Imaging Biomarkers Predict Response to Anti-HER2 (ErbB2) Therapy in Preclinical Models of Breast Cancer

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Abstract

Purpose: To evaluate noninvasive imaging methods as predictive biomarkers of response to trastuzumab in mouse models of HER2-overexpressing breast cancer. The correlation between tumor regression and molecular imaging of apoptosis, glucose metabolism, and cellular proliferation was evaluated longitudinally in responding and nonresponding tumor-bearing cohorts. Experimental Design: Mammary tumors from MMTV/HER2 transgenic female mice were transplanted into syngeneic female mice. BT474 human breast carcinoma cell line xenografts were grown in athymic nude mice. Tumor cell apoptosis (NIR700-Annexin V accumulation), glucose metabolism [2-deoxy-2-[18F]fluoro-D-glucose positron emission tomography ([18F]FDG-PET)], and proliferation [3'-[18F]fluoro-3'-deoxythymidine-PET ([18F]FLT-PET)] were evaluated throughout a biweekly trastuzumab regimen. Imaging metrics were validated by direct measurement of tumor size and immunohistochemical analysis of cleaved caspase-3, phosphorylated AKT, and Ki67.

Results: NIR700-Annexin V accumulated significantly in trastuzumab-treated MMTV/HER2 and BT474 tumors that ultimately regressed but not in nonresponding or vehicle-treated tumors. Uptake of [18F]FDG was not affected by trastuzumab treatment in MMTV/HER2 or BT474 tumors. [18F]FLT-PET imaging predicted trastuzumab response in BT474 tumors but not in MMTV/HER2 tumors, which exhibited modest uptake of [18F]FLT. Close agreement was observed between imaging metrics and immunohistochemical analysis.

Conclusions: Molecular imaging of apoptosis accurately predicts trastuzumab-induced regression of HER2+ tumors and may warrant clinical exploration to predict early response to neoadjuvant trastuzumab. Trastuzumab does not seem to alter glucose metabolism substantially enough to afford [18F]FDG-PET significant predictive value in this setting. Although promising in one preclinical model, further studies are required to determine the overall value of [18F]FLT-PET as a biomarker of response to trastuzumab in HER2+ breast cancer.

The American Cancer Society estimates that 178,480 women were diagnosed with breast cancer in the United States in 2007 (1). Twenty percent to 30% of breast cancers exhibit amplification or overexpression of the HER2/erbB2 oncogene, which is associated with more aggressive disease and poor prognosis (2, 3). HER2 is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, which includes HER1/erbB1 (EGFR), HER2/erbB2, HER3/erbB3, and HER4/erbB4. These receptors regulate a wide range of cellular processes, including proliferation, differentiation, motility, survival, angiogenesis, invasion, and antiapoptotic functions (4). To date, an endogenous ligand for the HER2 receptor has not been identified, but its activation is thought to occur through heterodimerization with other ligand-bound HER...
family members or by homodimerization when highly expressed (4).

The development of inhibitors targeting various portions of the HER signaling axis is an active and clinically important area of research. Trastuzumab (Herceptin) is a Food and Drug Administration–approved, recombinant, humanized monoclonal antibody that selectively binds to the extracellular domain of HER2. Objective means to assess early response to trastuzumab therapy remain undeveloped. To this end, we have evaluated three translational, noninvasive molecular imaging metrics to quantify and predict response to trastuzumab in preclinical mouse models of HER2-overexpressing breast cancer.

Translational Relevance

The development of inhibitors targeting various portions of the ErbB signaling axis is an active and clinically important area of research. Trastuzumab (Herceptin) is a Food and Drug Administration–approved, recombinant, humanized monoclonal antibody that selectively binds to the extracellular domain of HER2 (5). In combination with chemotherapy, trastuzumab extends overall survival and slows disease progression in HER2+ breast cancer patients. However, approximately 50% of HER2+ metastatic breast cancers exhibit de novo or acquired resistance to trastuzumab (6–9) and objective means to assess early response to trastuzumab therapy remain undeveloped.

