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Evaluation of the anti-HER2 C6.5 diabody as a PET radiotracer to monitor HER2 status and predict response to trastuzumab treatment

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Abstract

Purpose—The rapid tumor targeting and pharmacokinetic properties of engineered antibodies make them potentially suitable for use in imaging strategies to predict and monitor response to targeted therapies. This study aims to evaluate C6.5 diabody (C6.5db), a non-covalent anti-HER2 single chain-Fv dimer, as a radiotracer for predicting response to HER2-targeted therapies such as trastuzumab.

Experimental Design—Immunodeficient mice bearing established HER2-positive tumor xenografts were injected with radioiodinated C6.5db and imaged using PET/CT. Radiotracer biodistribution was quantified using biopsied tumor and normal tissues. Potential competition between trastuzumab and C6.5db was examined *in vitro* by flow cytometry and co-immunoprecipitations.

Results—Biodistribution analysis of mice bearing xenografts with varying HER2 density revealed that the tumor uptake of ¹²⁵I-C6.5db correlates with HER2 tumor density. *In vitro* competition experiments suggest that the C6.5db targets an epitope on HER2 that is distinct from that bound by trastuzumab. Treatment of SK-OV-3-tumored mice with trastuzumab for 3 d caused a 42% (P=0.002) decrease in tumor uptake of ¹²⁵I-C6.5db. This is consistent with a dramatic decrease in the tumor PET signal of ¹²⁴I-C6.5db after trastuzumab treatment. Furthermore, BT-474-tumored mice showed a ~60% decrease (P=0.0026) in C6.5db uptake after 6 d of trastuzumab treatment. Immunohistochemistry of excised xenograft sections and *in vitro* flow cytometry revealed that the decreased C6.5db uptake upon trastuzumab treatment is not associated with HER2 downregulation.

Conclusions—These studies suggest that ¹²⁴I-C6.5db-based imaging can be used to evaluate HER2 levels as a predictor of response to HER2-directed therapies.

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Conflict of Interest: Matthew K. Robinson is a member of the scientific advisory board of Avipep Pty, Ltd. Avipep has licensed the C6.5db from University of California, San Francisco. James D. Marks is an inventor of the C6.5db that was licensed to Avipep Pty, Ltd

Keywords

antibody; HER2; breast cancer; trastuzumab; PET imaging

Introduction

Our understanding of the molecular processes that drive both cancer formation and progression has increased dramatically in recent years. This has led to development of targeted therapeutics designed to disrupt specific cancer-associated processes. Development of companion diagnostics is hypothesized to aid in stratifying patients based on the molecular underpinnings of their disease and thus improve the clinical implementation of these targeted therapies. Molecular imaging agents capable of either detecting the levels of biomarkers or monitoring changes in the biomarkers in response to therapy have the potential to make an important contribution to effective disease management.

The human epidermal growth factor receptor family of receptor tyrosine kinases (RTKs) is known to play a critical role in the normal development and homeostasis of a variety of tissues (1). As such, inappropriate signaling through this family of RTKs is associated with formation and progression of a number of cancers (2). This is exemplified by the role of the human epidermal growth factor receptor 2 (HER2) RTK in breast cancer (3,4). Overexpression of the HER2 protein leads to inappropriate activation of signaling pathways downstream of the RTK (5) and is associated with poor clinical outcome and a high risk of relapse (6). This aggressive subtype of breast cancer (BrCa) accounts for approximately 20 – 30 % of all BrCa. The anti-HER2 monoclonal antibody (mAb) trastuzumab blocks unregulated signaling associated with HER2 overexpression (7). Diagnosis of HER2-positive BrCa is made through measuring either overexpression of HER2 protein by immunohistochemical staining (IHC) or gene-amplification via fluorescence *in situ* hybridization (FISH) techniques in biopsied primary tumor. Despite meeting current diagnostic criteria for HER2-positive BrCa, only a third of eligible patients in the metastatic setting respond to single-agent trastuzumab treatment (8). Although combining trastuzumab with chemotherapy increased response rates in both the adjuvant (9-11) and metastatic (12-14) setting, responders are seen to relapse despite continued treatment. This intrinsic and acquired resistance can in principle be due to a number of reasons including discordance in the HER2 expression in primary versus metastatic lesions as was seen by Zidan et al (15).

