Supporting Information

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SI Materials and Methods

Cell Lines. All cell lines were purchased from the American Type Culture Collection. Media and FBS were purchased from Invitrogen. The following growth media were used: for HCC1937 and HCC1954, RPMI-1640/10% FBS; for BT474, IMEM/10% FBS; for SKBR3, McCoy 5A/15% FBS; for UACC893, DMEM/10% FBS; for MDA453, DMEM/F12 (1:1)/20% FBS; and for SUM190, DMEM/F12 (1:1)/5% FBS. All cells were grown in a humidified 5% CO_2 incubator at 37 °C.

Cell Cycle Analysis. Cells were plated in 100-mm dishes in media containing 2.5% FBS with or without XL147. After 3 d, detached and adherent cells were pooled, fixed, and labeled with propidium iodide by using the APO-BrdU kit (Phoenix Flow Systems). Labeled cells were analyzed using the Becton Dickinson FACSCalibur system.

Xenograft Experiments. Animal experiments were approved by the institutional animal care committee of VUMC. Mice were housed in the accredited Animal Care Facility of the VUMC. A 17β-estradiol pellet (Innovative Research of America) was injected s.c. into each 6- to 7-wk-old athymic female mouse (Sprague–Dawley; Harlan) the day before tumor cell injection. BT474 cells (3×10^6) mixed 1:1 with Matrigel (BD Biosciences) were injected s.c. into the right flank of each mouse. Tumor diameters were measured with calipers twice per week and volume in mm^3 calculated as $width^2 \times length / 2$. When tumors had reached at least 200 mm^3, treatment was initiated with the following, either alone or in combination: trastuzumab 30 mg/kg twice per week i.p., lapatinib 100 mg/kg daily via orogastric gavage, and XL147 100 mg/kg daily via orogastric gavage. Mice were killed after 28 d of treatment.

Immunohistochemistry. All tumor samples (collected within 1 h of last treatment) were fixed in 10% neutral-buffered formalin for 24 h at room temperature, followed by dehydration and paraffin embedding. Immunostaining was done on 5-μm tissue sections. After deparaffinization in xylene and graded alcohols, heat-induced antigen retrieval was performed in a citrate buffer, pH 6.0, followed by incubation with 3% hydrogen peroxide for 20 min, protein block (Dako) for 10 min, and finally an overnight incubation with the primary antibody at 4 °C. The Envision Visualization System (Dako) was used followed by 3,3' diaminobenzidine as chromogen and hematoxylin as counterstain. Tumor sections were studied on a light microscope with an ocular magnification of 400× . Average percentage and intensity of tumor cell staining was calculated as a histoscore as described previously (1). An expert breast pathologist (M.G.K.) blinded to the type of treatment scored the sections.

Real-Time PCR Primers. Sequences for human HER3 primers are as follows: HER3F, GGGGAGTCTTGCCAGGAG; and HER3R, CATTGGGTGTAGAGAGACTGGAC. Primers for human actin, IGF1R, INSR, EPHA1, FGFR2, FGFR3, FoxO1, and FoxO3a were obtained from SABiosciences.

1. Goulding H, et al. (1995) A new immunohistochemical antibody for the assessment of estrogen receptor status on routine formalin-fixed tissue samples. *Hum Pathol* 26: 291–294.

