# Gedatolisib, a Pan-PI3K/mTOR Inhibitor, Shows Superior Potency and Efficacy Relative to Other PI3K/AKT/mTOR Pathway Inhibitors in Breast Cancer Models

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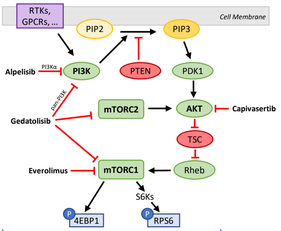
## BACKGROUND

- The PI3K, AKT, and mTOR (PAM) pathway (Figure 1) is one of the most commonly activated oncogenic pathways in breast cancer (BC). PIK3CA activating mutations and loss of PTEN function are especially frequent (1).
- Adaptive activation of the PAM pathway has also been associated with resistance to hormonal therapy (HT) with drugs targeting the estrogen receptor (ER) signaling pathway (2,3).
- Current standard-of-care therapy options for patients with advanced BC (ABC) include treatment with HT (e.g., letrozole, fulvestrant) and PAM inhibitors (PAMi; e.g., everolimus, alpelisib).
- Due to feedback loops between PI3K isoforms, AKT, and mTOR that cross-activate uninhibited sub-units, PAM inhibitors that selectively spare or weakly inhibit one or more key PAM pathway nodes (Figure 1) cannot achieve optimal therapeutic effect, even when combined with HT (4).
- We hypothesized that gedatolisib, which potently inhibits all Class I PI3K isoforms, as well as mTORC1 and mTORC2, can be more effective in BC cells than a PAMi targeting single PAM pathway components, both as a single agent and in combination with HT.
- The objective of this study was to compare the effects of gedatolisib and single node PAMi on PAM pathway activity as well as PAM-controlled functions (e.g., protein synthesis, DNA replication) that play a critical role in cancer cells proliferation.

### Figure 1. The PI3K, AKT, and mTOR (PAM) Pathway




## METHODS

**Cell Lines.** A panel of 28 BC cell lines was used in this study (Table 1). Cells were maintained according to American Type Culture Collection (ATCC) recommendations and authenticated by short tandem repeat (STR) profiling. Genetic alterations in PAM pathway genes were identified by cBioPortal (https://www.cbioportal.org) analysis of the Cancer Cell Line Encyclopedia (CCLE) (5). Cell line tumor type/subtype and Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2 status were based on Dai 2017 (6).

**Treatments with PAM Inhibitors.** Cells were seeded on 96-well plates coated with collagen-fibronectin, allowed to attach overnight, and treated with PAMi (Table 2) at increasing concentrations to obtain dose response curves (DRCs). The seeding density of each cell line was optimized to ensure untreated cells remained in the growth phase throughout the assay. For treatments in three-dimensional (3D) culture, cells were seeded in 96-well plates coated with 60% Cultrex Basement Membrane Extract (BME), overlayed with growth medium + 2% BME, and incubated for 72h before treatment.

**Viability and Proliferation-normalized Inhibition of Growth Rate (GR) Assays**. After treatment for the indicated time, cell viability was measured by RT-Glo MT assay (Promega) using a luminescence microplate reader. GR values, GR50 (i.e. concentration reducing growth rate by 50%), and GRMax (i.e. GR value at the highest tested dose) were calculated as described (7). 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 (7). The GR metrics results confirmed and expanded the results obtained by classical endpoint cell viability assays. Sensitivity cutoff for capivasertib, alpelisib, and everolimus were based on previously published studies (8, 9, 10).

**Flow Cytometry**. After treatment, 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. PAM pathway activity was assessed using antibodies against pRPS6 and p4EBP1, two markers that integrate PAM signaling pathway outputs from PI3K/mTORC1 and mTORC2/AKT (Figure 1). DNA replication and protein synthesis, two PAM-controlled functions required for cell proliferation, were assessed by 5-ethynyl-2′-deoxyuridine (EdU; 10 μM for 2h) and O-propargyl-puromycin (OPP; 5 μM for 30 min) incorporation followed by Click-IT reaction (Invitrogen).