Whole-body, non-invasive, molecular imaging strategies have the potential to extend the analysis of HER2-status to biopsy-inaccessible lesions. To that end, the diagnostic potential of positron emission tomography (PET) and single-photon emission computed tomography (SPECT) imaging with radiolabeled trastuzumab is being investigated in multiple phase I clinical trials (16,17). The pharmacokinetics (PK) of intact IgG molecules, although appropriate for therapeutic strategies, is not optimal for imaging. Their long half-life requires that imaging be performed multiple days post-injection in order to achieve sufficient blood clearance and optimal tumor:blood ratios. Advances in antibody engineering have facilitated the development of engineered antibody fragments that retain the antigen-binding specificity of mAbs but have tumor targeting and pharmacokinetic properties optimized for use as targeting vehicles for payloads, such as diagnostic or therapeutic radionuclides, chemotherapeutics, or toxins, based on the cell surface expression of tumor associated markers, like HER2 (18-23). We previously demonstrated that an anti-HER2 single chain Fv (scFv)-based antibody molecule, called C6.5db, can function as an effective PET radiotracer in xenograft models of HER2-positive disease (19). In this study, we expand our analysis of the C6.5db to understand both how antigen expression and

trastuzumab therapy impact on the function of the C6.5db in targeting HER2-positive tumors in our mouse models.

Materials and Methods

Cell culture

SK-OV-3 cells (ATCC# HTB-77), MDA-MB231 (ATCC# HTB-26), and BT-474 (ATCC# HTB-20) cells were purchased from the American Type Culture Collection. MDA-MB361/DYT2 was a kind gift from Dr. Dajun Yang (Georgetown University, Washington DC). SK-OV-3 cells were cultured in DMEM/HEPES, and MDA-MB361/DYT2, MDA-MB231, and BT-474 cells were cultured in DMEM/F12 medium under 5% CO₂ at 37°C. All media were supplemented with 10% fetal bovine serum (FBS).

Production and radioiodination of C6.5db

C6.5db was expressed in TG1 *Escherichia coli*, and purified by immobilized metal ion chromatography followed by high performance liquid chromatography size-exclusion chromatography over a Superdex 75 column (Amersham Pharmacia, Piscataway, NJ) as previously described (19). Protein was stored at -70°C at 0.5 mg/mL in PBS containing 10% glycerol until ready for use.

C6.5db was radioiodinated with Na¹²⁵I (cat # NEZ033H, DuPont NEN, Wilmington, DE) or Na¹²⁴I (RITVERC Isotope Products, St. Petersburg Russia) using either Iodogen-coated glass beads or with the water-soluble form of Bolton-Hunter reagent Sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate (SHPP, cat # 27712, Pierce Biotechnology) as described in Robinson et al (19). Immunoreactivity of the radiolabeled preparations were assayed by live-cell binding assays with SK-OV-3 cells under conditions of antigen excess as described previously (24).

Flow cytometry

SK-OV-3 and BT-474 cells were grown to subconfluence in T75 flasks, rinsed with Hank's solution containing 1 mM EDTA, and harvested with trypsin. Cells were pelleted down by centrifugation at 100 g for 5 min, resuspended in ice-cold FACS buffer (PBS containing 1% bovine and 0.1% sodium azide), and counted on a hemocytometer. About 250,000 cells were used per condition. Cells were exposed to 5 ug of rituximab, trastuzumab, or pertuzumab for 30 min on ice, washed with 1 mL of ice-cold FACS buffer, and centrifuged at 100 ×g for 5 min. Cells were then treated with 5 ug of C6.5db for 30 min on ice, washed with 1 mL of ice-cold FACS buffer, recovered by centrifugation and treated with secondary antibody for 30 min on ice, washed, centrifuged, and resuspended in 100 uL of 1% paraformaldehyde in PBS. In the reciprocal experiment, cells were first treated with either C6.5db or PBS on ice followed by treatment with rituximab, trastuzumab, or pertuzumab. C6.5db was detected with Alexa Fluor 488 conjugated anti-6×His secondary antibody (Qiagen, Inc, Valencia, CA) while human IgGs were detected with FITC-conjugated anti-human IgG (Milipore, Billerica, MA). Flow cytometry was conducted using a FACScan flow analyzer (BD BioSciences). Non-stained cells and cells stained with secondary antibody alone (no primary antibody) were included as negative controls in each experiment. Each experiment was repeated at least three times. Data were analyzed using FlowJo software (version 8.8.6; Stanford University).