Although the primary mechanism(s) of action of trastuzumab remains unclear (10), the importance of phosphatidylinositol 3-kinase (PI3K) signaling in HER2+ breast cancer (4) implies that perturbation of this pathway is important in order for HER2-directed therapies to exert an antitumor effect. This also implies that PI3K-regulated processes such as tumor cell apoptosis, proliferation, and glucose metabolism may be useful biomarkers of response to trastuzumab therapy. Indeed, a recent study in patients with HER2+ breast cancer showed that after 1 week of neoadjuvant treatment with trastuzumab as a single agent, a significant increase in tumor cell apoptosis was observed by cleaved caspase-3 immunostaining of tumor sections from core biopsies (11). HER2 signaling activates the PI3K/AKT/mammalian target of rapamycin cascade, and activated AKT stimulates the transport and metabolism of glucose (12). It has been shown that genes encoding most glycolytic enzymes are under dominant transcriptional control by AKT and target of rapamycin activities (13). Because trastuzumab inhibits PI3K activation in HER2+ breast cancer cells (14), glucose metabolism may be altered by trastuzumab therapy. Similarly, one might also predict that tumor cell proliferation would be inhibited by trastuzumab therapy, yet in a recent study, the proliferation marker Ki67 did not change in HER2+ tumors from patients treated with trastuzumab (11). Although these and other ex vivo assays are potentially valuable, procurement of tumor tissues through biopsies for the assessment of drug action is invasive and limited by sample bias stemming from tumor heterogeneity and other confounding factors such as inflammation. Serial biopsy, as would be required to assess the effects of therapy, is also clinically impractical in many cases. Alternatively, noninvasive molecular imaging biomarkers, which are capable of serial assessment of numerous relevant biological processes, could be particularly valuable toward clinical evaluation and prediction of response to trastuzumab in patients with HER2+ breast cancer.

In these preclinical investigations, we sought to evaluate and validate three noninvasive molecular imaging metrics as biomarkers of response to trastuzumab in two clinically relevant mouse models of HER2+ breast cancer. The imaging metrics evaluated include assessment of apoptosis with an optical imaging analogue of Annexin V, glucose uptake with 2-deoxy-2-[18F]fluoro-D-glucose positron emission tomography ([18F]FDG-PET), and cellular proliferation with 3-[18F]fluorodeoxythymidine-PET ([18F]FLT-PET). Data presented herein illustrate that molecular imaging of apoptosis can accurately predict trastuzumab-induced regression of both MMTV/HER2 transgenic mouse mammary tumors and BT474 human breast cancer cell line xenografts and may warrant further exploration clinically. Although modest overall uptake of [18F]FLT limited the predictive value of [18F]FLT-PET imaging in MMTV/HER2 tumors, [18F]FLT-PET accurately predicted trastuzumab response in BT477 xenografts. In both preclinical models, trastuzumab therapy did not seem to alter glucose uptake substantially enough to afford [18F]FDG-PET predictive value within this setting.

Materials and Methods

Animal model

The MMTV/HER2 transgenic mouse line, which expresses a transgene encoding MMTV promoter-driven human HER2 (15), was a gift from Sharon Erickson (Genentech). MMTV/HER2 females were used to generate mammary tumors. These mice develop mammary tumors with a latency of 8 to 12 mo. Tumors were harvested, divided in fragments, and transplanted s.c. (2 mm pieces) near the mammary fat pad #1 of 4- to 6-wk-old female FVB syngeneic mice as previously described (16). When the grafted tumors grew to >200 mm3, mice were imaged for baseline [18F]FDG-PET, and cellular proliferation with 3-[18F]FLT-PET. The mice were then treated with trastuzumab (Herceptin, 35 mg/kg; Vanderbilt University Hospital Pharmacy) or PBS vehicle twice per week by i.p. injection (0.2 mL). For the BT474 model, athymic nude female mice (4–5 wk of age; Harlan) were implanted s.c. with E2 pellets (0.72 mg, 60-d release; Innovative Research of America) on the dorsal flank. The next day, mice were injected s.c. on the upper back with 2 × 107 BT474-AZ cells (a gift from AstraZeneca) suspended in serum-free medium and mixed with Matrigel (BD Biosciences) at 1:1 ratio as described (17). Tumor diameters were measured before each treatment using calipers, and volume in mm3 was calculated as described below. All mice were maintained in a specific pathogen-free facility in accordance with the Institutional Animal Care and Use Committee of the Vanderbilt University Medical Center.

