SUPPLEMENTAL METHODS

Cell Binding Studies

Studies were performed in triplicate using a fixed concentration of either cetuximab or HER3 Ab105 and increasing concentration of corresponding PET probe on MDA-MB-468 cells with slight modification of previous studies. Briefly, increasing concentrations (0 to 100 nmol/L) of radiolabeled probes were incubated with 1 X 10^5 cells in 24-well plates at 4°C for 1 h. Unbound radioactivity was removed and the wells were rinsed three times with ice-cold PBS and detached with trypsin. The number of cells in each well was counted using an automated cell counter (Countess®, Invitrogen, Carlsbad, CA) and the total cell-bound radioactivity was measured in a gamma counter (Wizard 2480, Perkin Elmer, MA). The assay was repeated in the presence of 16 μmol/L of unlabeled cetuximab or HER3 Ab105 to measure non-specific binding at 4°C for 1h. Specific binding was calculated by subtracting non-specific binding from total binding and was plotted vs. the concentration of radiolabeled probe added. The resulting curve was fitted by non-linear regression to a one-site receptor-binding model by Prism Ver. 4.0 software (GraphPad, San Diego, CA). The dissociation constant and maximum number of receptors per cell was calculated.

Additionally, internalization dynamics of the EGFR PET probe was measured by comparing cell binding and uptake with both live and fixed cells at multiple time points. The MDA-MB-468 cell line was seeded in a 96-well pate (6x10^4 cells/well) and grown to 80% confluence. EGFR PET Probe was labeled with ^{64}Cu as described above and diluted to 1x10^6 counts per minute/mL in growth media with 1% (w:v) bovine serum

albumin. Prior to addition of EGFR PET probe, cells were washed with 100 μL PBS only, or were fixed with 4% paraformaldehyde. Following washing or washing and fixation, 100 μL of EGFR PET Probe was added to each well and incubated for either 1, 2, or 4h at either 4 or 37°C. Wells were then washed three times with PBS and total bound radioactivity extracted by incubation with 1% sodium hydroxide for 10 min. Total radioactivity for each sample was measured by gamma counter.

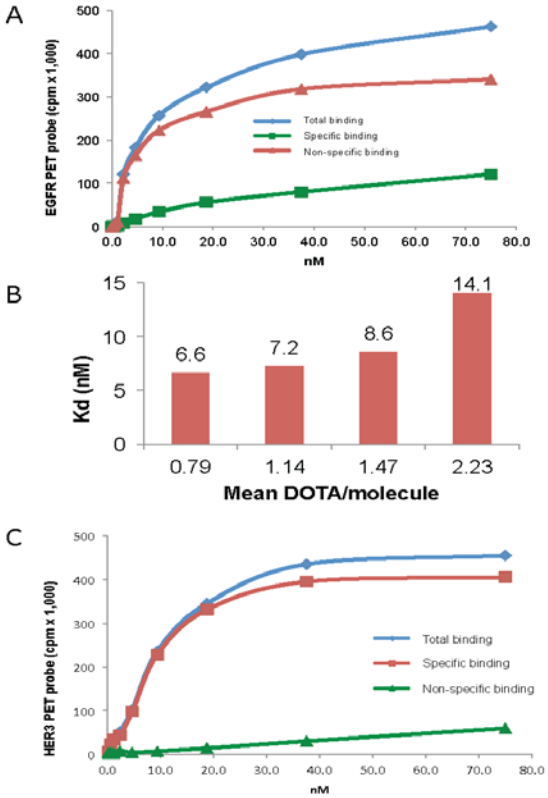
Surface Western

BT-474 and HCC-70 cells were grown to 80% confluence in 175 cm^2 flasks. Intact cells were labeled with biotin at 4ºC using a Pierce Surface Protein Isolation Kit (Pierce Biotechnology, Rockford, IL) according to manufacturer's protocol. Following biotinylation, cells were lysed and then incubated with Neutravidin Agarose beads. Surface fraction (agarose-bound) and intracellular fraction were separated by centrifugation. Western blots were performed as previously described on both surface and intracellular fractions.

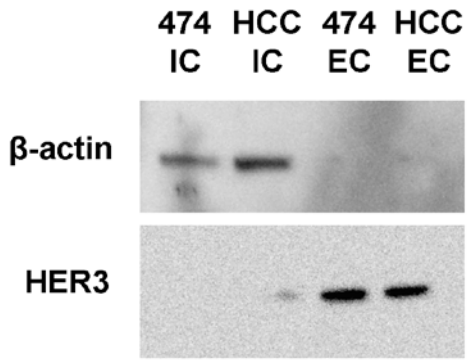
SUPPLEMENTAL DATA

Analysis of the binding kinetics of the EGFR PET probe demonstrates that accumulation of the EGFR PET probe is not significantly altered between 4ºC and 37ºC at 4 hours. These findings are confirmed by the comparison between live cells and paraformaldehyde fixed cells, in which cross-linking of cellular surface proteins eliminates receptor-mediated internalization of the probe. The binding of the radiolabeled antibody to live or fixed cells does not demonstrate significant differences. Studies at successive time points (1 hour, 2 hours, 4 hours) demonstrate increased

