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Utility of ¹⁸FLT-PET to Assess Treatment Response in Trastuzumab-resistant and Sensitive HER2-overexpressing Human Breast Cancer Xenografts

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Abstract

Purpose—Evaluate 3'-deoxy-3'-[¹⁸F]-fluorothymidine (¹⁸FLT) PET as an early marker of trastuzumab response in HER2-overexpressing xenografts.

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Procedures—Tumor-to-muscle ratios were compared between both trastuzumab-sensitive and resistant cohorts prior to and after one and two treatments.

Results—A significant difference ($P=0.03$) was observed between treated and control trastuzumab-sensitive xenografts after one treatment, which preceded between-group differences in tumor volume. Reduced Ki67 ($P=0.02$) and thymidine kinase 1 (TK1) ($P=0.35$) immunoreactivity was observed in the treated xenografts. No significant differences in volume, tumor-to-muscle ratio, or immunoreactivity were observed between treated and control trastuzumab-resistant cohorts. A significant difference ($P=0.02$) in tumor-to-muscle ratio was observed between trastuzumab-sensitive and resistant cohorts after two treatments; however, tumor volumes were also different ($P=0.04$). Ki67 ($P=0.04$) and TK1 ($P=0.24$) immunoreactivity was ~50% less in trastuzumab-sensitive xenografts..

Conclusions— ^{18}F FLT-PET provided early response assessment in trastuzumab-sensitive xenografts, but only differentiated between trastuzumab-resistant and sensitive xenografts concurrent with differences in tumor size.

Keywords

^{18}F FLT-PET; assessing treatment response; HER2+ xenografts; breast cancer; trastuzumab

1. Introduction

The human epidermal growth factor receptor 2 (HER2) is overexpressed in 20% to 25% of breast cancers [1,2], and positively correlates with a more aggressive disease and poor prognosis [2]. Several HER2-targeted drugs have been approved for the treatment of patients with HER2-overexpressing breast cancer [3,4]. Trastuzumab (Herceptin®, Genentech, San Francisco, CA) was the first HER2-targeted therapy that selectively binds to the extracellular domain of HER2 [5,6]. In combination with traditional chemotherapy, trastuzumab treatment extends overall survival and slows disease progression in patients with HER2-overexpressing breast cancer [7,8]. Despite the survival benefits observed with trastuzumab treatment, a majority of patients with HER2-overexpressing metastatic breast cancer that initially responds, eventually progress. Further, a cohort of patients treated with adjuvant trastuzumab, still recur with metastatic disease [9–13]. These outcomes emphasize the need for accurate and reliable assessments of treatment response early during the course of therapy.

A main tumor cell-autonomous mechanism of action of trastuzumab is inhibition of HER2 homodimerization and downstream signaling, namely phosphatidylinositol-3 kinase (PI3K), leading to inhibition of cell-cycle progression and survival [1]. This implies that PI3K-regulated processes (e.g., cellular proliferation and apoptosis [14]) may be useful biomarkers for assessing the clinical response to trastuzumab. Traditional means of quantifying expression of specific cellular processes are through tissue biopsies; however, serial biopsies used for the assessment of drug action are very invasive and often clinically impractical. Alternatively, noninvasive biomarkers derived from molecular imaging modalities could be valuable towards the clinical evaluation and prediction of response to trastuzumab.

Positron emission tomography (PET) with 3'-deoxy-3'-[¹⁸F]-fluorothymidine (¹⁸FLT) is a molecular imaging technique that provides a noninvasive estimate of cellular proliferation as its uptake is regulated by the expression and activity of thymidine kinase 1 (TK1), an enzyme that regulates the cell-cycle dependent thymidine salvage pathway of DNA synthesis [15]. ¹⁸FLT-PET has been shown to be useful for evaluating treatment efficacy with molecular targeted therapies in preclinical mouse models of colorectal, head and neck, and ovarian cancers [16–18], as well as assessing response early during the course of therapy in patients with head and neck and non-small cell lung cancers [19,20]. However, two recent studies provided evidence suggesting that ¹⁸FLT-PET might not always reflect the proliferative index of certain tumors [21,22]. These observations emphasize the need for a systematic evaluation of ¹⁸FLT-PET to assess response in a variety of cancer types and treatment regimens.