Immunoprecipitation

HER2 ECD was expressed and purified from stable clones of HEK 293 cells as described previously (25). HER2 was coupled to Aminolink resin following the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). HER2-coupled beads were mixed with

10-fold molar excess of rituximab, trastuzumab, or an equal volume of PBS, and rocked at room temperature for 1 h to allow binding to occur. Beads were then washed four times with vendor provided wash buffer, followed by incubation with 10-fold molar excess of C6.5db for 1 h. Beads were washed four times with wash buffer, pelleted down, mixed with 4× LDS sample buffer (Invitrogen, Carlsbad, CA) containing 40 mM dithiothreitol (DTT), and boiled at 70°C for 10 min. Samples were then spun down, equal volumes of the supernatants were loaded onto 4-12% gradient Bis-Tris gels (Invitrogen, Carlsbad, CA), and transferred onto nitrocellulose membranes. Blots were blocked with 5% nonfat milk in TBST (0.1% Tween 20 in Tris-buffered saline), and C6.5db was detected by sequentially probing the membrane with anti-His antibody (#34670 Qiagen, Valencia, CA) and horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (#NA931V Amersham, Piscataway, NJ). Proteins were detected using Supersignal West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). Blots were then stripped with Restore western blot stripping buffer (Pierce Biotechnology, Rockford, IL), and reprobed with HRP-conjugated sheep anti-human antibody (#NA933V Amersham, Piscataway, NJ) to detect rituximab and trastuzumab.

Biodistribution and PET/CT imaging

CB.17/ICR severe combined immunodeficient (SCID) mice aged 6-8 weeks were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. SK-OV-3, MDA-MB361/DYT2, and MDA-MB231 cells were implanted s.c in the inguinal region of mice at a density of 3×10^6 cells per mouse. At ~ 6 weeks post-implantation, 0.2% Lugol's solution was added to the drinking water to block thyroid accumulation of radioiodine and biodistribution studies were conducted as described previously (23). Cohorts of mice ($n = 5$) received radioiodinated C6.5db via tail-vein injection and blood samples ($\leq 70 \mu\text{L}$) were collected at 5 minutes p.i. and just prior to euthanasia. Animals were dissected and major organs were weighed and counted in a γ -well counter (Cobra Quantum, Packard Instruments, Meriden, CT). The retention of the radiolabel in tumor and normal tissues was expressed as a percentage of the injected dose per gram of tissue (% ID/gm). Values are reported as the mean and standard error of mean (SEM).

Individual cohorts of mice, injected with ^{124}I -C6.5db, were imaged on a clinical Discovery LS clinical PET/computed tomography (CT) scanner (GE Healthcare, Milwaukee, WI) using a custom-built acrylic holder mounted on a patient bed as previously described (19). In addition, to the CT scan for attenuation correction, a CT scan with 0.6 mm slice thickness was also acquired for image registration. PET was acquired for 10 minutes in 2-D mode. PET images were reconstructed on a 128×128 matrix for a 30 cm-diameter field of view using ordered subsets expectation maximization (OSEM) algorithm. The PET, CT and fused images were visualized with the MIM software package (MIMvista Corp., Cleveland, OH).

For trastuzumab/rituximab blockade experiments with SK-OV-3 xenografts, cohorts of mice ($n = 5$) were implanted s.c. with 3×10^6 cells per mouse. Mice were treated with trastuzumab (10 mg/ Kg) or rituximab (10 mg/ Kg) combined with excess IgG2A (50 mg/ Kg) using the following two dosing regimens: A) one dose administered 3 d prior to injection of radioiodinated C6.5db or B) two doses administered 6 d and 3 d prior to injection of radioiodinated C6.5db. Tumors were subjected to IHC in formalin-fixed paraffin-embedded sections using antigen retrieval and staining with the anti-HER2 mAb, CB 11 (Biogenex, San Ramon, CA).

Statistics

Statistical analyses to determine outliers (Grubbs test) and comparisons between different cohorts of mice (unpaired t test) were carried out using the online version of GraphPad

(GraphPad Software, Inc.). Animals with uptake values that were considered outliers by the Grubbs test were removed from the analysis.