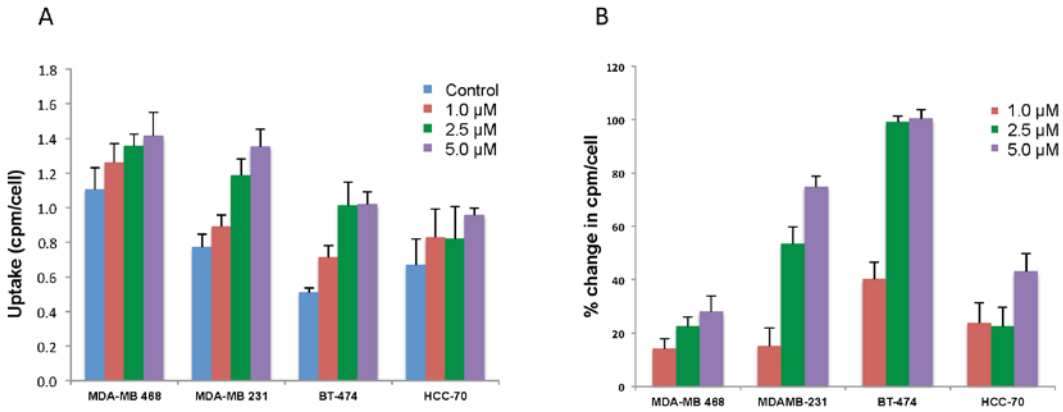
retention at 4ºC seen in both live and fixed cells. We believe this increase in binding at 4ºC relates to slower binding kinetics at 4ºC, rather than receptor mediated uptake. Cellular uptake of receptor:probe complex is not thought to contribute significantly to radiolabel retention. There is no significant increase in binding at successive time points at 37ºC as one would anticipate if cellular uptake machinery effects dominated tracer accumulation (Supplemental Fig. 4).



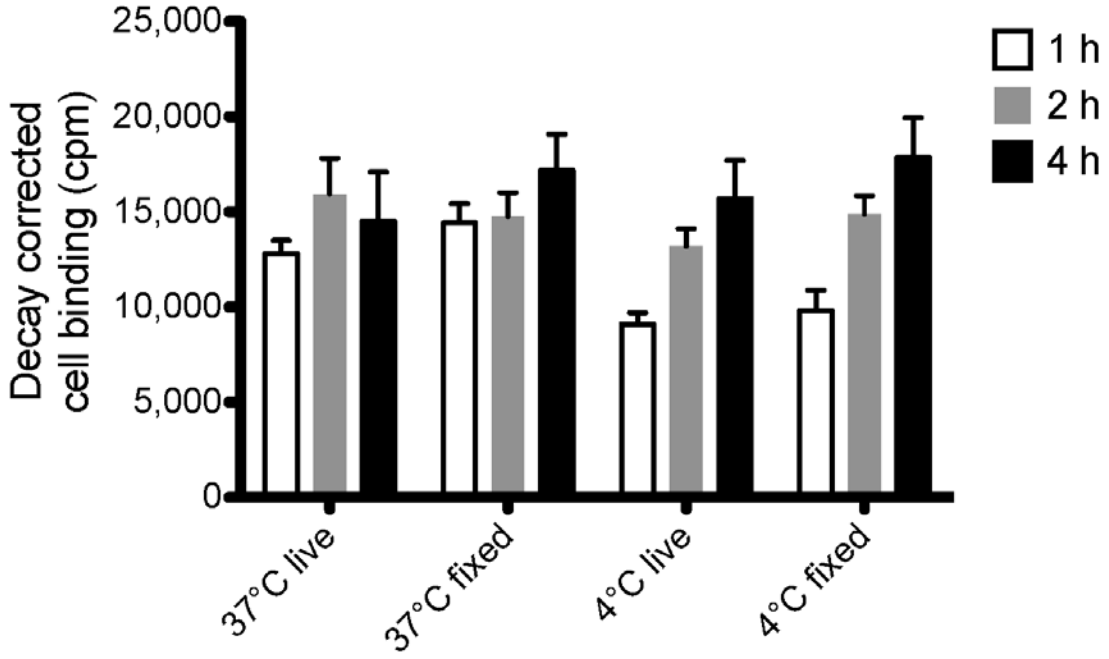
Supplemental Figure 1. EGFR and HER3 PET probe binding affinity. (A) *In vitro* studies with ^{67}Ga-DOTA-cetuximab F(ab′)2 demonstrate probe binding to EGFR with Kd of 8.6 nM. (B) Variation of mean number of bound DOTA equivalents demonstrates that an optimal number of equivalents is approximately 1.47. (C) *In vitro* studies with ^{67}Ga-DOTA-HER3 F(ab′)2 demonstrate probe binding to HER3 with Kd of 6.8 nM.



