

Supplementary Information

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Detailed Methods

Reagents and antibodies PD168393 was synthesized as previously described²⁹. Commercially available gefitinib and erlotinib were purified for *in vitro* use. AG825 and LY294002 were purchased from Calbiochem. Stocks of drugs were maintained in DMSO. Monensin sodium salt, Tempol (4-Hydroxy-TEMPO), DL- α -lipoic acid and streptavidin agarose CL-4B were purchased from Sigma-Aldrich. H₂DCFDA was from Molecular Probes. EZ-link Sulfo-NHS-SS-Biotin was from Pierce. pcDNA3-myr-Akt plasmid was a kind gift from David Stokoe. Anti- p-Ser473-Akt, p-Tyr202/Tyr204-MAPK, p-Thr183/Tyr185-JNK, p-Ser65-4E-BP1, p-Tyr1289-HER3, p-Tyr1068-EGFR, Akt, MAPK and JNK were from Cell Signaling. Anti-EGFR antibodies were from BD Transduction Laboratories. Anti-HER2, HER3, 4E-BP1 and anti-phosphotyrosine PY-99 antibodies were from Santa Cruz. Anti-rabbit AlexaFluor488-conjugated highly cross absorbed goat IgG antibodies were from Molecular Probes.

siRNA reagents were purchased from Dharmacon. These include pre-designed siGENOME SMARTpool siRNA reagents for HER2 and for HER3, and a control siGENOME non-targeting siRNA pool, ON-TARGETplus smartpool reagents for HER2 and for HER3, and the ON-TARGETplus siCONTROL non-targeting reagent. Specifically the reagents used were anti-HER2 siGENOME or control siGENOME SMARTpool siRNA (figure 2A and supplementary figure 3A), anti-HER2 ON-TARGETplus smartpool or ON-TARGETplus siCONTROL siRNA (supplementary figure 3B), anti-HER3 ON-TARGETplus smartpool or ON-TARGETplus siCONTROL siRNA (figure 2F,G, and supplementary figure 4A), and anti-HER3 siGENOME smartpool or control siGENOME smartpool siRNA (supplementary figure 4B). The siRNA pools contained the following sequences:

M-003126-01, ERBB2, NM_004448

D-003126-05; GGACGAAUUCUGCACAAUG

D-003126-06; GACGAAUUCUGCACAAUGG

D-003126-07; CUACAACACAGACACGUUU

D-003126-08; AGACGAAGCAUACGUGAUG

L-003126-00, ERBB2, NM_004448

J-003126-17; UGGAAGAGAUCACAGGUUA

J-003126-18; GAGACCCGCUGAACAAUAC

J-003126-19; GGAGGAAUGCCGAGUACUG

J-003126-20; GCUCAUCGCUCACAACCAA

M-003127-02, ERBB3, NM_001982

D-003127-05 AAGAGGAUGUCAACGGUUA

D-003127-06 GAAGACUGCCAGACAUUGA

D-003127-08 GCAGUGGAUUCGAGAAGUG

D-003127-09 GGACCGAGAUGCUGAGAUUA

L-003127-00, ERBB3, NM_001982

J-003127-10 GCGAUGCUGAGAACCAAUA

J-003127-11 AGAUUGUGCUCACGGGACA

J-003127-12 GCAGUGGAUUCGAGAAGUG

J-003127-13 UCGUCAUGUUGAACUAUAA

Cell culture and blotting. Tumor cells were obtained from the American Type Culture Collection (ATCC) and maintained in 1:1 mixture of DME:F12 media supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 4mM glutamine, and 10% heat inactivated fetal bovine serum and incubated at 37 C in 5%CO₂. For western blotting, cells were harvested in modified RIPA buffer and 50 ug of total cellular lysate separated by SDS-PAGE, transferred to membrane, and immunoblotted using the indicated primary antibodies, appropriate secondary antibodies, and visualized by enhanced chemo luminescence (ECL). Phospho-immunoblots in figure 1 were done by anti-EGFR, anti-HER2, and anti-HER3 immunoprecipitation followed by anti-phosphotyrosine immunoblotting. Other immunoblots were done by phospho-specific immunoblotting.

