Early adaption and acquired resistance to CDK4/6 inhibition in ER-positive breast cancer

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Supplementary Materials and Methods

Antibodies and reagents

Antibodies used were phospho-AKT Ser473 (4058), phospho-AKT T308 (2965), AKT (4691), phospho-ERK1/2-Thr202/Tyr204 (4370), ERK1/2 (9102), phospho-pRb S807/811 (8516), phospho-pRb Ser 780 (9307), pRb (9313), cyclin D1 (2978), cyclin E2 (4132), cyclin E1 (4129), CDK2 (2546), CDK4 (12790), S6 (2317), phospho-S6 Ser235/6 (2111), phospho-S6 Ser240/4 (2215), PARP cleavage (5625 and 9441) (all Cell Signalling Technology, Danvers, MA), β-actin (A5441) and Tubulin (T-9026)(Sigma), CDK2 (sc163), Cyclin E1 (sc198), Human GAPDH (sc137179) (Santa Cruz Biotechnology), Cyclin E2 (ab40890) (AbCAM), Cyclin D1 (RM9104) (Thermo-Scientific). PD-0332991 (palbociclib) was provided by Pfizer. GDC-0941 and fulvestrant were purchased from Selleckchem. NVP-AEW541 from Cayman Chemicals. siRNA were from Dharmacon. Unless otherwise stated palbociclib was used at 500nM and GDC-0941 at 250nM.

Western Blotting

Cell lines were grown on 35mm plates, treated as indicated, and lysed in NP40 lysis buffer, (1% v/v NP40, 10mM Tris.Cl pH8, 150mM NaCl, 1mM EDTA, 1mM DTT) supplemented with phosphatase inhibitor cocktail (Cell Signalling Technology, 5872)

and protease inhibitor cocktail (Roche, 11697498001). Western blots were carried out with precast TA or Bis-Tris gels (Life Technologies).

Immunoprecipitation

Cells were treated as indicated and lysates prepared using Triton X-100 lysis buffer (0.1% Triton X-100, 50mM Tris-HCl pH 7.4, 150mM Na-Cl, 1mM EDTA) supplemented with phosphatase inhibitor cocktail and protease inhibitor cocktail. The antibodies used for immunoprecipitation (IP) were CDK2 (sc-163, Santa Cruz Biotechnology), cyclin D1 (2978) and CDK2 (2546) from Cell Signaling Technology. 500-1000mg of total cellular protein was incubated with primary antibodies and the protein complexes precipitated using Protein G coated Dynabeads (Life Technologies, 10001D). Non-specific control was performed with normal rabbit IgG (SC2027, Santa Cruz Biotechnologies). Secondary antibody was anti-rabbit IgG (ab131366) HRP conjugated.

In vitro cell line assessment of viability and proliferation

Clonogenic assays were conducted in 6-well plates, with 1000 to 3000 cells seeded per well, and 24 hours later cells were exposed to vehicle, or the indicated treatments, followed by growth in media for at least 2 weeks as indicated to allow colony growth. Treatments were replaced every 3-4 days. Then cells were fixed with ethanol and staining with sulforhodamine B.

For short-term survival assays, cells were exposed to the indicated compounds for 5 to 6 days with survival assessed with CellTiter-Glo®. To assess synergy cell lines were plated in 384-well plates, and the following day exposed to fixed-ratio combinations of indicated drugs for 5 days, with combination index assessed according to Chou and Talalay [26] using Calcusyn v2.1 (BIOSOFT, UK).

S phase fraction was assayed after 24 or 72 hours exposure to compounds, with the addition of 10 μM BrdU for the indicated times. BrdU incorporation was assessed

with Cell Proliferation chemiluminescent ELISA-BrdU assay (Roche 11 669 915 001) according to manufacturer's instructions and adjusted for viable cells in parallel wells assessed with CellTiter-Glo®.