Synthesis of NIR700-Annexin V imaging probe

Synthesis of NIR700-Annexin V has been reported previously (18). Briefly, Annexin V (Sigma-Aldrich) was reconstituted in ice-cold 1× PBS (pH 7.4, 0.2 g/L). Near-IR (NIR) dye (IRDye 700DX NHS ester, LI-COR Biosciences), dissolved in dry DMSO per manufacturer’s specifications, was added to the Annexin V solution with vortex mixing (dye to protein stoichiometry, 9:1). The conjugation reaction vessel was protected from light and gently agitated for 2 h at room temperature. Labeling progress was monitored via gel filtration chromatography (Superdex 200HR, Amersham Pharmacia), eluting with 1× PBS. Following conjugation, labeled probes were purified exhaustively by dialysis (1× PBS, 4°C) using 7000 molecular weight cutoff Slide-A-Lyzer dialysis cassettes (Pierce of Thermo Fisher Scientific). The purity of the conjugates was
assessed chromatographically, and dye to protein ratio (routinely 1:1) was quantified by spectrophotometry.

**Synthesis of [18F]FLT**

[18F]FLT was prepared from [18F]fluoride in a two-step, one-pot reaction as previously described using a GE TRACERlab FX-FN automated module (19). Aqueous [18F]fluoride from an H2[18O]O target was trapped by ion exchange (QMA, Waters) and then eluted with Kryptofix-222 (K222) and K2CO3 in CH3CN/H2O into the reaction vessel. Three sequences of heating (110°C) with He(g) flow resulted in dry [18F]fluoride/K222/K2CO3. The cyclic precursor 2,3'-anhydro-5'-O-benzoyl-2'-deoxythymidine (ABX Advanced Biochemical Compounds) was added in DMSO and reacted for 10 min at 160°C. The benzoyl protecting group was removed from the labeled intermediate by basic hydrolysis (0.25 mol/L NaOH, 50°C, 10 min). The reaction mixture was purified on a semipreparative C-18 high-performance liquid chromatography column eluting with 10% ethanol/10 mmol/L sodium phosphate buffer and sterilized by 0.2-μm membrane filtration. Radiochemical identity, purity, and specific activity were determined by analytic high-performance liquid chromatography. Product was obtained with average radiochemical purity of 98.3% and specific activity of 3,480 Ci/mmol.

**Procurement of [18F]FDG**

[18F]FDG was synthesized in the Vanderbilt University Medical Center Radiopharmacy and distributed by PETNET. The average radiochemical purity of the product was 98.5% and specific activity was >1,000 Ci/mmol.

**In vivo imaging**

Mice were imaged with NIR700-Annexin V, [18F]FLT, and [18F]FDG weekly before and within 24 h of administration of trastuzumab or PBS for up to 3 wk or until complete tumor regression was observed. Inclusion criteria were baseline tumor size between 200 and 600 mm3. During the imaging sessions, the mice were kept under general anesthesia by inhalation of 1% to 3% isoflurane with 2% oxygen (and on a warm water pad during PET imaging).

**NIR700-Annexin V imaging.** Before imaging, the fur was removed (MMTV/HER2 only) from the right dorsal half of the mouse using a commercial depilatory cream (Nair, Church & Dwight Co., Inc.) to

![Fig. 1. Accumulation of NIR700-Annexin V is enhanced in MMTV/HER2 tumors responding to trastuzumab.](image-url)
reduce scatter and absorbance from overlying fur. NIR images were acquired using a Maestro FLEX In Vivo Imaging system (CRI) equipped with the F-filter set (spectral range of 680-950 nm, 500 ms exposure time, stage height of 1, and 2.8 f-stop). Preinjection autofluorescence images were acquired with mice in the lateral decubitus position with the tumor facing the camera lens. The mice were then retroorbitally injected with NIR700-Annexin V (0.5 nmol). Longitudinal images were acquired immediately after the injection and subsequently at regular intervals for up to 44 h after injection. Time-fluorescence activity curves were generated for each mouse at each imaging session as described below.

