Dual mTOR Kinases MLN0128 Inhibitor Sensitizes HR+/HER2+ Breast Cancer Patient-derived Xenografts to Trastuzumab or Fulvestrant

SUPPLEMENTAL MATERIALS AND METHODS

Cell culture

As described previously (1), MCF-7aro/HER2 cancer cell line as described previously was maintained in the Minimum Essential Medium (MEM) supplemented with 2 mM L-Glutamine, 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, and 100 µg/mL G418. BT-474 cells were maintained in the Dulbecco's Modified Eagle Medium (DMEM) with 4 mM L-Glutamine, 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 1 mM sodium pyruvate. BT/HER0.2R cells were cultured in DMEM media with 30 µg/ml trastuzmab (2). In estrogen-driven assays, cells were changed to estrogenic deprivation conditions for 48 hr in prior to treatment. Estrogen, fulvestrant, and MLN0128 were purchased from Sigma Chemicals (St. Louis, MO, USA), Tocris (Ellisville, MO, USA), and Selleck Chemicals (Houston, TX, USA), respectively.

Western blot analysis

To examine the molecular changes driven by the drug of interest *in vivo*, three-day treatment scheme of administrating MLN0128 (1mg/kg/gavage daily) and/or fulvestrant (subcutaneous injection of 2 mg, 1 mg, and 1 mg, respectively, in three-consecutive days) was conducted and

tumors were collected 2 hours in posterior to the last treatment (3). Total protein extracted using RIRA buffer supplied with 100 mM PMSF (Cell Signaling Technology, Danvers MA) was quantified by BCA assay (Pierce, Rockford, IL) and then resolved on 10% SDS-PAGE, following transferred to PVDF membrane. Antibodies used in this study included ERα (sc-543) from Santa Cruz Biotechnology (Santa Cruz CA); AKT (#9272), p-AKT (Ser473; #9271), NDRG1 (#9272), p-NDRG1 (Thr346; #8947), 4E-BP1 (#9452), p-4E-BP1 (Thr37/46; #9459), and GAPDH (#2118) from Cell Signaling Technology; p-ERα (Ser118; #32396) and PDZK1 (ab92491) from Abcam. Antibody-protein complexes were detected using HRP-conjugated secondary antibodies (GE Healthcare) and ECL substrate (Bio-Rad, Hercules CA). Images were captured using ChemiDoc MP Imaging System (Bio-Rad). Image J (National Institutes of Health, Bethesda, MD) was used to quantify the expression levels of ERα protein.

RNA-seq and pathway analysis

Total RNA extracted from COH-SC1 (n=6), COH-SC31(n=3), and the reference (n=2) PDX tumors were subjected to RNA sequencing conducted by the Integrative Genomics Core at City of Hope. Briefly, 100 ng of total RNA per sample was subjected to library preparation using the KAPA Stranded mRNA-Seq Kit (KK8421; Kapa Biosystems) following the manufacturer's protocol with 12-cycle PCR amplification and validation by the Agilent Bioanalyzer High Sensitivity DNA Kit (Agilent). Clusters generated by the HiSeq SR Cluster V4 Kit (GD-401-4001; Illumina Inc. San Diego, CA) and sequencing run using the HiSeq SBS V4 Kit (FC-401-4002;

Illumina) were performed on the Illumina HiSeq 2500 platform with 51-bp per read.

Reads aligned to the Human Genome Assembly (NCBI build hg19) by TopHat2 were tabulated using HTSeq-count with UCSC known gene annotations (TxDb Hsapiens UCSC hg19.knownGene) and quantified separately to avoid discounting reads in overlapping genes with different names in the different reference sets (4-6). False discovery rate (FDR) was determined by the method of Benjamini and Hochberg and p-values by edgeR of raw counts (7, 8). To define the HR+/HER2+-specific gene signature, differential expression levels was calculated from Fragments Per Kilobase per Million reads (FPKM) normalized expression values with a scaling factor of 0.1 and FDR < 0.05 in relation to the reference dataset generated from two biological replications from an ER+/HER2- PDX established in our laboratory (indicated as REF in Figure 3A). Ingenuity Pathway Analysis (Ingenuity® Systems) was used to explore signaling pathways involved in driving HR+/HER2+ tumor growth at the systems-level. Log2(FPKM+0.1) values of differentially expressed loci were subjected to gene set enrichment analysis (GSEA) for MSigDB signatures (c2, c5, c6, and Hallmark) (9, 10).

In vitro drug administration

Cells (4,000 per well) were plated in 96-well plates and then subjected to drug treatment. Fulvestrant and MLN0128 dissolved in 100% dimethyl sulfoxide (DMSO) with escalating concentration were diluted with culture media and then added to the cells. DMSO was used as negative control. Media containing treatment components was refreshed after 3 days of culture.

Cell viability was assayed with MTS (Promega, Fitchburg, Wisconsin, USA) or MTT (Sigma, St. Louis) after 6 days when cell growth reached the exponential phase following the manufacturer's instructions.   Growth % = (OD at Day 6th of treatment sample)/(OD at Day 6th of DMSO treated sample) × 100 %.   IC50 was calculated by Calcusyn 2.1 software (Biosoft, Cambridge, UK).

Statistical analysis

Two-way ANOVA analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA) to compare differences among treatment and vehicle control groups.   Error bars represent the SEM.

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Table S1. Clinical characteristics of COH-SC1 and COH-SC31 PDXs

	COH-SC1	COH-SC31
Age at diagnosis	34	72
Race	Caucasian	Caucasian
Pathology	Invasive ductal carcinoma	Invasive ductal carcinoma
Stage	II	IV
Grade	2	3
Metastatic site	N/A	Chest wall
ER status	Positive	Positive
PR status	Negative	Positive
HER2 status	Positive	Positive
BRCA status	No mutation	No mutation
Systemic treatment history	- Grafted as COH-SC1 in 2013 - Chemotherapy (Taxotere and Carboplatin) plus trastuzumab in 2013 - Trastuzumab in 2013 - Tamoxifen in 2013 - Radiation in 2013-2014 - Trastuzumab in 2014 - Disease-free in Dec 2015	- Neoadjuvant Navelbine and trastuzumab in Sep 2018 - Trastuzumab in 2009 - Progression, Lapatinib and trastuzumab in 2012 - Nab-paclitaxel and trastuzumab in Feb 2014-Jun 2014 - Surgery in 2014; grafted as COH-SC31 - Progression of disease in the chest wall despite of Letrozole in Dec 2014 - Hospice and passed away in 2015