The evaluation of ¹⁸FLT-PET as a biomarker of treatment response in HER2-overexpressing breast cancer has been limited. Monazzam et al. showed that ¹⁸FLT-PET could be used to monitor treatment efficacy of heat shock protein 90 inhibitors in multicellular tumor spheroids [23]. Shah et al. showed that ¹⁸FLT-PET could differentiate between trastuzumab and vehicle treated HER2-overexpressing xenografts after one week of therapy; this difference in ¹⁸FLT retention occurred prior to significant changes in tumor size that occurred at two weeks [24]. However, neither of these studies investigated the ability of ¹⁸FLT-PET to assess therapeutic resistance. This of great clinical significance as it would inform the early use of combinations of anti-HER2 therapies, thus abrogating resistance to single agent trastuzumab.

Our study builds on the previous study performed by Shah et al. as we investigated the utility of ¹⁸FLT-PET to assess response prior to one week of trastuzumab therapy before significant changes in tumor size occurred in trastuzumab-sensitive xenografts. Additionally, we explored the sensitivity of ¹⁸FLT-PET to differentiate trastuzumab resistance between sensitive and resistant HER2-overexpressing xenografts after exposure to trastuzumab. We also performed immunohistochemistry analyses that reflect on cellular proliferation in order to investigate the relationship between the imaging data and the underlying biology.

2. Materials and Methods

2.1 Cell lines

Trastuzumab-sensitive (BT474) and resistant (HR6) cell lines were cultured in improved minimal essential medium (IMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37 °C in a humidified, 5% CO₂ incubator. The HR6 cell line was developed *in vivo* as described previously [25]. Briefly, female athymic mice with established BT474 xenografts were treated twice per week with 20 mg/kg trastuzumab i.p. diluted in sterile saline. Tumors that initially responded completely and then recurred in the presence of continuous trastuzumab treatment were harvested, minced, and digested to generate the HR6 cell line [25]. To maintain trastuzumab resistance, HR6 cells were also cultured with 10 µg/ml trastuzumab as previously described [25]. Trastuzumab was purchased from the Vanderbilt University Medical Center Outpatient Pharmacy (Nashville, TN). Both cell lines

were harvested with trypsin at approximately 85% confluence for inoculation as previously described [25].

2.2 Xenograft models

Our institution's Animal Care and Use Committee approved all animal procedures. Female athymic mice (n=27, 4–6 weeks old, Harlan, Indianapolis, IN) were implanted with 0.72 mg, 60-day release, 17 β -estradiol pellets (Innovative Research of America, Sarasota, FL). Twenty-four hours later, approximately 10⁷ BT474 or HR6 cells suspended in a 1:10 ratio of growth factor-reduced Matrigel and IMEM were injected subcutaneously into the right flank. Tumor volumes were measured once per week *via* calipers. A 26-gauge jugular catheter was surgically implanted for radiotracer delivery two days prior to start of PET imaging. Mice were anesthetized with 2% isoflurane in pure oxygen mixture for all surgical procedures.

Mice bearing tumors were grouped into four cohorts: trastuzumab and vehicle treated BT474 cohorts, and trastuzumab and vehicle treated HR6 cohorts. (The specific number of mice for each cohort at each time point during the study is listed in Table 1). Imaging and treatment was initiated when tumor volumes reached 200 mm³, which was typically 4 to 8 weeks post cell inoculation. Imaging was conducted at three time points, days 0 (i.e., baseline before treatment), 1, and 4. Therapy consisted of two treatments that were administered immediately following imaging at day 0 and at day 3. Each treatment included an intra-peritoneal injection (total volume of 100 μ L) of trastuzumab (10 mg/kg) or saline vehicle. The imaging and treatment schema is diagrammed in Figure 1.

To evaluate the sensitivity of BT474 tumors to trastuzumab, a preliminary dose-response analysis was performed. Mice bearing BT474 xenografts were imaged at baseline and on days 1, 2, 4, 5, 7, and 8 after baseline. Mice were treated immediately following imaging at baseline and days 3 and 6 with either trastuzumab (10 mg/kg; n=4) or saline (n=4) vehicle *via* an i.p. injection (total volume, 100 μ L).

2.3 Radiotracer synthesis

¹⁸FLT was prepared as a service by our institution's radiochemistry core using a two-step, one pot reaction as described previously [26,27]. ¹⁸FLT was obtained with average radiochemical purity of 98.3% and specific activity of approximately 3480 Ci/mmol.