**[18F]FDG-PET and [18F]FLT-PET imaging.** Animal handling methods in preparation for and during [18F]FDG-PET imaging were similar to published protocols (20, 21). Briefly, before imaging, the mice were fasted overnight and allowed to acclimate to the PET imaging facility environment for at least 1 h while on a warm water pad. [18F]FDG and [18F]FLT were administered on different days via a single retroorbital injection of 200 μCi (100 μl). Following a 40-min distribution period, 20-min static PET scans were collected on a Concorde Microsystems microPET Focus 220 (Siemens). Mice were awake during the uptake period and maintained on a warm water heating pad. PET images then were reconstructed using the ordered subsets expectation maximization algorithm.

**Immunohistochemistry**

Immediately following imaging, mice were euthanized and tumor tissues were collected for immunohistochemical analysis. Tissues were fixed in 4% formalin for 24 h and subsequently transferred to 70% ethanol before processing and paraffin embedding. Tissues were examined by H&E staining to verify that adequate tumor tissue was present. Subsequently, tissues were immunostained using antibodies against cleaved caspase-3, S473 phosphorylated AKT (p-AKT; Cell Signaling), and Ki67 (Biocare). Cleaved caspase-3+ cells were counted from five randomly selected fields at ×100 magnification, and results were expressed as percentage positive cells per field. Ki67 and S473 p-AKT were evaluated by an expert pathologist (M.G.O.) as described previously (22).

**Data analysis**

**Tumor volume.** Tumor volume was calculated using the formula

\[
\text{Volume} = \frac{\text{length} \times \text{length} \times \text{width}}{2},
\]

where length is the smaller dimension. Percentage change in tumor volume from baseline was then calculated using the following formula:
Trastuzumab-treated tumors were defined as “responding” to therapy if the total tumor volume decreased by 20% or more from baseline by either the end of 2 wk of treatment (BT474) or 3 wk of treatment (MMTV/HER2).

**Optical imaging analysis.** Images acquired with a Maestro FLEX In Vivo imaging system were spectrally unmixed using CRI-supplied software version 0.9.3. Any animals exhibiting postinjection dose infiltration were excluded from imaging analysis. The average intensity of NIR700-Annexin V in the manually defined tumor region of interest was quantified and compared with that in the right thigh muscle region of interest, which served as an internal reference at each imaging time point for each mouse. Time versus NIR700-Annexin V intensity plots were calculated for both tumor and muscle at each imaging time point using a trapezoidal integration. The ratio of tumor AUC and muscle AUC (T/M AUC) was calculated for each imaging session, and the percentage change in T/M AUC from baseline was subsequently calculated using the formula:

$$\% \Delta \text{Volume} = \frac{\text{Vol}_{t-x} - \text{Vol}_{t=0}}{\text{Vol}_{t=0}} \times 100$$

The T/M ratio at each imaging time point for each mouse was then plotted with respect to the number of treatments received, and statistical analyses were done using a paired t test. The percentage change in T/M AUC was also plotted relative to the percentage change in tumor size and fitted to a one-phase exponential fit to assess the degree of correlation.

**[18F]FDG-PET and [18F]FLT-PET analysis.** Any animals exhibiting significant postinjection dose infiltration were excluded from imaging analysis. The maximum standard uptake values (SUV) were quantified by manually drawing region of interests around the tumor using ASIPro VM (CTI Concorde Microsystems) and normalizing the signal intensity to the injected dose and mouse mass. SUV\text{max} was selected for PET imaging analysis to eliminate potential bias in the quantified data due to tumor heterogeneity. The percentage change in SUV\text{max} from baseline was subsequently calculated using the formula:

$$\% \Delta \text{SUV}_{\text{max}} = \frac{\text{max SUV}_{t-x} - \text{max SUV}_{t=0}}{\text{max SUV}_{t=0}} \times 100$$

and plotted with respect to the weeks of treatment received. For display purposes, PET images are presented on similar normal tissue activity scales. Statistical analysis was done using a paired t test.