**Animal Studies.** *MCF7 xenograft study:* Estrogenized female SCID mice were inoculated in the mammary fat pad with $5x10^6$ MCF7 cells. When tumors reached ~230 $mm^3$, mice (N=10/arm) were randomly assigned to either a control vehicle group; or treatment groups that received gedatolisib, fulvestrant, or gedatolisib + fulvestrant. Fulvestrant was dosed subcutaneously (s.q.) once a day (QD) for 3 days, then once every 3 days (Q3D). Gedatolisib was dosed intravenously (i.v.) once every 4 days (Q4D). Tumor volumes were measured twice a week with a digital caliper. *Mini-PDX study* (LIDE Biotech): Patient-derived xenograft (PDX) tumor tissue was digested and transferred into hollow fiber mini-capsules. The mini-PDX capsules were inoculated into both flanks of BALB/c nude mice and treated for 7 days. Gedatolisib was dosed i.v. Q4D; alpelisib was dosed orally (p.o.) QD; capivasertib was dosed p.o. twice a day (BID) on a 4 days on/3 days off schedule; everolimus was dosed p.o. QD. These PAMi concentrations and dosing schedules were previously reported to be efficacious in xenograft models (11, 12, 13, 14). At Day 7, the encapsulated tumor cell number was evaluated by CellTiter-Glo Luminescent 3D Cell Viability Assay (Promega).

### Table 1. Cancer Cell Lines Tested

| Cell line | Source | Tumor type | Tumor subtype | ER+ | HER2+ | AKT1 | AKT2 | AKT3 | PIK3CA | PIK3CB | PIK3R1 | PIK3R2 | PTEN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AU565 | ATCC | AC | H | - | Yes | - | - | - | - | - | - | - | HOMDEL |
| BT20 | ATCC | IDC | TNA | - | - | - | - | - | Mut | - | - | - | HOMDEL |
| BT474 | ATCC | IDC | LB | Yes | Yes | - | - | AMP | Mut | - | - | HOMDEL | - |
| BT549 | ATCC | IDC | TNB | - | - | - | - | - | Mut | - | - | - | Mut |
| CAL51 | DSMZ | IDC | TNB | - | - | - | - | - | Mut | - | - | - | Mut |
| CAMA1 | ATCC | AC | LA | Yes | - | - | - | - | Mut | - | - | - | Mut |
| EFM19 | DSMZ | IDC | LA | Yes | - | - | - | - | Mut | - | - | - | - |
| EFM192A | DSMZ | AC | LB | Yes | Yes | - | - | - | Mut | - | - | - | - |
| HCC1419 | ATCC | IDC | NA | - | Yes | - | - | - | - | - | - | - | - |
| HCC1428 | ATCC | AC | LA | Yes | - | - | - | - | - | - | - | - | - |
| HCC1500 | ATCC | IDC | - | Yes | - | - | - | AMP | - | - | - | - | - |
| HCC1954 | ATCC | IDC | H | - | Yes | - | - | - | Mut | - | - | - | - |
| HCC38 | ATCC | DC | TNB | - | - | - | - | AMP | - | - | - | - | - |
| HS578T | ATCC | IDC | TNB | - | - | - | - | - | - | - | Mut | - | - |
| JIMT1 | DSMZ | IDC | NA | - | Yes | - | - | - | Mut, AMP | AMP | - | - | - |
| KPL1 | DSMZ | IDC | LA | Yes | - | AMP | - | - | Mut | - | - | - | - |
| MCF7 | ATCC | IDC | LA | Yes | - | - | - | - | Mut | - | - | - | - |
| MDAMB134VI | ATCC | IDC | LA | Yes | - | - | - | - | - | - | - | - | - |
| MDAMB175VII | ATCC | IDC | LA | Yes | - | - | - | - | - | - | - | - | - |
| MDAMB231 | ATCC | AC | TNB | - | - | - | - | - | - | - | - | - | - |
| MDAMB361 | ATCC | AC | LB | Yes | Yes | - | - | - | Mut | - | - | - | - |
| MDAMB415 | ATCC | AC | LA | Yes | - | - | - | AMP | AMP | - | - | - | Mut |
| MDAMB453 | ATCC | AC | H | - | Yes | - | - | AMP | Mut | - | - | - | - |
| MFM223 | Sigma | C | TNA | - | - | - | - | - | Mut | - | - | - | - |
| SKBR3 | ATCC | AC | H | - | Yes | - | - | - | - | - | - | - | HOMDEL |
| T47D | ATCC | IDC | LA | Yes | - | - | - | - | Mut, AMP | - | - | - | - |
| ZR751 | ATCC | IDC | LA | Yes | - | - | - | AMP | - | - | - | - | Mut |
| ZR7530 | ATCC | IDC | LB | Yes | Yes | - | - | AMP | - | - | - | - | - |