2.4 Volumetric imaging and analysis

Longitudinal tumor volume was measured from *T*₂-weighted magnetic resonance images using a 7T scanner (Agilent Technologies (formally Varian), Palo Alto, CA) equipped with a 38-mm quadrature radiofrequency coil (Doty Scientific, Columbia, SC). Anesthesia was induced and maintained *via* 2% isoflurane in pure oxygen. Animal respiration rate was monitored, and animal body temperature was maintained at an external temperature of 32°C by means of a flow of warm air directly into the bore of the magnet. Each animal was placed in a custom built restraint, and the tumor region was first localized *via* gradient echo scout images in three dimensions (3D). *T*₂-weighted images covering the entire tumor volume were acquired using a fast spin-echo pulse sequence with the following parameters:

repetition time=5500 ms, effective echo time=35.6 ms, 1 mm slice thickness, and an acquisition matrix of 128×128 over a 28×28 mm² field of view, which yielded a voxel size of 0.22×0.22 mm². Data were imported into MATLAB® version R2013b (The MathWorks, Natick, MA), and tumor volumes were measured at each time point by manually drawing regions of interest (ROIs) along tumor boundaries from all slices consisting of tumor tissue. One investigator (J.G.W.) delineated all tumor ROIs to ensure consistency.

2.5 ¹⁸FLT-PET imaging and analysis

After volumetric imaging, mice were transferred under anesthesia to a dedicated small-animal microPET Focus 220 system (Concorde Microsystems Inc, Knoxville, TN [28]) where animal body temperature was then maintained by a heated water pad and heat lamp. Anesthesia was continued, and list-mode data were collected for 60 (n=2) or 90 (n=25) minutes before, during, and after injection of 293 ± 7.00 µCi (mean ± standard error, SE) of ¹⁸FLT diluted in 100 µL of sterile saline. Images collected for 90 minutes were reconstructed into a 46-frame dynamic sequence (12×5 s, 6×10 s, 4×40 s, 4×60 s, 5×120 s, 4×180 s, and 12×300 s) using a three-dimensional ordered-subsets expectation maximization (OSEM3D) algorithm followed by a maximum *a posteriori* (MAP) algorithm [29]. OSEM3D-MAP reconstruction was performed using two OSEM3D iterations with nine subsets, followed by 18 MAP iterations with a beta value 0.038 [29]. Images collected for 60 minutes were reconstructed similarly but with a 64-frame dynamic sequence (5×12 s, 59×60 s). The resulting 3D reconstruction had a voxel size of 0.475×0.475×0.796 mm³. Attenuation and scatter correction were not performed as the amount of attenuation in PET studies of mice is small compared to human studies and differences between animals are not expected to be significant [28].

Standardized uptake value (SUV) parametric maps were calculated by normalizing the average activity concentration from the last 30 minutes of each dynamic acquisition by animal weight and injected dose. All time activity curves reached a plateau by the last 30 minutes of data acquisition (data not shown). MATLAB was used to manually draw 3D ROIs around the entire tumor volume on the SUV maps. ROIs were also drawn across three slices within the contralateral muscle. Again, one investigator (J.G.W.) delineated all ROIs to ensure consistency. To eliminate the dependency on injected activity [30], tumor-to-muscle ratios were used to compare ¹⁸FLT retention in the tumors before and after treatment. Tumor-to-muscle ratios were calculated by dividing the maximum SUV from the tumor ROI by the mean SUV from the muscle ROI. The maximum SUV from the tumor ROI was selected to eliminate potential bias in the quantified data due to tumor necrosis, which would ultimately lead to an increase in heterogeneity of radiotracer uptake throughout the whole tumor volume. The mean was chosen for the muscle ROI as it is a homogenous tissue in terms of ¹⁸FLT retention; thus, the mean provides a robust value by which the tumor maximum SUV can be normalized.