**Results**

**Trastuzumab-induced regression of MMTV/HER2 and BT474 mammary tumors.** Similar to our prior observations (16), a significant fraction of tumors derived from MMTV/HER2 transgenic mice responded to trastuzumab therapy, defined as a reduction of tumor volume ≥20% by the end of the 3-week regimen. For analysis, MMTV/HER2 tumor mice were stratified as responders (16 of 30) or nonresponders (14 of 30) according to the change in tumor size from baseline. Tumors that were initially responsive regressed proportionately with successive

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Fig. 3. MMTV/HER2 tumor uptake of [18F]FDG is similar across responding and nonresponding cohorts. Representative [18F]FDG-PET images collected before and following 2 wk of trastuzumab therapy in responding (A) and nonresponding (B) mice.
trastuzumab treatments. In contrast, nonresponding MMTV/HER2 tumors grew significantly over the course of treatment (Supplementary Fig. S1A/B). We found BT474 tumors to be considerably more sensitive to trastuzumab treatment, noting that all trastuzumab-treated tumors, but not vehicle-treated tumors, showed significant regression following 2 weeks of treatment (Supplementary Fig. S2A/B).

Imaging apoptosis with NIR700-Annexin V in MMTV/HER2 model predicts tumor regression. Before and 1 week following trastuzumab treatment, no significant accumulation of NIR700-Annexin V was observed in MMTV/HER2 tumors. However, after 2 weeks of treatment, significant accumulation of NIR700-Annexin V was observed in the tumors of responding but not nonresponding mice (Fig. 1). Immediately following injection of NIR700-Annexin V, renal accumulation was noted in all mice (Fig. 1). Clearance profile analysis was done longitudinally on each mouse to assess the relative accumulation of NIR700-Annexin V in tumors and in reference muscle tissue (Supplementary Fig. S3). From this analysis, tumor probe accumulation was significantly increased after 2 weeks of treatment and thereafter compared with baseline in responding mice ($P < 0.0005$; Fig. 2A). The mean NIR700-Annexin V tumor uptake (T/M AUC ratio) in responding tumors increased by 9.7%, 46%, and 69% after 1, 2, and 3 weeks of trastuzumab treatment, respectively. In contrast, NIR700-Annexin V did not accumulate in MMTV/HER2 tumors that did not shrink with treatment (Fig. 2B). Interestingly, we noted increased NIR700-Annexin V accumulation in tumors after 2 weeks of therapy in all but one MMTV/HER2 responder (Fig. 2C), yet we actually noted decreased accumulation of the imaging probe over the same time frame in nonresponders of the same model (Fig. 2D). Importantly, analysis of all MMTV/HER2 imaging data from responder and nonresponder mice over the entire 3-week treatment course indicated that the magnitude of NIR700-Annexin V uptake in tumors was inversely proportional to the change in tumor size from baseline (Fig. 2E). Nonlinear regression analysis of all NIR700-Annexin V imaging data points showed a significant negative correlation between

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**Fig. 4.** Longitudinal assessment of $[{^{18}}F]$FDG uptake in MMTV/HER2 tumors suggests that overall tumor glucose metabolism is not altered by trastuzumab therapy. No significant difference was observed between serial $[{^{18}}F]$FDG-PET images in either responding (A) or nonresponding (B) MMTV/HER2 tumor-bearing mice. C, on an individual basis, tumor $[{^{18}}F]$FDG uptake (pretreatment imaging compared with 2-wk posttreatment) was almost identical following treatment in a vast majority of responding mice. D, however, some nonresponding cohorts showed a trend toward increased $[{^{18}}F]$FDG uptake after 2 wk of trastuzumab treatment. E, nonlinear regression analysis showed that no correlation was observed between changes in $[{^{18}}F]$FDG uptake and overall changes in tumor volume from baseline.
change in tumor size and NIR700-Annexin V tumor uptake ($R^2 = 0.375$). Thus, tumors exhibiting increased accumulation of NIR700-Annexin V regressed, whereas tumors with reduced NIR700-Annexin V accumulation progressed on therapy.

