**Fox et al. Additional file 2**

Autocrine IGF-I/Insulin receptor axis compensates for inhibition of AKT in ER-positive breast cancer cells with acquired resistance to estrogen deprivation

**Supplementary figures S1-S9**

**Figure S1**

<table>
<thead>
<tr>
<th>MCF-7</th>
<th>ZR75-1</th>
<th>HCC-1428</th>
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<tr>
<td>0</td>
<td>1</td>
<td>2</td>
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<tr>
<td>P-AKT&lt;sub&gt;6475&lt;/sub&gt;</td>
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<tr>
<td>P-PRAS40</td>
<td>P-GSK-3</td>
<td>P-S6</td>
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Figure S1. Catalytic AKT inhibitor AZD5363 reduces phosphorylation of AKT/TORC1 substrates in ER+ breast cancer cells. Cells were treated with 10% FBS ± 0-20 µM AZD5363 for 24 h. Protein lysates were analyzed by immunoblot using the indicated antibodies.
Figure S2. Inhibition of AKT with AZD5363 prevents the emergence of hormone-independent ER+ breast cancer cells. Parental cells in 10% DCC-FBS were treated with 0.4 µM AZD5363 or 1 µM selumetinib. Media and inhibitors were replenished every 3 days. When control wells reached 60-80% cell confluence [after 15 (MCF-7), 30 (ZR75-1), 25 (MDA-361) or 39 (HCC-1428) days, respectively], cells were fixed and stained with crystal violet. Representative images and quantification of integrated intensity (% control) are shown (*p<0.05 vs. control, t-test).
Figure S3. Inhibition of PI3K with BKM120 upregulates ER expression and activity. ER+ MCF-7, T47D, and MDA-361 breast cancer cells were treated with medium containing 10% FBS +/- 1 µM BKM120 for the indicated time points. MCF-7 and T47D cells were also selected for growth in the presence of 1 µM BKM120 for approximately 8 weeks. Protein lysates were prepared, separated by SDS-PAGE, and analyzed by immunoblot with the indicated antibodies.
Figure S4. Treatment with AZD5363 and fulvestrant synergistically inhibits proliferation in vivo. MCF-7 cells were injected s.c. into athymic mice supplemented with 14-day release 17β-estradiol pellets. Mice bearing tumors ≥150 mm³ were randomized to vehicle, AZD5363 (150 mg/kg/day bid p.o.), fulvestrant (5 mg/wk i.p.), or both drugs for six weeks. Quantification of Ki67+ tumor cell nuclei by IHC is shown (*p<0.001 vs. vehicle; # p<0.05 vs. AZD or fulv, t-test).
Figure S5. AKT inhibition suppresses the growth of HBCx-3 ER+ luminal B breast cancer xenografts. A) Patient derived xenografts (HBCx-3) were transplanted into nude mice as described in Methods. Representative images from IHC for PTEN, ER, HER2, and HER3 are shown. B) Mice bearing tumors 60-200 mm$^3$ were randomized to treatment with vehicle, AZD5363 (150 mg/kg bid p.o.), or tamoxifen (4 mg/kg x3 each week p.o.), for 21 days. Data are presented as mean tumor volume ± SEM. C) Mice bearing HBCx-3 xenografts 60-200 mm$^3$ were randomized to treatment with vehicle, AZD5363 (100 mg/kg bid p.o.), or fulvestrant (150 mg/kg x3 each week i.p.), for 18 days. Data are presented as mean tumor volume ± SEM. D) Xenografts from C) were homogenized and tumor lysates were analyzed by immunoblot. Quantitation of immunoblots are presented as ER expression percent of control ± SEM (n=5).
Figure S6. Src inhibitor dasatinib suppresses AZD5363-induced upregulation of HER3 phosphorylation and enhances its growth inhibitory effects. A) 2.5x10^5 MCF-7 or MCF-7/LTED cells were plated in 6-well plates. Cells in 10% DCC-FBS were pre-treated for 1 h with 1 µM AZD0530 or 1 µM dasatinib, followed by 24 h ± 2 µM AZD5363. Protein lysates were analyzed by immunoblot using the indicated antibodies. B) LTED cells were treated with 10% DCC-FBS ± 2 µM AZD5363, 1 µM AZD0530 or 1 µM dasatinib. Media and drugs were replenished every 3 days. Cells were counted after 5 days. Data are presented as percent of control; each bar, mean ± SEM (n=3; *p<0.0001 vs. Con, #p<0.05 vs. AZD or Das, one-way ANOVA). C) MCF-7/LTED cells were treated with 10% DCC-FBS ± 1 µM AEW541 or BKM120 for 1 h, followed by addition of 2 µM AZD5363 for 24 h. Protein lysates were analyzed by immunoblot with the indicated antibodies.
Figure S7. Inhibition of AKT is followed by phosphorylation of multiple RTKs. LTED cells in 10% DCC-FBS were treated ± 2 µM AZD5363 for 0, 3, 6, and 24 hr. Cell lysates were prepared and analyzed by phospho-RTK arrays as described in Methods. A darker exposure is shown here.
Figure S8. Inhibition of AKT with AZD5363 upregulates IGF-I and IGF-II protein levels. 3x10^5 (MCF-7) or 5x10^5 (ZR75-1, MDA-361, HCC-1428) LTED cells were plated in each well of a 6-well plate and treated with 1.5 ml of 10% DCC-FBS ± 2 µM AZD5363 for 24 or 48 h. Cell culture supernatants were collected and ELISAs for IGF-I (A) or IGF-II (B) were performed as described in the Methods. Concentrations in pg/ml were calculated using a standard curve. Recombinant IGF-I (4 or 6 ng/ml) or IGF-II (4 or 6 ng/ml) were used as controls (data not shown). The standard curve for the IGF-I kit ranged from 0-6 ng/ml, and the standard curve for the IGF-II kit ranged from 0-4 ng/ml. The experimental concentrations were within the range of the standard curve.
Figure S9. Mice exhibit minimal weight loss when treated with pharmacological inhibitors. MCF-7 cells were injected s.c. into athymic mice supplemented with 14-day release 17β-estradiol pellets. Mice bearing tumors ≥150 mm³ were randomized to vehicle, AZD5363 (100 mg/kg/day bid p.o.), AZD9362 (25 mg/kg/day p.o.), AZD5363 + AZD9362, AZD4547 (12.5 mg/kg/day p.o.), or AZD5363 + AZD4547 for six weeks. Data are presented as the average percent change in weight on week 1-6 for each treatment group.