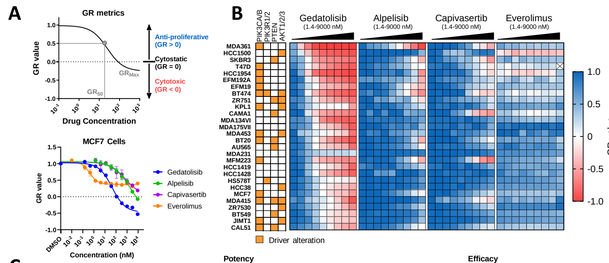
AC=adenocarcinoma; AMP=amplified; ATCC=American Type Culture Collection; C=carcinoma; DC=ductal carcinoma; DSMZ=German Collection of Microorganisms and Cell Cultures; H=HER2+; HOMDEL=deep deletion; IDC=invasive ductal carcinoma; LA=luminal A; LB=luminal B; Mut=mutated; TNA=triple neg A; TNB=triple neg B; Red=driver alteration

### Table 2. PAM Inhibitors Tested

| Drug | PAM specificity | Cell-free Assay Ki (nM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PI3Kα | PI3Kβ | PI3Kγ | PI3Kδ | mTOR | AKT1 | AKT2 | AKT3 |
| **Gedatolisib** | Pan-PI3K/mTOR | 0.4 | 6 | 5.4 | 6 | 1.6 | - | - | - |
| **Alpelisib** | PI3Kα | 5 | >1000 | 250 | 290 | - | - | - | - |
| **Capivasertib** | AKT | - | - | - | - | - | 3 | 8 | 8 |
| **Everolimus** | mTORC1 | - | - | - | - | 1.6 | - | - | - |

## RESULTS

### Figure 2. Analysis of Cytostatic and Cytotoxic Effects of Gedatolisib and Single Node PAMi

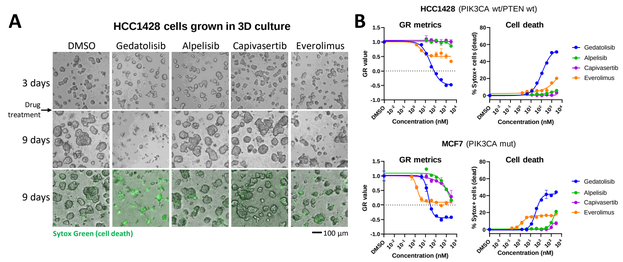



[1]Sensitive cell lines: GR50 <100 nM for gedatolisib; GR50 <3000 nM for alpelisib, capivasertib; GR50 <50 nM for everolimus; [2]Based on 1.4-9000 nM titration; [3]High efficacy cell lines: GRMax <0; [4]Bimodal distribution

GR metrics were used to assess potency and efficacy of gedatolisib and single node PAMi (alpelisib, capivasertib, everolimus). **(A)** The GR metrics represent an improvement over classical viability assays as they allow identification of both cytostatic and cytotoxic drug effects independent of cell doubling time. Drug potency can be assessed by GR50 and drug efficacy by GRMax (top). An example of DRCs in response to 72h PAMi treatment in the MCF7 cell line is shown at the bottom. Data are relative to DMSO-treated cells (set as 1) and represent mean +/- standard deviation (SD). **(B)** Heatmap summarizing the DRCs of all the cell lines tested (n=28). **(C)** Average potency and efficacy of gedatolisib and single node PAMi in BC cell lines (n=28). alt = altered; wt = wild type.

- **Gedatolisib exerted potent anti-proliferative and cytotoxic effects in BC cells lines, regardless of the PAM pathway mutational status**
- **Alpelisib and capivasertib were more potent and efficacious in cell lines with altered vs wild type PIK3CA/PTEN**
- **Gedatolisib was more potent and efficacious than single node PAMi, regardless of the PAM pathway mutational status**

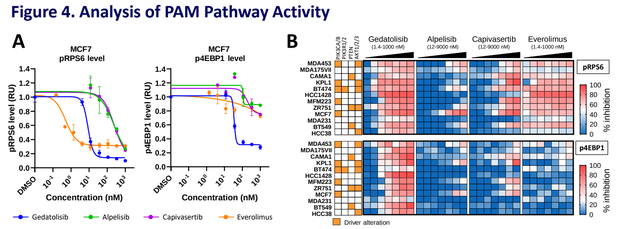
### Figure 3. Analysis of Cytostatic and Cytotoxic Effects of Gedatolisib and Single Node PAMi in 3D Culture