2.6 Immunohistochemistry

Immediately following imaging at day 4, animals were sacrificed and tumors were immediately fixed in 10% formalin for at least 24 hours. Samples were then transferred to 70% ethanol and stored at 4°C. Samples were processed and embedded in paraffin, and 4-

µm sections were prepared for immunostaining with Ki67 (DAKO, Catalog # m7240, antigen retrieval: HpH Buffer pH 8 (Decloaking Chamber), dilution 1:100, overnight incubation, Envision, chromogen DAB (DAKO), and TK1 (Abcam, Catalog # ab57757, antigen retrieval: Citrate Buffer pH 6 (Decloaking Chamber), dilution 1:1000, overnight incubation, Envision, chromogen DAB (DAKO). Stained sections were imaged at ×20 magnification using a Leica SCN400 Slice Scanner (Leica, Buffalo Grove, IL). For assessment of Ki67 and TK1, the total numbers of positive and negative cells were counted from four random fields uniformly spaced throughout the tissue section. The scores for each histological marker were expressed as the ratio of the number of percent positive cells to total cell number. Tumor sections for a subset of animals from each cohort were analyzed for comparison with the *in vivo* data.

2.7 Statistical analysis of data

Three statistical comparisons were performed in this study: 1) trastuzumab versus vehicle treated BT474 cohorts, 2) trastuzumab versus vehicle treated HR6 cohorts, and 3) treated trastuzumab-sensitive (BT474) versus trastuzumab-resistant (HR6). Tumor volume and tumor-to-muscle ratio were compared within and between cohorts at each imaging time point. Ki67 and TK1 percent positive scores were compared between cohorts. All data are presented as mean ± SE. All statistical comparisons were evaluated using the nonparametric Wilcoxon rank sum test in MATLAB version 2010b, and significance was assumed if $P < 0.05$.

In order to determine if ^{18}FLT retention measured at day 1 correlated with tumor volume at the end of the study (i.e., day 4), tumor-to-muscle ratios and tumor volumes from all mice in the study were analyzed with a linear regression model. The number of observations for the model was 25 as two mice died before day 4 due to issues unrelated to the study. Tumor volume at day 4 serves as the outcome variable and tumor-to-muscle ratio at day 1 is the predictor variable while adjusting for the effect of tumor volume at baseline. The following linear regression model was employed:

$$\text{Volume}_{\text{day4}} = \beta_0 + \beta_1 \cdot \text{Volume}_{\text{day0}} + \beta_2 \cdot \text{tumor-to-muscle}_{\text{day1}} + \varepsilon, \quad (1)$$

where β_1 and β_2 are regression coefficients corresponding to tumor volume at baseline and tumor-to-muscle ratio at day 1, respectively, and ε incorporates random experimental errors (assuming a normal distribution with mean=0 and variance= σ^2). The performance of the model was assessed *via* the R^2 value. The linear association between outcome and predictor variables was evaluated by quantifying the statistical significance of the cubic and quadratic terms of tumor-to-muscle ratio at day 1 in the model; i.e., (tumor-to-muscle ratio at day 1) 2 and (tumor-to-muscle ratio at day 1) 3 .

The model in Eq. (1) was validated by computing the inflation of R^2 due to overfitting *via* 100 bootstrapping samples as described in reference [31]. With each bootstrapped sample, we computed the difference between the R^2 based on the bootstrapped sample and the R^2 from the estimated model when applied to the original data. The mean of the difference (referred to as optimism in reference [31]) was then subtracted from the R^2 of our final model to adjust for overfitting.

3. Results

3.1 Sensitivity of BT474 and HR6 xenografts to trastuzumab

With trastuzumab treatment two times per week, the trastuzumab treated mice experienced a 61% [range: 23% – 82%] regression in tumor volume by day 8 while the vehicle treated mice experienced a 43% [range: 34% – 49%] increase in tumor volume, as displayed in Figure 2. On day 4, a marginal significant difference in tumor volume was observed ($P=0.057$) between the animal cohorts. Thus, we selected days 1 and 4 for the longitudinal treatment study so that we could test the hypothesis that ^{18}F -FLT-PET could separate trastuzumab-sensitive from resistant tumors before changes in tumor volume.

Longitudinal changes in tumor volume for the trastuzumab and vehicle treated BT474 cohorts are displayed in Figure 3a. In the vehicle treated group, tumor size increased slightly over a short period of four days; however, the increase was not statistically significant compared to baseline (day 1: $P=0.42$; day 4: $P=0.11$). Tumor growth was inhibited in the trastuzumab treated group, yet the decrease from baseline was marginally significant at day 4 ($P=0.059$). Difference in tumor volume between the trastuzumab and vehicle treated BT474 mice was also not statistically significant at day 1 ($P=0.28$). However, the difference between cohorts at day 4 was statistically significant ($P=0.019$), indicating that this model is sensitive to trastuzumab.