To assess the effect of siRNA on drug sensitivity cells were reverse transfected at final siRNA concentration 20nM, at 24 hours post transfection plates were exposed to compound for the indicated time.

Exome sequencing analysis

Samples were prepared from 1-5 μg DNA according to Agilent's SureSelect Protocol Version 1.2 with Agilent SureSelect Human All Exon V5. Sequencing was performed on the Illumina HiSeq2000 platform using TruSeq v3 chemistry at Oxford Gene Technology (OGT) to a median depth of 100X. Reads were aligned to the human reference genome (GRCh37) using BWA (v0.7.5a) [19]. PCR duplicates were filtered out from the subsequent analysis using Picard-tools (v1.94) and variants were called using the GATK pipeline (v2.3.9) best practices [20]. Somatic changes between parental and resistant cell lines were further investigated using MuTect (v1.1.4) [21]. Mutations were annotated using Annovar [6]. Other analysis was performed using bedtools (v2.17) and samtools (v0.1.19) [22]. Copy number alterations were analysed using ASCAT [23] after adaptation of read depth and b allele frequency data.

Apoptosis assessment

Caspase 3/7 activity was tested using Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, G7790) according to manufacturer's instructions and results were adjusted for viable cells in parallel wells with Cell Titre-Glo®. Assessment of PARP cleavage in western blots was with cleaved PARP antibody.

Tumor xenograft experiments

Experiments were conducted following the European Union's animal care directive (86/609/EEC) and were approved by the Ethical Committee of Animal Experimentation of the Vall d'Hebron Research Institute and patient consent. Fresh primary or metastatic human breast tumors were obtained from patients at time of surgery or biopsy. Fragments of 30 to 60 mm^3 were immediately implanted into the mammary fat pad (surgery samples) or the lower flank (metastatic samples) of female athymic NMRI nu/nu mice. Mice were continuously treated with 17 beta-Estradiol (Sigma-Aldrich) in the drinking water at 1μM. Upon growth of the engrafted tumors, the model was perpetuated by serial transplantation. In each passage, flash-frozen and formalin-fixed paraffin embedded samples were taken for genotyping and histological studies.

To evaluate the sensitivity to the drugs one tumor of each PDX model was expanded to 6-24 recipient mice. When the tumors reached an average volume of 200 mm^3, mice were separated into four/five treatment arms: vehicle (0.5% methylcellulose in PBS), BYL719 (35mg/kg QD, 6 days/week), LEE011 (75mg/kg QD, 6 days/week) and BYL719 plus LEE011. These doses are comparable to 75% of the human maximum tolerated dose. The treatment was prolonged for at least 40 days. In acquired resistance experiments performed with PDX244 (PDX244LR1), treatments were prolonged up to 100 days with the same dosing regimen. At relapse (tumor volume >800mm^3), one acquired-resistance LEE011-treated tumor was excised and tumor pieces were implanted in 8 recipient nude mice. Like in the previous experiments, when the tumors reached 200 mm^3, mice were separated into two different groups (vehicle- and LEE011-treated) and the treatments followed as described.

In all experiments, mouse weight and tumor dimensions were recorded twice weekly with a digital caliper starting with the first day of treatment. The tumor volume was calculated as $V = 4\pi/3(Lxl^2)$, “L” being the largest diameter and “l” the smallest.

PDX protein isolation and western-blotting

Flash-frozen pieces of tumor were lysed and WB were performed as described in: Shunqiang L, et al. Endocrine-Therapy-Resistant *ESR1* variants revealed by genomic characterization of breast-cancer derivated xenografts. Cell Reports, 2013

PDX sequencing

Tumor samples of one vehicle-treated tumor of each PDX model were pre-screened at VHIO for adequacy as defined by an initial requirement of paraffin-embedded (FFPE) cancer tissue block with greater than 20% tumor content. Tissue was sent to a Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP)-accredited laboratory (Foundation Medicine, Cambridge, MA) for NGS. NGS was performed as described elsewhere [27] [28].