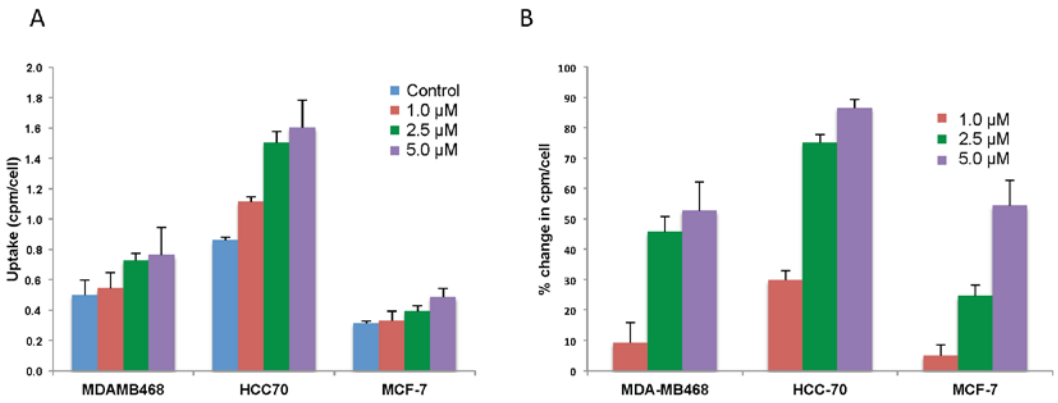
Supplemental Figure 2. Location of HER3 expression. In BT-474 and HCC-80 cells that have been surface-biotinylated and intracellular (IC) and surface (EC) fractions separated, comparison of HER3 found in the two fractions shows that most HER3 is found on the cell surface



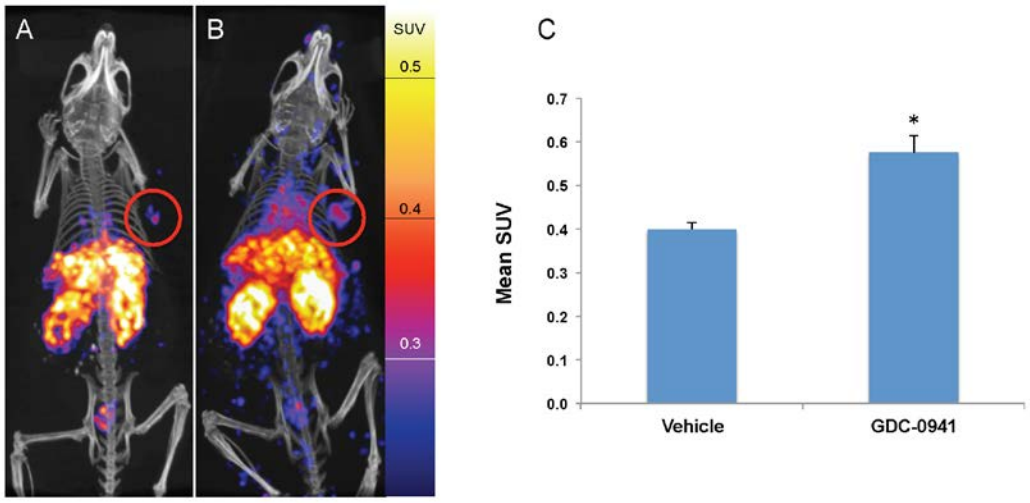
Supplemental Figure 3. EGFR PET probe measures the effects on cellular EGFR surface-expression after treatment with PI3Kalpha inhibitor BYL-719. (A) Following 48h treatment with vehicle or increasing concentrations of BYL-719 at specified doses, cell lines were incubated with the EGFR PET probe for 2h and binding measured by gamma counting. (B) Percentage change in probe binding relative to control.



Supplemental Figure 4. Time course of EGFR PET probe cell binding. To analyze the internalization of the EGFR PET probe, cells were plated in 96 well plates (N=6 wells per condition) and treated with either 4% paraformaldehyde or PBS prior to addition of $1x10^6$ CPM of probe. Cells were incubated for 1 (white bars), 2 (gray bars) or 4h (black bars) at either 4 or 37°C. Following washing, bound radioactivity was eluted and quantified by gamma counter. The results do not demonstrate significant changes in accumulation for decreased temperature or cell surface protein cross-linking.



Supplemental Figure 5. HER3 PET probe measures the effect on cellular HER3 surface-expression after treatment with PI3Kalpha inhibitor BYL-719. (A) Following 48h treatment with vehicle or increasing concentrations of BYL-719 at specified doses, cell lines were incubated with HER3 PET probe for 2h and binding measured by gamma counting. (B) Percentage change in probe binding relative to control.



Supplemental Figure 6. EGFR PET probe visualizes changes in EGFR expression with treatment of MDAMB231 tumors. (A) MDA-MB-231 xenografts imaged with EGFR PET probe after treatment with vehicle, or (B) GDC-0941. Images normalized to 0.55 SUV. (C) SUVmean of MDA-MB-231 xenografts imaged with EGFR PET probe after treatment demonstrates change in SUV of 44% in comparison to vehicle, respectively, n=4 for both groups, * = $p<0.01$.