Longitudinal changes in tumor volume for the trastuzumab and vehicle treated HR6 cohorts are displayed in Figure 3b. Tumor size steadily increased over four days in the vehicle treated group yet differences in tumor volume after treatment were not statistically different compared to baseline (day 1: $P=0.39$; day 4: $P=0.24$). Tumor size also increased in the trastuzumab treated group; although, similar to the vehicle treated cohort, differences were not significant at any time point (day 1: $P=0.80$; day 4: $P=0.96$). Additionally, the difference in tumor volume between the trastuzumab and vehicle treated HR6 cohorts was not statistically different at any imaging time point (baseline: $P=0.18$; day 1: $P=0.14$; day 4: $P=0.18$), indicating that this model is resistant to trastuzumab.

To evaluate the impact of trastuzumab on xenografts with different trastuzumab sensitivities, differences in tumor volume between the trastuzumab treated BT474 and trastuzumab treated HR6 were evaluated. A difference in tumor volume was not observed at baseline ($P=0.38$) or day 1 ($P=0.23$), but a statistically significant difference was observed at day 4 ($P=0.035$).

3.2 ^{18}F FLT-PET assesses early treatment response in BT474 xenografts

Sequential ^{18}F FLT-PET images of a mouse from the trastuzumab and vehicle treated BT474 cohorts are displayed in Figure 4a. It can be observed from these images that ^{18}F FLT retention increases slightly after baseline in the vehicle treated animal and decreases after the initiation of treatment in the trastuzumab treated animal. Longitudinal changes in ^{18}F FLT accumulation (i.e., tumor-to-muscle ratios) in response to trastuzumab are shown for each cohort in Figure 4b. More precisely, tumor-to-muscle ratios in the vehicle treated BT474 mice were 1.4 ± 0.044 at baseline, 1.5 ± 0.053 at day 1, and 1.6 ± 0.10 at day 4, showing a marginal increase, although not significant, in ^{18}F FLT retention (compared to baseline: day 1,

$P=0.45$; day 4, $P=0.063$). In contrast, the tumor-to-muscle ratios in the trastuzumab treated BT474 mice were 1.5 ± 0.064 at baseline, 1.3 ± 0.030 at day 1, and 1.4 ± 0.041 at day 4, showing a gradual, yet not significant, decrease in ^{18}FLT retention (compared to baseline: day 1, $P=0.10$; day 4, $P=0.73$). No statistical difference was observed between the trastuzumab and vehicle treated BT474 cohorts at baseline ($P=0.72$). However, the tumor-to-muscle ratios were statistically significant between groups at days 1 ($P=0.030$) and 4 ($P=0.038$).

Ki67 immunohistochemistry (Figures 4c, d) supported the imaging findings at day 4 as a significant difference ($P=0.019$) in Ki67 score was observed between the trastuzumab and vehicle treated BT474 cohorts. While the TK1 staining was greater in the vehicle treated group, the difference between cohorts was not statistically significant ($P=0.35$).

3.3 No difference in ^{18}FLT -PET between trastuzumab and vehicle treated HR6 xenografts

Sequential ^{18}FLT -PET images of a mouse from the trastuzumab and vehicle treated HR6 cohorts are displayed in Figure 5a. It can be observed from these images that ^{18}FLT retention is similar across all time points for the vehicle treated animal, while ^{18}FLT retention appears to at first decrease and then increase in the trastuzumab treated animal. Longitudinal tumor-to-muscle ratios are displayed in Figure 5b for the trastuzumab and vehicle treated HR6 cohorts. More precisely, tumor-to-muscle ratios in the vehicle treated HR6 mice were 1.8 ± 0.16 at baseline, 1.5 ± 0.026 at day 1, and 1.7 ± 0.16 at day 4, showing no statistical differences in ^{18}FLT retention (compared to baseline: day 1, $P=0.18$; day 4, $P=0.59$). Similarly, the tumor-to-muscle ratios in the trastuzumab treated HR6 mice were 1.6 ± 0.17 at baseline, 1.5 ± 0.15 at day 1, and 1.7 ± 0.10 at day 4, showing no statistical differences in ^{18}FLT retention (compared to baseline: day 1, $P=0.34$; day 4, $P=0.20$). Additionally, differences in tumor-to-muscle ratios between the trastuzumab and vehicle treated HR6 groups were not significant at any time point during the study (baseline: $P=0.28$; day 1: $P=0.36$; day 4: $P=0.84$).