**Trastuzumab does not alter tumor $^{[18]}$F-FDG uptake in MMTV/HER2 model.** Before trastuzumab treatment, MMTV/HER2 tumors of responding and nonresponding mice were differentiable from background by PET imaging of $^{[18]}$F-FDG uptake (Fig. 3A/B). However, throughout the 3-week treatment course, no statistically significant difference was observed between baseline $^{[18]}$F-FDG uptake and uptake following successive trastuzumab treatments in responding or nonresponding mice (Fig. 4A/B). Over the first 2 weeks of therapy, the majority of responding mice (71%) showed similar $^{[18]}$F-FDG uptake after trastuzumab treatment when compared with baseline (Fig. 4C), suggesting that $^{[18]}$F-FDG uptake is not a suitable predictive biomarker in this setting. Although tumor $^{[18]}$F-FDG uptake in nonresponding mice seemed to trend upward when comparing baseline and 2-week images (Fig. 4D), the trend was nonsignificant. Furthermore, in the overall population of treated and untreated MMTV/HER2 tumor-bearing mice, no correlation was observed between tumor size and $^{[18]}$F-FDG uptake (Fig. 4E).

**MMTV/HER2 tumors exhibit poor $^{[18]}$F-FLT uptake.** Only modest tumor uptake of $^{[18]}$F-FLT was observed before trastuzumab treatment in both responding and nonresponding mice. (Fig. 5).

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![Image](https://clincancerres.aacrjournals.org/)

**Fig. 5.** *In situ* markers of drug action correlate with noninvasive imaging biomarkers in MMTV/HER2 tumors. Representative tumor sections from formalin-fixed, paraffin-embedded transgenic tumor transplants stained with H&E and p-AKT, cleaved caspase-3, and Ki67 antibodies as described in Materials and Methods. Compared with vehicle-treated controls, elevated cleaved caspase-3 staining was observed in MMTV/HER2 tumor tissues collected from responding, but not nonresponding, mice. Nuclear and cytoplasmic p-AKT immunoreactivity was observed in vehicle-treated control. Tumor tissues collected from responding mice showed a trend toward decreased nuclear p-AKT immunoreactivity, but staining was elevated significantly in the cytoplasm of many tumor cells. Similarly, nonresponding cohorts showed elevated cytoplasmic p-AKT, but also nuclear p-AKT, which was similar to vehicle-treated cohorts. Ki67 immunoreactivity of tumor tissues was elevated following treatment with trastuzumab compared with vehicle-treated controls.
MMTV/HER2-bearing cohorts (Supplementary Fig. S5A). Subsequent treatment with trastuzumab had no discernible effect on tumor uptake of \(^{18}F\)FLT (Supplementary Fig. S5B) through 2 weeks of treatment, over which time many responding tumors exhibited significant regression. Ki67 immunoreactivity of tumor tissues collected from treated mice showed a modest increase compared with untreated or untreated mice (Fig. 5). Consequently, \(^{18}F\)FLT-PET imaging was discontinued in MMTV/HER2 tumor-bearing mice after the second week of treatment and not considered further in this model.

Trastuzumab-induced tumor regression correlates with increased apoptosis in MMTV/HER2 tumors. MMTV/HER2 tumor tissues were evaluated for cleaved caspase-3, Ki67, and p-AKT immunoreactivity. Responding MMTV/HER2 tumors collected after 3 weeks of treatment exhibited significantly increased cleaved caspase-3 immunoreactivity compared with untreated or non-responding cohorts (Fig. 5). Furthermore, MMTV/HER2 tumor tissues that were collected before or after each week of trastuzumab treatment revealed a proportionately increased number of apoptotic cells over time on treatment (Supplementary Fig. S4), in agreement with NIR700-Annexin V imaging.

On average in responding MMTV/HER2 tumors, 0.69%, 1.1%, and 1.3% caspase-3+ cells were detected per tumor field following 1, 2, and 3 weeks of trastuzumab treatment, respectively \((P < 0.05)\), compared with 0.24% detected at baseline. Although MMTV/HER2 tumors seemed proliferative, we did not observe a consistent correlation between percentage Ki67-immunoreactive cells and trastuzumab treatment or response in this model. We also observed variability in p-AKT immunoreactivity between all MMTV/HER2 tumors and, thus,
did not find a correlation between p-AKT and response. These results seem to be consistent with tumor [18F]FDG uptake in this model, which was also variable.