Animal studies. NCR nude mice were purchased from Taconic. For BT474 tumor growth, mice were preimplanted with estrogen pellets. Mice were injected subcutaneously with 20 million

tumor cells in matrigel, monitored daily, and allowed to grow till approximately 200-300 mm³ in size. The animals were treated with gefitinib 150 mg/kg once daily by oral gavage. At the indicated timepoints, animals were sacrificed and tumors rapidly removed and snap frozen. Frozen tumors were crushed and lysates prepared for western blotting.

Apoptosis. Cells were seeded at 300,000-500,000 per well in 12-well or 6-well clusters.

Apoptotic cells were identified and quantified by analysis of Annexin V binding or by their sub-G1 DNA content. Annexin V binding was assayed using the Annexin V-FITC Apoptosis Detection Kit (Calbiochem) according to the manufacturer's instructions, and quantified by FACS analysis. In other experiments, subG1 DNA content was quantitated as a measure of apoptosis. Briefly cell nuclei were prepared and labeled as previously described³⁰, DNA content was quantified by FACS analysis, and apoptotic cells identified by their subG1 DNA content and quantified. All experimental arms were done in duplicate and displayed as averages with standard of deviation error bars.

Transfections. Cells were seeded at a density of 300,000 cells per well in 12-well plates and transfected the following day. For siRNA transfections 100-300nmol of siRNA (Dharmacon) was premixed with Lipofectemine2000 in Opti-MEM media and then added to each well for 5h. For plasmid transfections, 2 ug of plasmid DNA was premixed with Lipofectamine2000 in Opti-MEM media and added to wells for 6 hours.

Cell surface biotinylation Cells were chilled on ice and rinsed twice with ice-cold PBS. The freshly prepared cell impermeable reagent sulfo-NHS-SS-biotin was added to the final concentration of 0.5 mg/ml in PBS. Following 45min incubation at 4°C cells were lysed in RIPA buffer. 300 ug of cell lysate was precipitated with streptavidin-agarose beads (Sigma), boiled in sample buffer, separated by SDS-PAGE, and immunoblotted as indicated.

Immunofluorescence studies. Cells were seeded on fibronectin coated cover slips and allowed to enter log phase. After the indicated period of drug treatment, cells were fixed in 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton-X in PBS, blocked in 2% goat serum in PBS, and stained with the indicated primary antibodies in blocking buffer. Cells were then stained with FITC conjugated secondary antibodies, washed and mounted on glass slides and visualized using a Zeiss Axioplan 2 fluorescence imaging microscope .

Reactive Oxidation Species Assay

Cells were rinsed twice with PBS and incubated with 10 μ M of freshly prepared H₂DCFDA in phenol-red free media for 45 min at 37°C. Gefitinib treatment was continued during H₂DCFDA labeling. Cells were then trypsinized and ROS levels were detected by flow cytometry. An increase in ROS levels corresponds to the increase in fluorescence as seen as a right shift of the curve.

Supplementary Figure legends

Figure 1

HER TK inhibitors fail to induce sustained inhibition of HER3 signaling in HER2-driven breast cancer cells *in vitro* (A) Au565 (B) MDA-453 (C) MDA-361 and (D) HCC1954 cells grown *in vitro* were treated with 5 μ M gefitinib for the indicated times. The drug was refreshed at the 71 hour time point sample 1h before collection. Phospho- and total protein levels were detected by immunoblotting with the appropriate antibodies.

Figure 2

HER TK inhibitors fail to induce sustained inhibition of HER3 signaling in HER2-driven breast cancer cells *in vivo* (A) BT474 and (B) MDA-361 xenografts were grown in nude mice. Gefitinib was administered daily at 150 mg/kg (BT474 experiment) or 300 mg/kg/day (MDA-361 experiment). Animals were sacrificed at the indicated timepoints after initiation of therapy, tumor rapidly harvested and lysates immunoblotted with the antibodies as indicated.