Immunohistochemistry results (Figures 5c, d) supported the imaging findings at day 4 as the Ki67 and TK1 scores between the trastuzumab and vehicle treated HR6 cohorts were not statistically different ($P=0.17$, $P=0.26$, respectively) even though the Ki67 and TK1 scores were almost 50% higher in the trastuzumab treated cohort. We hypothesize that the 50% increase in histological markers relating to cellular proliferation (i.e., Ki67 and TK1) is the effect of overcoming trastuzumab. We return to this point in the Discussion.

3.4 ^{18}FLT -PET differentiates trastuzumab-sensitive and resistant xenografts

Tumor-to-muscle ratios were not statistically different at baseline ($P=0.65$) or day 1 ($P=0.52$) between trastuzumab-sensitive and resistant cohorts. However, differences at day 4 were statistically significant ($P=0.020$), suggesting that ^{18}FLT -PET can differentiate between tumors that are sensitive and resistant to trastuzumab. Ki67 immunohistochemistry supported the imaging findings at day 4 as a significant difference ($P=0.041$) was observed between the trastuzumab-sensitive and resistant cohorts. The expected trend in TK1 staining (i.e., trastuzumab-sensitive tumors having a lower TK1 score) was observed, though the difference between cohorts was not statistically significant ($P=0.24$).

3.5 Early ^{18}FLT -PET correlates with late tumor volume

We were not able to find a significant effect of polynomial covariates (i.e., the cubic and quadratic forms of the tumor-to-muscle ratios) on prediction of tumor volume at day 4 using the likelihood ratio test. This assures that there is a strong linear association between tumor volume at day 4 and ^{18}FLT retention at day 1; thus, the selected linear regression model (i.e., Eq. (1)) is appropriate for modeling the present data. The results of the linear regression model are tabulated in Table 2. The overfitting-corrected R^2 value of the model fit was 0.94. Tumor-to-muscle ratio at day 1 is significantly correlated to tumor volume at day 4 ($P=0.0011$), thus suggesting that the tumor-to-muscle ratio at day 1 is significantly predictive of tumor volume at day 4.

4. Discussion

The objective of this study was to evaluate the potential utility of ^{18}FLT -PET to offer an earlier response assessment in HER2-overexpressing breast cancers treated with trastuzumab. We demonstrated the utility of ^{18}FLT -PET to assess early response to trastuzumab in the BT474 human breast cancer model. Shah et al. performed ^{18}FLT -PET before and after 1 week of trastuzumab therapy, and observed a significant difference between trastuzumab and vehicle treated BT474 cohorts [24]. Our study builds on the previous effort and suggests that ^{18}FLT -PET can detect a significant change as early as 24 hours after one trastuzumab treatment.

We are, however, the first to present longitudinal ^{18}FLT -PET data in a xenograft model that was engineered to exhibit trastuzumab resistance. We demonstrated that differences in ^{18}FLT retention were not observed between trastuzumab and vehicle treated mice with trastuzumab-resistant xenografts (i.e., HR6 cohorts). This result then allowed for the comparison between the so-called “responders” and “nonresponders”, or the trastuzumab treated BT474 and HR6 cohorts, respectively. We observed a significant difference in ^{18}FLT retention between cohorts, suggesting that ^{18}FLT -PET can differentiate between trastuzumab-resistant and sensitive xenografts. However, the detected change occurred at the same time point as a significant difference in tumor volume (i.e., at day 4); thus, ^{18}FLT -PET did not provide an early biomarker of treatment response when comparing these cohorts.

The objective of the immunohistochemistry analyses was to provide validation of the *in vivo* data by investigating the relationship between the imaging parameter and the underlying biology. Both the Ki67 and TK1 scores for the BT474 cohorts corresponded with the imaging data (Figure 4d); although, only the difference in Ki67 between the cohorts was statistically significant ($P=0.019$). Significant differences in Ki67 or TK1 scores were not observed between the trastuzumab and vehicle treated HR6 cohorts ($P>0.05$), which also corresponded with the imaging data (Figure 5d). The hypothesized trends in Ki67 and TK1 score of the trastuzumab-sensitive (treated BT474) and trastuzumab-resistant (treated HR6) were observed and corresponded with the imaging data, yet only the difference in Ki67 was significant ($P=0.041$).