**Apoptosis imaging and [18F]FLT-PET imaging predict response to trastuzumab in BT474 xenografts.** All trastuzumab-treated BT474 tumor-bearing mice carried out as far as 2 weeks of therapy exhibited significant tumor regression (Supplementary Fig. S2). We therefore evaluated the potentially predictive imaging metrics following just 1 week of trastuzumab treatment in this model. Similar to our observations in responding MMTV/HER2 tumor-bearing mice, we found that N1R700-Annexin V accumulation was predictive of response to treatment. In BT474 tumor-bearing mice, the difference between N1R700-Annexin V accumulation in vehicle- and trastuzumab-treated tumors was statistically significant following just a single week of therapy (Fig. 6A-D). Also similar to the MMTV/HER2 studies, trastuzumab treatment did not seem to have any observable effect on [18F]FDG uptake in BT474 tumors (Fig. 6E-H). We found that baseline [18F]FLT uptake in BT474 tumors was considerably higher compared with MMTV/HER2 tumors. Additionally, 1 week of trastuzumab treatment significantly reduced [18F]FLT uptake in this model (Fig. 6I-L). In similar fashion to the MMTV/HER2 studies, we found good agreement between each of the imaging metrics and histology (Supplementary Figs. S6 and S7), where we noted significantly elevated caspase-3 staining and significantly decreased Ki67 staining in trastuzumab-treated BT474 tumors following both 1 and 2 weeks of therapy. As with MMTV/HER2 tumors, trastuzumab treatment did not seem to significantly affect p-AKT staining in BT474 tumor tissues.

### Discussion

In contrast with predominantly anatomic imaging modalities such as computed tomography, planar X-ray, or magnetic resonance imaging, noninvasive molecular imaging aims to visualize and quantify cellular and physiologic processes in vivo. Because specific biological changes may occur in tumors within hours to days of treatment and therefore precede clinical regression, imaging biomarkers capable of assessing the most relevant of these molecular events may be highly suitable for detecting early responses and potentially predicting treatment outcome. Validation of such imaging metrics forms the basis of important research occurring at the interface between imaging science and cancer biology.

Clinically, the PET tracer [18F]FDG is recognized as a tool for cancer detection and staging in most organ sites. In addition to [18F]FDG, additional tracers, such as [18F]FLT-PET to assess proliferation and Annexin V conjugates to assess apoptosis, offer great promise as a means to evaluate response to conventional and molecularly targeted therapeutic interventions (23–25). Selection criteria for appropriate molecular imaging biomarker(s) are highly dependent on the underlying tumor cell biology and its modulation following therapeutic intervention. For example, capitalizing on the role that EGFR serves in mediating tumor cell proliferation and apoptosis, we recently illustrated that molecular imaging assessments of these events are potentially biomarkers of response to EGFR-directed therapy in colorectal cancer (26).

In this study, we have evaluated three independent molecular imaging metrics as potential biomarkers of response to the HER2 antibody trastuzumab in a mouse model of HER2+ breast cancer. Each imaging metric was selected to report a type of cellular response and was evaluated within the context of responding and nonresponding tumor-bearing cohorts. Data presented here illustrate that accumulation of an Annexin V–based optical imaging probe in trastuzumab-treated, MMTV/HER2 transgenic mouse tumor allografts, as well as in BT474 human xenografts, correlates with response and predicts clinical regression before evident by physical means. In the more responsive BT474 model, [18F]FLT-PET also seemed to be a potential biomarker of response to trastuzumab. The variability we noted in tumor [18F]FLT uptake when comparing these two preclinical models studied likely has biological significance. Theoretically, [18F]FLT and other nucleoside-based tracers serve as a surrogate markers of proliferation by reporting the activity of the thymidine salvage pathway, a cellular mechanism that uses uptake of deoxynucleobases from the extracellular environment to provide dividing cells with DNA precursors. However, thymidine salvage is not a prerequisite for cell survival in replicating mammalian cells. In fact, thymidine salvage is a complementary route for providing cells with nucleosides and de novo synthesis of nucleosides is fully capable of providing all the DNA precursors that are needed for cell growth (13). Thus, [18F]FLT uptake (if observed) in tumors relying predominantly or completely on de novo nucleoside synthesis may poorly inform proliferation status compared with tumors that rely more heavily on thymidine salvage. Although beyond the scope of these studies, this could explain differences in [18F]FLT uptake between MMTV/HER2 and BT474 tumors. At present time, the prognostic and therapeutic implications for tumors that use de novo nucleoside synthesis versus thymidine salvage are not well understood and additional research within this area should be conducted.