3D culture on BME was used to assess PAMi cytostatic/cytotoxic effects under conditions that mimic key aspects of *in vivo* tumor tissue architecture. **(A)** Micrographs showing the effect of gedatolisib and single node PAMi (333 nM) on HCC1428 3D spheroids after a 6-day treatment (from Day 3 to Day 9). At the end of the treatment, spheroids were stained with Sytox Green to identify dead cells (bottom panel). **(B)** GR metrics were used at the end of the treatment to quantify PAMi cytostatic and cytotoxic effects in HCC1428 and MCF7 cells (left panels). Cell death was also quantified by using Sytox Green (right panels). mut=mutant; wt=wild type.

- **Gedatolisib inhibited tumor spheroid growth and induced tumor spheroid cell death and regression in both wild type and mutant PIK3CA cell lines**
- **Gedatolisib exerted a greater cytostatic and cytotoxic effect than the single node PAMi evaluated in 3D cultures**

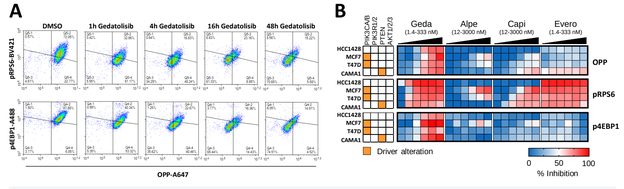
### Figure 4. Analysis of PAM Pathway Activity




A panel of 12 BC cell lines was treated for 48h with escalating doses of PAM inhibitors and analyzed by flow cytometry for markers of PAM signaling activity (pRPS6 and p4EBP1). **(A)** Examples of PAMi DRCs in MCF7 cells. Data are relative to DMSO-treated cells (set as 1) and represent mean +/- SD. **(B)** Heatmaps summarizing PAMi DRCs for pRPS6 and p4EBP1 in cell lines tested.

- **Gedatolisib suppressed PAM signaling in BC cells independent of their PAM pathway status more effectively than single node PAM inhibitors**

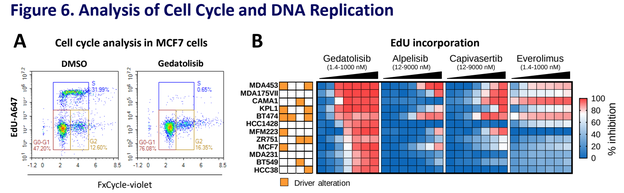
### Figure 5. Analysis of Protein Synthesis




Protein synthesis is a PAM-controlled cellular function required for cancer cell proliferation. BC cell lines were treated with PAM inhibitors and analyzed by flow cytometry for protein synthesis by OPP incorporation. **(A)** Gedatolisib (333 nM) decreased pRPS6, p4EBP1, and OPP incorporation within 1-4h of treatment in T47D cells. **(B)** Heatmaps summarizing DRCs for OPP incorporation, pRPS6, and p4EBP1 in response to 24h PAMi treatment. % inhibition is relative to DMSO-treated cells.

- **Gedatolisib suppressed protein synthesis in BC cells independent of their PAM pathway status more effectively than single node PAM inhibitors**

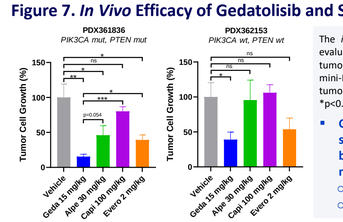
### Figure 6. Analysis of Cell Cycle and DNA Replication




BC cell lines were treated for 48h with PAM inhibitors and analyzed by flow cytometry for DNA replication (EdU incorporation). **(A)** Gedatolisib (333 nM) inhibited DNA replication during the S phase of the cell cycle in MCF7 cells. **(B)** Heatmaps summarizing DRCs for EdU incorporation in a panel of 12 BC cell lines treated with PAMi. % inhibition is relative to DMSO-treated cells.