The objective of analyzing the ^{18}FLT -PET and tumor volume data with a linear regression model was to determine if a relationship exists between ^{18}FLT retention at day 1 (i.e., after the first treatment dose) and tumor volume at day 4. The coefficient of determination is 0.94, suggesting that we are able to explain 94% of total variation in the tumor volume at day 4 with this model. Furthermore, the tumor-to-muscle ratios at day 1 significantly correlated with tumor volume at day 4 ($P=0.0011$), suggesting that early changes in ^{18}FLT correlated with later changes in tumor volume.

Our study had several limitations. It can be seen in Figures 4a and 5a that these tumors had large regions of necrosis, which is why we chose the maximum SUV instead of the mean of the ROI mean. We note that the ^{18}FLT uptake (as quantified by the SUV; see Figures 4a and 5a) is approximately 25% lower than what investigators have observed in different xenograft models [17,32]; however, the tumors in this study are composed of viable sections of tissue as assessed by Ki67 IHC (See Figures 4c and 5c). Additionally, it is well known that ^{18}FLT retention is dependent on the salvage pathway of thymidine synthesis [33,34], which is activated when a cell cannot carry out *de novo* synthesis of thymidine during DNA replication. Thus, investigators have recently suggested the importance of determining the contribution of the thymidine salvage pathway when ^{18}FLT -PET is used to image and monitor tumor proliferation [21,22]. Further evaluation on the dependence of the thymidine salvage pathway in our HER2-overexpressing breast cancer tumor models is needed in order to further validate ^{18}FLT -PET as an early marker of treatment response to trastuzumab. We did, however, perform TK1 IHC on a subset of animals from each vehicle-treated cohort. There was no statistically significant difference in TK1 score between the vehicle BT474 and HR6 cohorts ($P=0.057$, Wilcoxon Signed Rank), though there was a strong trend in that direction.

We noted (see Results above) that the trastuzumab treated HR6 tumors showed a marked (~50%) increase in Ki67 and TK1 staining as compared to the tumors from the vehicle treated HR6 cohort. This increase is indicative of enhanced proliferation. Our hypothesis for these observations is that the resistance of HR6 tumors to trastuzumab may be the result of aberrant activation of cell signaling networks that enhance proliferation and survival. It was proposed previously that the HR6 cell line might escape trastuzumab action through the activation of PI3K/AKT pathway [25], which would enhance cellular survival, growth, and proliferation [35]. A subsequent report of theirs indicated that inhibition of mTOR (a major downstream effector of the PI3K/AKT pathway) suppressed growth of HR6 cells, and that inhibition of PI3K and mTOR were required for the growth-inhibitory effects of HER2 antagonists including trastuzumab [36]. Future studies envisage testing the hypothesis that trastuzumab resistance results from the activation of proliferation and survival pathways by, for example, quantifying Ki67, TK1, and other markers of cellular proliferation as a function of trastuzumab dose (with and without inhibitors of PI3K and/or mTOR). Such studies are anticipated to elucidate the postulated relationship between TK1 expression and trastuzumab resistance in HER2-overexpressing tumors used in this study.

^{18}FLT -PET is an emerging molecular imaging technique that offers noninvasive measurements of cellular proliferation. However, the practical utility of ^{18}FLT -PET to assess early treatment response in HER2-overexpressing breast cancer has not yet been

adequately addressed, thus emphasizing the need to systematically evaluate this technique in different cancer types and treatment regimens. Our data suggest that ^{18}F -FLT-PET is sensitive to early molecular changes in trastuzumab-sensitive HER2-overexpressing breast cancer xenografts, and can also differentiate mouse models of HER2-overexpressing breast cancer with varying trastuzumab sensitivities. The latter result potentially has significant clinical impact as the development of imaging methods that can identify nonresponders earlier during the course of therapy is of great interest in order to spare patients a delay in initiation of effective combination therapies.

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Figure 1.

Longitudinal imaging and trastuzumab treatment study schema. Mice were treated twice in four days with either trastuzumab (10 mg/kg) or saline vehicle. Imaging data (both PET and MRI) were acquired at baseline and 24 hours post the first and second treatments.

Figure 2.