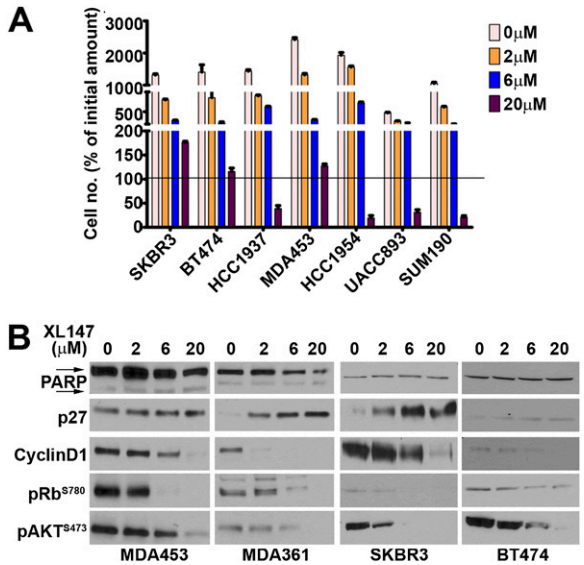

Fig. S1. Treatment with XL147 induces cell cycle arrest. (*A*) Percent of cell number relative to the initial amount of plated cells was calculated for each breast cancer cell line treated with the indicated concentrations of XL147 (Fig. 1*A*). Numbers below 100% (straight black line) are indicative of apoptosis. (*B*) Cells were treated with 0 to 20 μM XL147 for 24 h. Cell lysates were prepared and subjected to immunoblot with the antibodies indicated to the left. Upper arrow indicates total PARP and lower arrow indicates cleaved PARP.

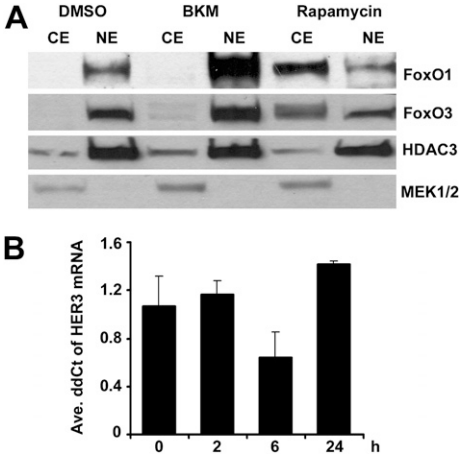

Fig. S2. Rapamycin does not induce FoxO-dependent upregulation of HER3 mRNA. (*A*) BT474 cells were treated with DMSO or 1 μM BKM120 or 55 nM rapamycin for 2.5 h. Cytosolic and nuclear extracts (CE and NE, respectively) were prepared and subjected to IB with the indicated antibodies. HDAC3, nuclear loading control; MEK1/2, cytosolic loading control. (*B*) Time-course experiment with BT474 cells treated with 55 nM rapamycin. Cells were harvested at indicated time points and subjected to qPCR analysis with primers specific for HER3. Results were normalized to actin expression and expressed as average ddCt ± SE of triplicates.

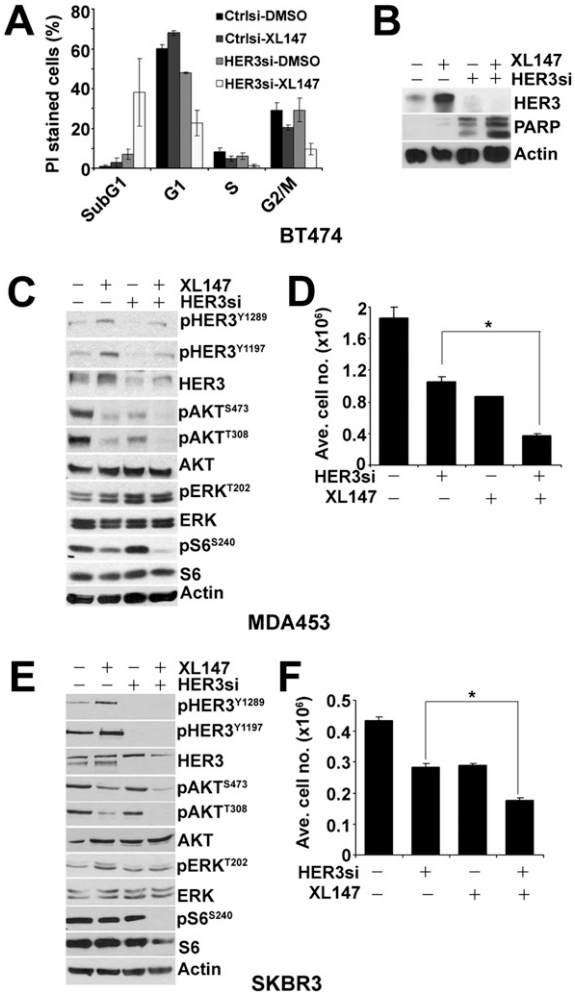

Fig. S3. HER3 knockdown and XL147 synergize against HER2-overexpressing cells. (*A* and *B*) BT474 cells were transfected with HER3 siRNA or control duplexes and then treated with 6 μM XL147 for 3 d. At this time, cells were washed, harvested, and subjected to cell cycle analysis (*A*) and IB for HER3 and PARP (*B*) as indicated in *Materials and Methods*. Each bar represents the mean ± SD of duplicate wells (*A*). *(C–F)* MDA453 and SKBR3 cells were transfected with HER3 siRNA and subjected to treatment with 6 mM XL147 (as in Fig. 4). After 24 h, cells were lysed and subjected to IB with the indicated antibodies (*C* and *E*). In other cases, cells were allowed to grow while replenishing fresh medium and XL147 every 3 d, and counted on day 10 (*D* and *F*). Each bar represents the mean ± SE of six replicates (*$P < 0.05$, paired *t* test).