Results

Radiotracer preparations

The C6.5db was radiolabeled directly on tyrosine residues using Iodogen or indirectly on lysine residues using SHPP with efficiencies of 20 – 38% and 13 - 16%, respectively. Preparations ranged from 95% - 97.5% radiochemical purity as measured by instant thin layer chromatography (TLC) and the purified radiotracer had a specific activity of 0.5 – 0.57 $\mu\text{Ci}/\mu\text{g}$ protein. Immunoreactivity of all the preps, except that used for Figure 1, ranged from 69% - 78% active. The ^{124}I -SHPP-C6.5db preparation used to evaluate the impact of tumor size on targeting had a 43.5% immunoreactivity.

C6.5db uptake correlates with antigen expression

Effective use of the C6.5db as an immunoPET radiotracer requires that it accumulate to sufficiently high levels within a tumor to be detectable by the PET scanner, while maintaining a high tumor: normal tissue contrast. Antibody-based radiotracers target and are retained in tumor based on their binding to their cognate antigen on the tumor cell surface. Here, we examined the impact of two variables, tumor size and the density of HER2 on the tumor cell surface, on the ability of C6.5db to target and image subcutaneous xenografts in a SCID mouse model.

SCID mice received either two or four s.c. injections of SK-OV-3 tumor cells (1×10^6 copies HER2 / cell) at discrete anatomical locations at combinations of 2, 4, 6, and 8 weeks prior to intravenous (i.v.) injection of radioiodinated C6.5db. These implantations resulted in tumor sizes that ranged from 25 - 701 mg. In order to minimize non-specific uptake in iodine metabolizing tissues such as the thyroid and stomach, C6.5db was indirectly radiolabeled with ^{124}I -SHPP and administered to animals and mice were then imaged 24 or 48 hours (h) post injection (p.i.) on a clinical PET/CT scanner (Discovery LS PET/CT, GE Healthcare) as described previously (19). As seen in Figure 1, at 48 h post-injection [^{124}I -SHPP]-C6.5db demonstrated sufficient targeting of tumors in this model to allow for effective imaging of tumors weighing at least 50 mg ($\sim 4 \text{ mm}^3$). Quantification of tumor uptake, based on biodistribution analysis and the specific activity of the radiotracer, showed that 0.02, 0.06, 0.12, and 0.58 μg [^{124}I -SHPP]-C6.5db accumulated in the 25, 50, 95, and 360 mg tumors, respectively, at the time of imaging.

The impact of antigen density on tumor targeting of C6.5db was examined in a series of experiments using three different tumor cell lines (SK-OV-3, MDA-MB361/DYT2, and MDA-MB231) that differ in their levels of HER2 expression. The IHC staining patterns of tumor xenografts grown in SCID mice corresponds with the level of HER2 found on the surface of these cells when grown *in vitro* (data not shown). At 24 h p.i., tumor uptake of the ^{125}I -C6.5db into each of the xenografts positively correlated with levels of HER2 expressed on the tumor cell surface (Table 1). At 24 h p.i. the approximate 3-fold higher level of HER2 on the surface of SK-OV-3 (1×10^6 HER2 per cell) as compared to MDA-MB361/DYT2 (3.7×10^5 HER2 per cell) positively correlated with a statistically significant 1.7-fold higher uptake ($P = 0.002$) of C6.5db in SK-OV-3 xenografts. The tumors in these two groups did not differ significantly ($P = 0.06$) in size. Consistent with the very low level of HER2 expression on the surface of MDA-MB231 cells (2.3×10^4 HER2 per cell), tumor uptake of the C6.5db in these xenografts did not differ significantly from that seen in blood, and was 6.2-fold ($P < 0.0001$) and 3.6-fold lower ($P < 0.001$) than that seen in SK-OV-3 and MDA-MB361/DYT2 xenografts, respectively. Using blood levels as a measure of clearance

rates, no significant difference was seen between the clearance rates of the C6.5db from animals in the three groups. The increased tumor uptake in SK-OV-3 (0.76 ± 0.25 %ID/g) as compared to MDA-MB361/DYT2 (0.41 ± 0.04 %ID/g) was maintained at 48 h p.i., but was not statistically significant ($P = 0.20$), perhaps due to the fact that the MDA-MB361/DYT2 tumors were larger than the SK-OV-3 tumors analyzed at this time point (0.69 ± 0.2 g vs. 0.25 ± 0.1 g). Consistent with uptake seen at 24 h p.i., uptake into MDA-MB231 tumors at 48 h p.i. did not differ from that seen in blood (0.13 ± 0.01 vs 0.1 ± 0.01 %ID/g, $P = 0.07$) and was 7.6-fold ($P = 0.02$) and 4.1-fold ($P = <0.001$) lower than uptake into SK-OV-3 and MDA-MB361/DYT2 tumors, respectively.