In both MMTV/HER2 and BT474 tumors, [18F]FDG showed significant uptake ($\geq 2 \times$ background), but this was unchanged in response to trastuzumab treatment. This finding is significant because recent human studies have shown higher [18F]FDG uptake in triple-negative compared with hormone receptor–positive breast cancers (27). Furthermore, human studies have suggested that adequate measures of treatment response with [18F]FDG PET imaging are more difficult in tumors exhibiting modest pretreatment uptake of [18F]FDG (28).

Importantly, trends observed between imaging metrics and standard histologic markers [i.e., cleaved caspase-3, activated AKT (p-AKT), and Ki67] exhibited temporal agreement, suggesting that the observations made by imaging were reflective of molecular and cellular events in tumors in situ. Cleaved caspase-3 immunoreactivity was directly proportional to, and temporally correlated with, N1R700-Annexin V tumor uptake and response to trastuzumab treatment in both MMTV/HER2 and BT474 tumors. In the analysis of p-AKT staining of MMTV/HER2 tumor tissues, a portion of the cells collected from the tumors of treated, responding mice showed decreased p-AKT, whereas other cells showed significantly increased cytoplasmic p-AKT. Thus, we did not observe a consistent net difference in p-AKT staining across treated, untreated, responding, and nonresponding cohorts in this model. Similarly, we did not observe differential p-AKT staining across trastuzumab- or vehicle-treated BT474 tumors. Although numerous biological factors affect glucose metabolism, the lack of sensitivity observed with [18F]FDG-PET
imaging in these studies seems consistent with the lack of modulation of p-AKT (12). In agreement with previous clinical findings that showed no correlation between Ki67 immunoreactivity and response to neoadjuvant trastuzumab in patients with HER2+ locally advanced breast cancer (11), neither Ki67 immunoreactivity nor [18F]FLT-PET was reflective of response to trastuzumab in the MMTV/HER2 model. These results mirror our recent findings in the evaluation of [18F]FLT-PET imaging as a biomarker of response to the EGFR therapeutic antibody cetuximab (mAb-C225) in human colorectal cancer xenografts. In these studies, we noted excellent correlation between [18F]FLT-PET imaging and tumor Ki67 immunoreactivity, yet similarly to the trastuzumab results, cetuximab was ineffective at reducing tumor cell proliferation in vivo (18). In contrast, trastuzumab treatment was effective at reducing proliferation in the more responsive BT474 tumors as assessed by Ki67 immunoreactivity. This effect was predictable with [18F]FLT-PET imaging following just 1 week of therapy.

In conclusion, molecular imaging of apoptosis seems to be a promising noninvasive method for prediction and evaluation of early response to trastuzumab therapy. Although the Annexin V analogue reported here used an optical imaging label and may be best suited for preclinical imaging, our data suggest that pharmacodynamic clinical evaluation of apoptosis in real time in breast cancer patients is warranted and feasible via functionalization with 99mTc for SPECT imaging (29). Data presented in these studies also suggest that [18F]FLT-PET imaging is potentially an informative biomarker in this setting, especially for some exquisitely sensitive tumors. However, the discrepancy observed between baseline uptake of [18F]FLT across the MMTV/HER2 and BT474 preclinical models indicates that additional studies are needed to further elucidate the biological basis of FLT-PET imaging as a biomarker in breast cancer. Although single-agent trastuzumab did not consistently alter glucose metabolism substantially enough to afford [18F]FDG-PET predictive value in these studies, this metric remains potentially appropriate for use in evaluating other novel, molecularly targeted regimens in breast cancer that may include trastuzumab combined with other novel therapies.

References