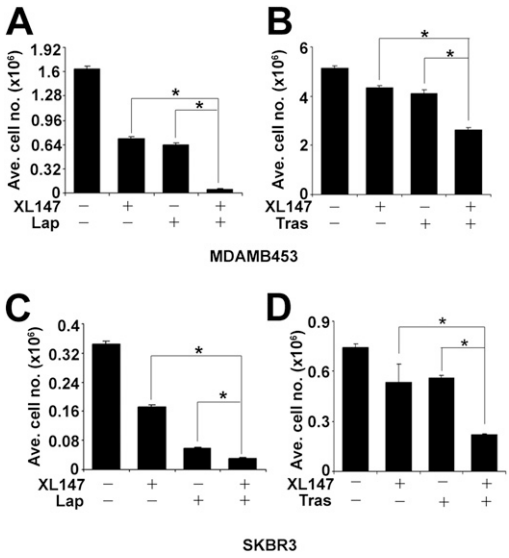

Fig. S4. XL147 and HER2 inhibitors synergized against HER2-dependent cells. MDA453 (*A*, *B*) and SKBR3 (*C*, *D*) cells were plated as indicated in *Materials and Methods* and then treated with 0.1 μM lapatinib, 10 μg/mL trastuzumab, or 2 μM XL147 alone or in the indicated combinations for a total of 6 d. Fresh medium and drugs were replenished every 3 d. Each bar represents the mean ± SE of six replicates (*$P < 0.05$, paired *t* test).

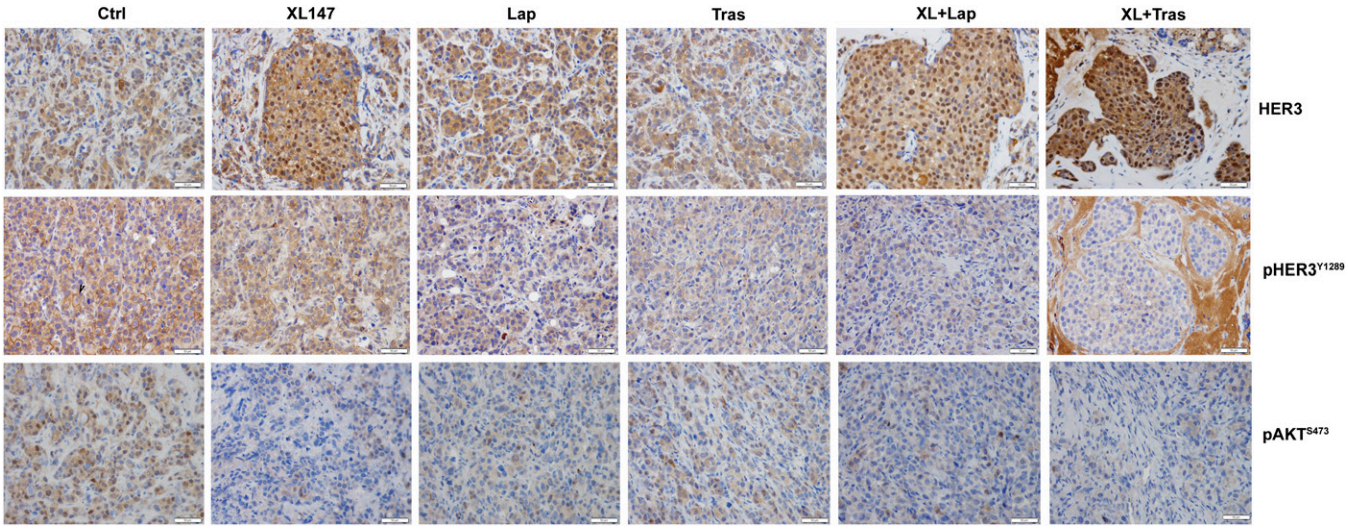

Fig. S5. Combined inhibition of HER2 and PI3K is synergistic in vivo. Nude mice were transplanted with BT474 cells. When tumors had reached at least 200 mm^3, mice were treated with vehicle (Ctrl), XL147, lapatinib, trastuzumab, or the indicated drug combinations for 28 d. IHC of sections from formalin-fixed, paraffin-embedded tumor blocks hybridized with indicated antibodies (Scale bar: 50 μm.).