Longitudinal tumor volume (mean $\pm$ SE) from the preliminary dose-response analysis is displayed at each imaging time point for trastuzumab and vehicle treated cohorts. Tumor volume increased by an average of 43% over the eight days in the vehicle treated group, yet differences in tumor volume compared to baseline were not significant. Tumor volume decreased with an average of 61% in the trastuzumab treated group, although not significantly when compared to baseline. The difference in tumor volume between the cohorts at day 4 was marginally significant ($P=0.057$).

Figure 3.

Longitudinal tumor volume (mean $\pm$ SE) data for: (a) trastuzumab and vehicle treated BT474 cohorts, (b) trastuzumab and vehicle treated HR6 cohorts. Note the drug sensitivity of the trastuzumab treated BT474 cohort as tumor volume decreased after initiation of therapy, while tumor volume in the vehicle treated BT474 group increased. Also note the insensitivity to trastuzumab in the trastuzumab treated HR6 cohort as tumor volume increased throughout the course of the study. * $P < 0.05$

Figure 4.

Longitudinal ^{18}FLT -PET images of representative vehicle (top row) and trastuzumab (bottom row) treated animals from the BT474 cohorts (panel a). Tumor xenografts are denoted in each image with a dashed circle. ^{18}FLT retention increased longitudinally in the vehicle BT474 mouse, in contrast to the observed decrease in ^{18}FLT retention in the BT474 mouse that received trastuzumab. The region with high ^{18}FLT uptake in the bottom of each subpanel is the bladder. Differences in ^{18}FLT uptake between the trastuzumab and vehicle treated BT474 cohorts were observed as early as 24 hours after initiation of therapy (panel b), which preceded the observed difference in tumor volume (see Figure 3a); thus, ^{18}FLT -PET provided an early assessment of tumor response in the BT474 model of HER2-overexpressing breast cancer. Representative tumor sections for Ki67 ($\times 20$) and TK1 ($\times 20$) are displayed for each cohort in panel c. A quantitative analysis of the immunohistochemistry data (d, mean $\pm$ SE) showed that the observed difference in Ki67 score was significant ($P=0.019$); thus, validating the observed *in vivo* ^{18}FLT -PET data at day 4. The hypothesized trend in TK1 score was observed (i.e., lower TK1 expression in the

trastuzumab treated cohort), but the difference was not statistically significant between groups ($P=0.35$). $*P<0.05$

Figure 5.

Longitudinal ^{18}FLT -PET images of representative vehicle (top row) and trastuzumab (bottom row) treated animals from the HR6 cohorts (panel a). Tumor xenografts are denoted in each image with a dashed circle. ^{18}FLT retention appeared to stay relatively constant from baseline to day 1 and increased at day 4 in the vehicle treated HR6 mouse, whereas ^{18}FLT retention appeared to decrease at day 1 and then increase at day 4 in the trastuzumab treated HR6 mouse. The region with high ^{18}FLT uptake in the bottom of each subpanel is the bladder. No statistical difference in ^{18}FLT retention was observed between the trastuzumab and vehicle treated HR6 cohorts at any time point during the study (panel b; baseline: $P=0.28$; day 1: $P=0.36$; day 4: $P=0.84$), even though one cohort was receiving a therapy that was meant to alter cell proliferation. Representative tumor sections for Ki67 ($\times 20$) and TK1 ($\times 20$) are displayed for each cohort (c). Statistically significant differences between cohorts were not observed with either Ki67 ($P=0.17$) or TK1 ($P=0.26$) scores (d). Note that both Ki67 and TK1 scores are approximately 50% higher in the trastuzumab treated HR6 cohort as compared to the vehicle treated cohort. We hypothesize that the

observed increase in histological markers relating to cellular proliferation is the effect of these tumors overcoming trastuzumab. The interested reader should look to the Discussion for a more detailed description of this hypothesis.

Table 1

Tabulated list of animal number for each cohort and time point

Time point	BT474 treated	BT474 vehicle	HR6 treated	HR6 vehicle
Baseline	8	5	8	6
Day 1	8	5	8	6
Day 4	6	4	7	6

Table 2

Results of the linear regression model

	Estimate	Standard Error	<i>p</i> -value
Intercept	−353	75	< 0.001
Volume at day 0	1.2	0.074	< 0.001
Tumor-to-muscle ratio at day 1	204	54	0.0011