The C6.5db Binds to an Epitope on HER2 Distinct from that Bound by Trastuzumab

Because of trastuzumab's role in the treatment of HER2-positive disease, its HER2-binding activity was taken into account as part of the development of the C6.5db for use as either a diagnostic radiotracer or therapeutic. We examined the ability of C6.5db and trastuzumab to compete for binding to HER2 in a series of *in vitro* experiments. Pretreatment of either SK-OV-3 or BT-474 cells (Figure 2A) with a saturating concentration of trastuzumab at 4°C did not inhibit the ability of C6.5db to bind to the cells as compared to treatment with an equal concentration of rituximab, the FDA-approved anti-CD20 mAb (26). In a reciprocal experiment, pretreatment with C6.5db also failed to compete for subsequent trastuzumab binding (Figure 2B). Co-immunoprecipitation experiments using purified HER2 extracellular domain (ECD; (25)) provided further support for this finding (Figure 2C). Immobilized HER2 ECD pretreated with a saturating concentration of trastuzumab was able to bind to and co-precipitate C6.5db at levels equal to that seen with HER2 ECD beads pretreated with either an equal amount of rituximab or vehicle as controls. In contrast, the anti-HER2 mAb pertuzumab (27) effectively competed the binding of C6.5db to both SK-OV-3 and BT-474 cells (Figure 2D) as compared to the rituximab control. In the reciprocal experiment C6.5db was able to partially compete the binding of pertuzumab (Figure 2E). Taken together, these data suggest that the C6.5db binds to an epitope distinct from that bound by trastuzumab (28). Rather C6.5db appears to bind to HER2 near, but probably distinct from, the epitope recognized by pertuzumab (27). This interpretation is further supported by the observation that intact IgG molecules based on the C6.5 scFv do not exhibit the therapeutic activity associated with pertuzumab (29).

Trastuzumab Treatment Inhibits Tumor Targeting by the C6.5db *In Vivo*

Based on our *in vitro* experiments that indicate trastuzumab and the C6.5db do not compete for HER2 binding we hypothesized that the C6.5db could effectively target HER2-positive tumors in mice pretreated with trastuzumab. Cohorts of SCID mice ($n = 5$) bearing SK-OV-3 tumor xenografts (mean tumor size=147 mg) were pretreated with a single dose of trastuzumab three days prior to administration of radioiodinated C6.5db. A second cohort of animals was pre-treated with the anti-CD20 IgG rituximab to control for non-specific effects associated with bulk IgG levels in the SCID mice. Tumor regression was not seen in response to trastuzumab-therapy over the 3-day treatment regimen (data not shown) but biodistribution analysis of ^{125}I -C6.5db at 24 h p.i. (Table 2) revealed a statistically significant 42% decrease ($P = 0.002$) in tumor uptake of the radiotracer in animals pretreated with trastuzumab (1.58 ± 0.08 %ID/g) as compared to those pretreated with rituximab (2.71 ± 0.24 %ID/g). This decrease in radiotracer tumor targeting is evident in PET images of mice receiving ^{124}I -C6.5db and imaged 24 h p.i. (Figure 3). Tumor uptake in animals treated with either trastuzumab or rituximab for six days showed the same statistically significant decrease ($P = 0.002$) in tumor uptake in the trastuzumab treated cohort (1.16 ± 0.20 %ID/g) as compared to the rituximab-treated controls (2.29 ± 0.16 %ID/g). This decrease in C6.5db uptake was also seen in SCID mice bearing s.c. BT-474 BrCa xenografts pretreated with trastuzumab for six days (Table 3). Animals pretreated with trastuzumab showed a ~60%