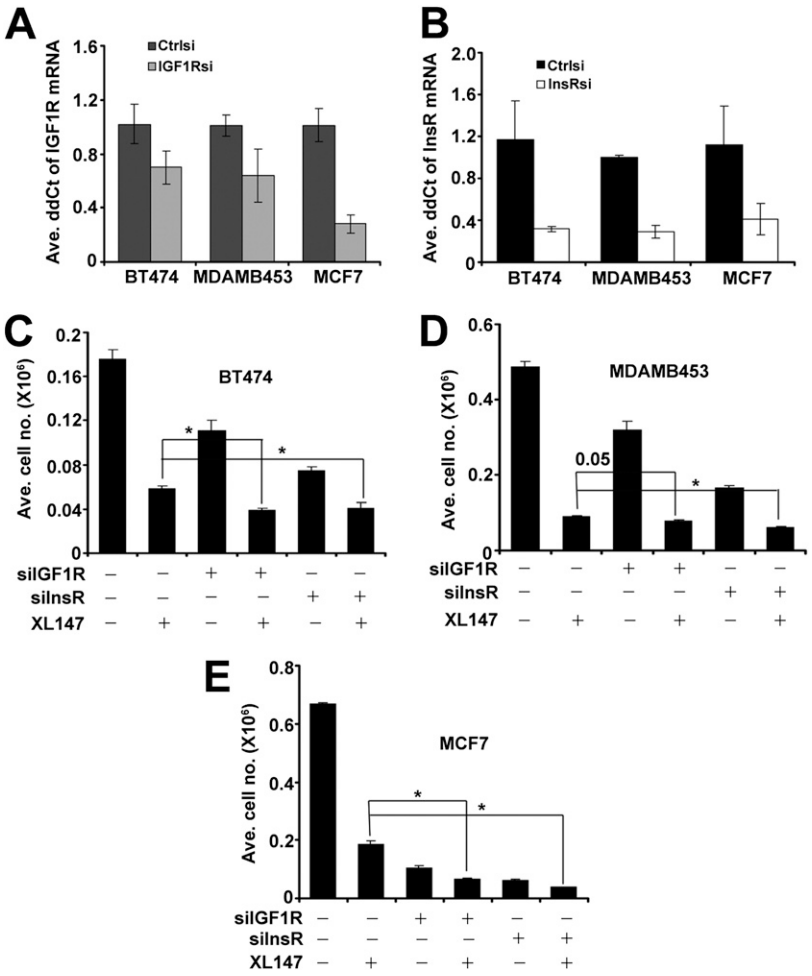

Fig. S6. RNAi-mediated knockdown of IGF1R or InsR sensitizes cancer cells to XL147. (*A* and *B*) BT474, MDA453, and MCF7 cells were transfected with IGF1R (*A*) or InsR (*B*) siRNA or control duplexes. Forty-eight hours later, RNA was isolated and subjected to qPCR analysis for IGF1R (*A*) and InsR (*B*) mRNA levels. Each bar represents the mean ± SE of triplicate wells. (*C*–*E*) BT474 (*C*), MDA453 (*D*), and MCF7 (*E*) cells were transfected as in *A* and *B*. Forty-eight hours later, they were treated with 10 μM XL147 for a total of 4 d. At this time, cells were trypsinized and counted by using a Coulter counter. Each bar represents the mean ± SE of triplicate wells (*$P < 0.05$, paired *t* test).