- **Gedatolisib suppressed DNA replication in BC cells independent of their PAM pathway status more effectively than single node PAM inhibitors**

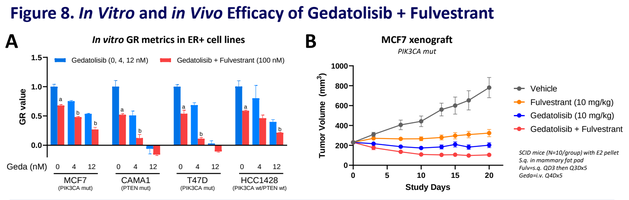
### Figure 7. *In Vivo* Efficacy of Gedatolisib and Single Node PAMi




The *in vivo* efficacy of gedatolisib and single node PAMi was evaluated in mini-PDX models derived from human ER+ breast tumors with wild type (wt) or mutant (mut) PIK3CA/PTEN. The mini-PDX were treated for 7 days as indicated in the methods, and tumor cell growth was assessed by a luciferase viability assay. *p<0.05, **p<0.01, ***p<0.001; ns=not significant.

- **Gedatolisib was the only PAMi inducing significant tumor cell growth inhibition (TCGI) in both wild type and mutant PI3K/PTEN PDX models**
  - **85% TCGI in PIK3CA/PTEN mutant model**
  - **61% TCGI in PIK3CA/PTEN wild type model**

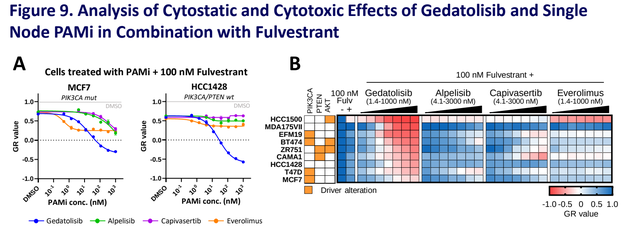
### Figure 8. *In Vitro* and *in Vivo* Efficacy of Gedatolisib + Fulvestrant




**(A)** A panel of ER+ BC cell lines was treated with low dose gedatolisib +/- 100 nM fulvestrant for 6 days and analyzed for cell viability. The data represent mean +/- SD (n=2) and are relative to DMSO-treated cells (set as 1). Statistical significance was calculated by two-tailed, unpaired t-test. **(B)** SCID female mice were xenografted with MCF7 cells in the mammary fat pad and treated with gedatolisib +/- fulvestrant for the indicated time. mut=mutant; wt=wild type.

- **Fulvestrant increased the growth-inhibitory effects of gedatolisib in ER+ BC cells *in vitro***
- **In the MCF7 xenograft model, gedatolisib induced robust tumor growth inhibition (TGI) (105%), which was increased by co-treatment with fulvestrant (123%, p<0.001)**

### Figure 9. Analysis of Cytostatic and Cytotoxic Effects of Gedatolisib and Single Node PAMi in Combination with Fulvestrant




A panel of ER+ BC cells with various PAM pathway mutational status was treated with escalating doses of PAMi +/- 100 nM fulvestrant for 6 days and analyzed by GR metrics. **(A)** Examples of PAMi DRCs in the presence of 100 nM fulvestrant. The data represent mean +/- SD (n=2) and are relative to DMSO-treated cells (set as 1). **(B)** Heatmaps summarizing DRCs for PAMi + 100 nM fulvestrant. mut=mutant; wt=wild type.

- **Gedatolisib + fulvestrant is more efficacious than single node PAMi + fulvestrant regardless of the PAM pathway mutational status**

## SUMMARY AND CONCLUSIONS

- The pan-PI3K/mTOR inhibitor, gedatolisib, exerted potent cytostatic and cytotoxic effects in BC cell lines regardless of PTEN, PI3K, or AKT mutational status.
- Gedatolisib exhibited superior potency and efficacy across BC cell lines with different PAM pathway mutational status *in vitro* relative to single node PAM inhibitors.
- Gedatolisib repressed PAM pathway activity and PAM-controlled cellular functions required for cancer cell proliferation (protein synthesis, DNA replication) more effectively than the single node PAM inhibitors.
- Gedatolisib was the only PAMi that induced significant tumor cell growth inhibition in both wild type and mutant PIK3CA/PTEN PDX models.
- Gedatolisib exerted greater cytotoxic effects than single node PAMi both as single drug and in combination with fulvestrant.
- Gedatolisib may effectively address potential drug resistance mechanisms associated with narrowly targeted PAMi.
- Gedatolisib in combination with HT has previously demonstrated promising preliminary clinical efficacy and safety data in patients with HR+/HER2- ABC who had received prior therapy with CDK4/6i. 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 while on treatment with a CDK4/6 inhibitor.

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