test. Similar results were observed in cells adapted to palbociclib (data not shown).

BC, breast cancer; DMSO, dimethylsulfoxide; EdU, 5-Ethynyl-2'-deoxyuridine; fulv, fulvestrant; geda, gedatolisib; palbo, palbociclib; PARP, poly (ADP-ribose) polymerase.

- **BC cells adapted to fulvestrant and/or palbociclib were developed to model BC progression after treatment with ET and/or CDK4/6 inhibitors**
- **Gedatolisib inhibited cell proliferation and induced apoptotic cell death as a single agent in BC cells adapted to fulvestrant and/or palbociclib**
- **The gedatolisib/fulvestrant/palbociclib triplet was generally more effective than gedatolisib alone or the drug doublets at inhibiting cell proliferation and/or inducing apoptotic cell death. In most cases, the gedatolisib doublets were also more effective than gedatolisib alone or the fulvestrant/palbociclib doublet.**
- **The gedatolisib triplet was effective in both *PIK3CA* mutated and *PIK3CA* wild-type BC cells**

## Figure 6. Gedatolisib Versus Single-Target Inhibitors in Combination With Fulvestrant and/or Palbociclib in BC Cells Adapted to Fulvestrant/Palbociclib

A



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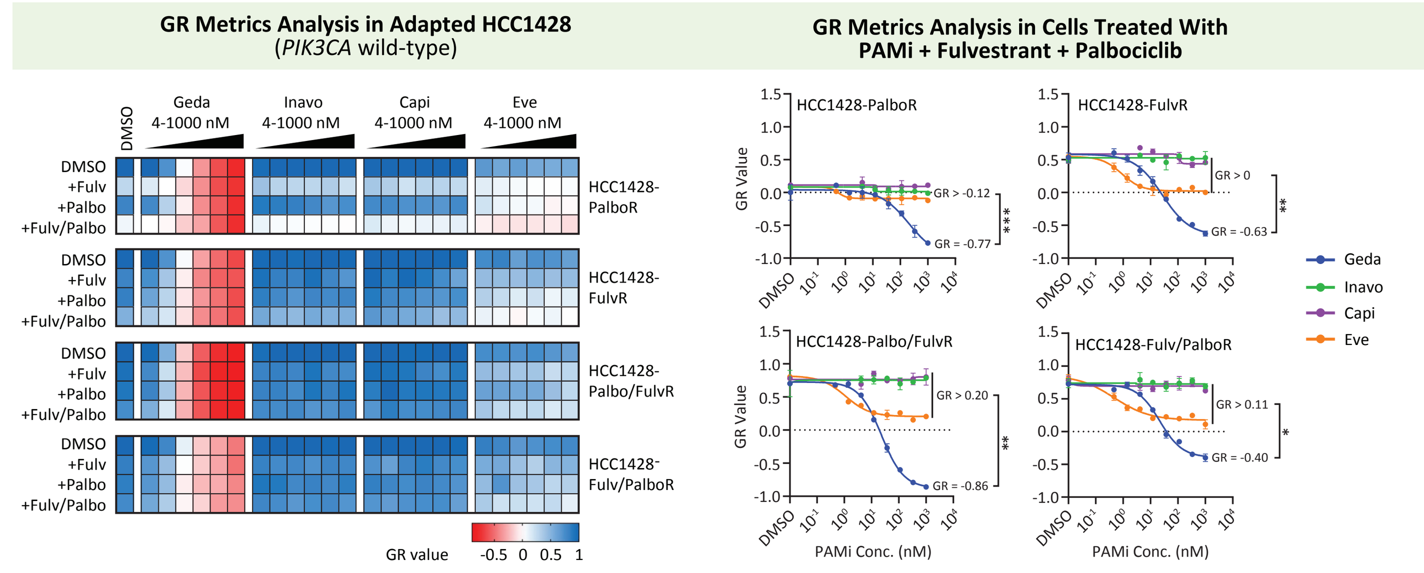

GR metrics analysis of MCF7 **(A)** and HCC1428 **(B)** cell lines adapted to fulvestrant (FulvR), palbociclib (PalboR), or both fulvestrant and palbociclib (Fulv/PalboR, Palbo/FulvR) after 6-day treatment with gedatolisib (geda), invaolisib (inavo), capivasertib (capi), or everolimus (eve), alone or in combination with fulvestrant (100 nM) and/or palbociclib (100 nM for MCF7 and 250 nM for HCC1428). The heatmaps on the left summarize the GR values in all the adapted cell lines treated with all drug combinations. The graphs on the right show dose response curves for gedatolisib, inavolisib, capivasertib, and everolimus, each combined with fulvestrant and palbociclib. Data represent the mean +/- standard deviation (n=2) and are relative to DMSO-treated cells (set as 1). * p< 0.05, ** p<0.01, *** p<0.001 by two-sided, unpaired t-test.

BC, breast cancer; Conc., concentration; DMSO, dimethylsulfoxide; GR, growth rate; PAMi, PAM inhibitor.

- **The gedatolisib/fulvestrant/palbociclib triplet exerted growth-inhibitory effects in both *PIK3CA* mutated (MCF7) and *PIK3CA* wild-type (HCC1428) cells adapted to fulvestrant and/or palbociclib**
- **The single-target inhibitor triplets were mostly effective in the *PIK3CA* mutant cell models**
- **The gedatolisib triplet was more effective than the single-target inhibitor triplets, regardless of *PIK3CA* mutational status or drug adaptation**

## SUMMARY AND CONCLUSIONS

- **The combination of gedatolisib with fulvestrant and/or palbociclib was generally more effective at inhibiting cell proliferation and/or inducing apoptotic cell death relative to the single drugs or the fulvestrant/palbociclib doublet in both treatment-naïve BC cell lines and BC cell lines adapted to treatment with fulvestrant and/or palbociclib.**
- **The gedatolisib/fulvestrant/palbociclib triplet was similarly effective in *PIK3CA* mutated and wild-type cell models, while the corresponding single-target inhibitor triplets were mostly effective in the *PIK3CA* mutant models.**
- **Gedatolisib was more effective than the single-target inhibitors both as a single agent and in combination with fulvestrant and/or palbociclib in all cell models tested, regardless of drug adaptation or *PIK3CA* mutational status.**
- **Gedatolisib has previously demonstrated promising preliminary clinical efficacy and safety in patients with HR+/HER2- ABC in combination with ET and palbociclib.**
- **A phase 3 study (VIKTORIA-1, NCT05501886) evaluating gedatolisib plus fulvestrant with and without palbociclib is underway in patients with HR+/HER2- ABC whose disease progressed after CDK4/6 inhibitors (see Presentation # RF4-03).**
- **Another phase 3 trial (VIKTORIA-2, NCT06757634) is also ongoing to evaluate the combination of gedatolisib with fulvestrant and a CDK4/6 inhibitor as first-line therapy.**

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