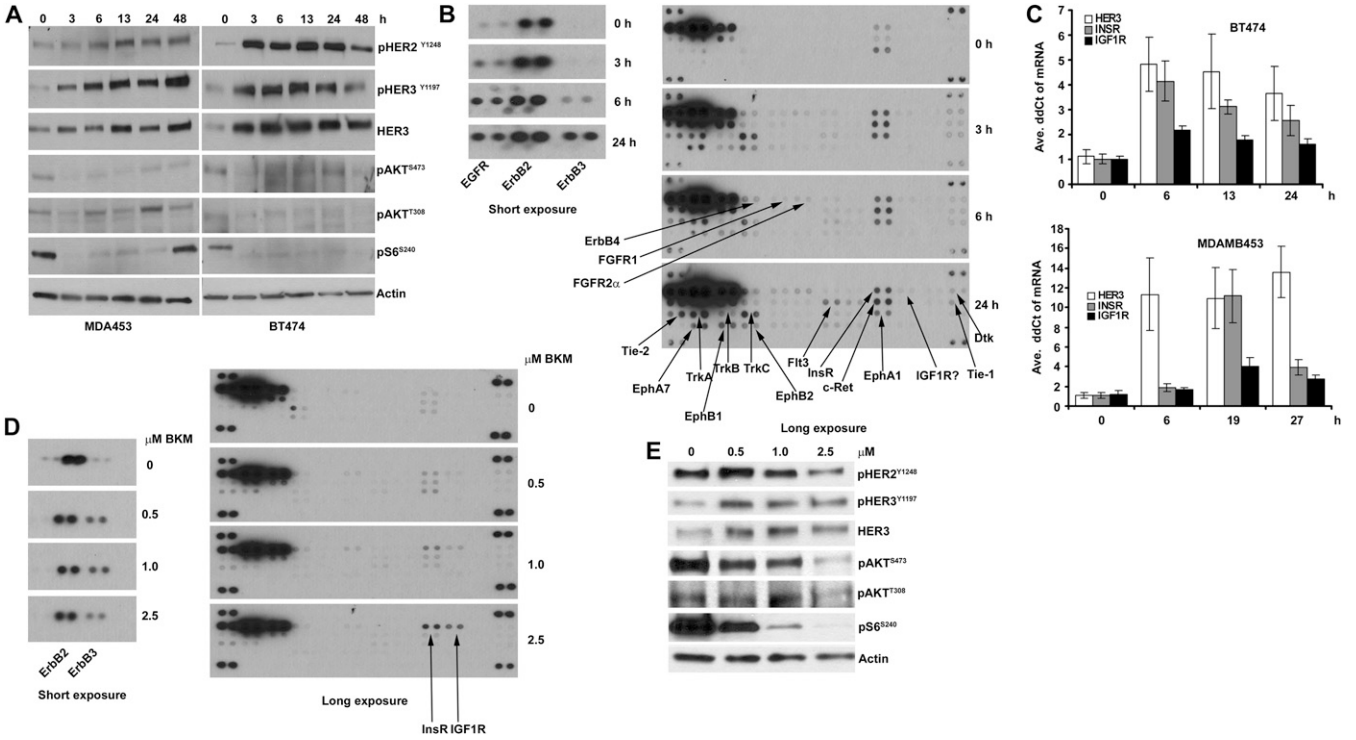

Fig. S7. BKM120-mediated inhibition causes compensatory up-regulation of RTKs in HER2-overexpressing cells. (*A*–*C*) Time-course experiment with BKM120. (*A*) Immunoblot with BT474 and MDA453 cells treated with 1 μM BKM120 for 0, 3, 6, 13, 24, and 48 h. (*B*) BT474 cells were treated with 1 μM BKM120 for as long as 24 h as indicated. Cell lysates were prepared and 0.5 mg of total protein was applied to pRTK arrays. (*C*) BT474 and MDA453 cells were treated with 1 μM BKM120 for indicated times. RNA was isolated and subjected to qPCR with primers specific for HER3, InsR, and IGF-1R. Results were normalized to actin expression and expressed as average ΔΔCt (ddCt) ± SE of triplicates. (*D* and *E*) Dose–response experiment with BT474 cells treated with BKM120. Cells were treated with 0, 0.5, 1, and 2.5 μM BKM120 for 24 h; at this time, cells were harvested and cell extracts were prepared for pRTK arrays (*D*) or immunoblot (*E*). Arrows indicate RTKs whose phosphorylation were up-regulated upon treatment with BKM120 (*B* and *D*).