Figure 3

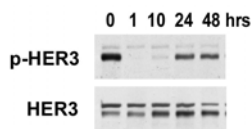
HER2 knockdown enables durable TKI suppression of HER3 signaling. SkBr3 cells were left untransfected, or were transfected with anti-HER2 siRNA pool (indicated by H) or control siRNA pool (indicated by C) and subsequently treated with 5 μ M gefitinib or control for the indicated times. In figure 3A cells were transfected with anti-HER2 siGENOME SMARTpool and control SMARTpool siRNA reagents. In figure 3B cells were transfected with anti-HER2 ON-TARGETplus smartpool siRNA and ON-TARGETplus siCONTROL non-targeting reagents. The two smartpools consist of different siRNA sequences.

Figure 4

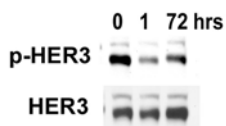
Addition of HER3 siRNA to HER TKI induces apoptotic cell death. Cells were either left untransfected or transfected with anti-HER3 siRNA pool or control siRNA pool and subsequently treated with 5uM gefitinib or control. After 48 hours of drug treatment apoptotic cells were identified and quantified as described in methods. Figure 4A shows BT474 cells transfected with anti-HER3 ON-TARGETplus smartpool siRNA or ON-TARGETplus smartpool siCONTROL non-targetting siRNA. Figure 4B shows SkBr3 cells transfected with anti-HER3 siGENOME smartpool or control non-targetting smartpool siRNA. The two HER3 smartpools consist of different siRNA sequences.

Supplementary figures

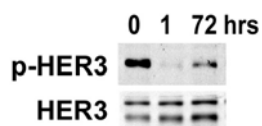
1A



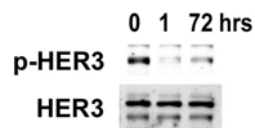
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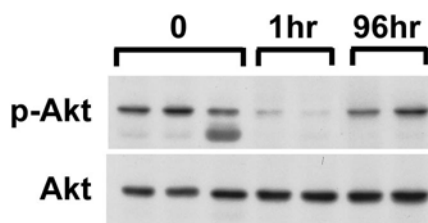
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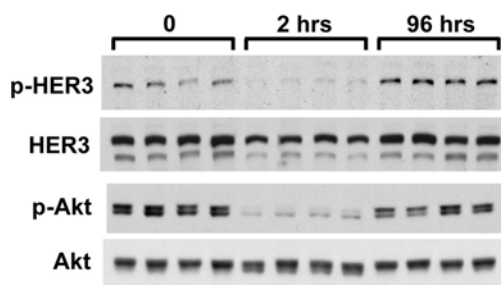
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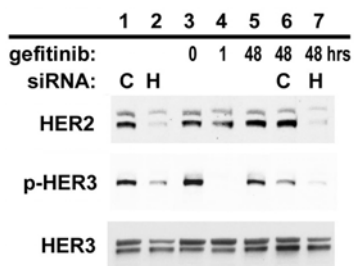
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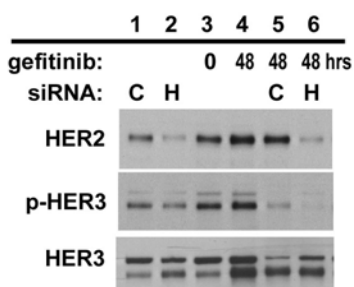
2B



3A

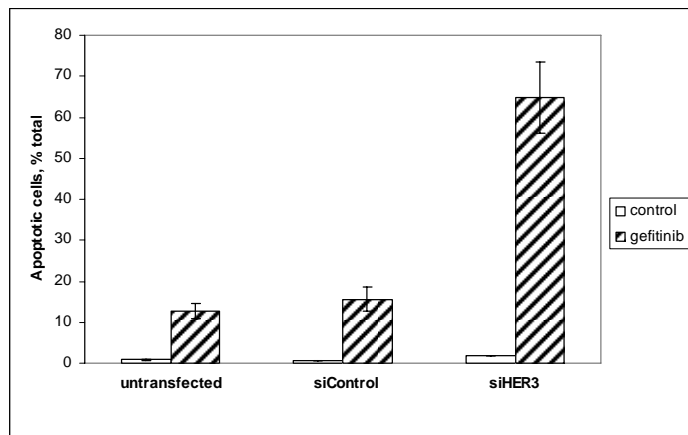


3B



Supplementary figures

4A



4B