decrease in tumor uptake of ^{125}I -C6.5db at 24 h p.i. as compared to rituximab treated controls (%ID/g of 1.42 ± 0.18 for trastuzumab vs. 3.55 ± 0.39 for rituximab, $p=0.003$). This loss of tumor targeting does not correlate with a large decrease in HER2 levels on the surface of the tumor cells. SK-OV-3 tumor xenografts from mice treated for up to one week with either trastuzumab or rituximab were subjected to IHC. As seen in Figure 4A, SK-OV-3 and BT-474 tumors from trastuzumab treated animals exhibited similar HER2 staining patterns to those from animals treated with rituximab. Consistent with these results, treatment of SK-OV-3 and BT-474 (Figure 4B) cells *in vitro* with trastuzumab (10 $\mu\text{g/mL}$) over a time course of 72 hours failed to decrease the levels of HER2 on the cell surface when measured by FACS with a non-competing anti-HER2 antibody. Interestingly, despite a lack of physical competition for binding epitopes, trastuzumab treatment inhibited C6.5db binding, as measured by FACS, compared to non-treated controls over the same 72-hour time frame. The exact mechanism by which trastuzumab treatment inhibits C6.5db binding is not yet understood, and studies are underway to investigate this further.

Discussion

The quantitative nature of PET facilitates the accurate measurement of tracer concentration within a lesion and such measurements correlate well with those obtained through standard biodistribution studies (19,30,31). Monoclonal antibodies, combined with flow cytometry, have long been used to quantitatively measure the expression of cell surface proteins. This has led us, and others, to hypothesize that antibody-based radiotracers, coupled with PET, can be used to measure antigen expression *in vivo*. In addition, significant data in the literature suggests that smaller antibody fragments, affibodies, or engineered antibody fragments are poised to be more effective than intact mAbs as PET radiotracers due to their faster blood clearance and higher tumor:background ratios (32-34)).

At the most basic level, response to mAb-based therapies requires that the target protein be expressed on the surface of tumor cells, and that the therapeutic mAb effectively target and accumulate to sufficient levels within the tumor. The ability to monitor each of these variables has the potential to guide patient selection and treatment plans. In the setting of HER2-positive breast cancer, response to trastuzumab positively correlates with the level of HER2 expression. High-level overexpression in biopsied tumor tissue, as measured by IHC or FISH, is the critical criteria for treatment eligibility. Thus we and others (34) have speculated that a molecular imaging based approach to evaluate HER2 expression across a patient's entire tumor burden could provide a more complete analysis of HER2 expression, potentially providing a better prediction of initial response to trastuzumab-based therapy or even obviating the need for invasive biopsy procedures. The data we present here demonstrates that tumor uptake of the C6.5db is dependent upon antigen density on the surface of the tumor cells and suggest that C6.5db-based radiotracers may be useful for evaluating the levels of HER2 expression on tumor cells *in vivo*, and by extension predicting initial response to trastuzumab therapy. Data from Cai et al (35) suggests that this approach may be applicable to target antigens beyond HER2. Uptake of [^{64}Cu -DOTA]-cetuximab correlated with the level of EGFR expression across a number of tumor models, suggesting that immunoPET-based determination of antigen density could be applied to a broader range of target antigens.

Biological properties of the target antigen, the strategy employed to radiolabel the tracer, and the intended imaging application are all critical components in radiotracer design. ImmunoPET images obtained with a residualizing radionuclide, such as the ^{64}Cu or ^{89}Zr used to label cetuximab by Cai (35) and Aerts (36), depict the cumulative antibody bound to, and internalized by the cells over the course of the experiment. This is ideal for the purpose of lesion detection but potentially suboptimal for monitoring antigen levels. When targeting

rapidly internalizing antigens, such as EGFR or HER2, the long half-life of intact mAbs coupled with residualizing radionuclides would be predicted to obscure internalization rates, and thereby provide an inaccurate estimate of level of antigen expression. The radiohalogen ^{124}I has a physical half-life that pairs appropriately with the biological half-lives of mAb-based tracers (33). However, it has been speculated that ^{124}I is inappropriate for labeling of mAbs because internalization and degradation leads to rapid loss of the iodine from cells resulting in both insufficient tumor:normal tissue contrast for imaging (37) and unwanted uptake by tissues, such as the thyroid, that express the Na/I symporter. Engineered antibodies such as the C6.5db have the potential to function as effective radiotracers, in part, because their rapid systemic clearance leads to positive tumor-to-blood ratios early after administration (19). Our results demonstrate that sufficient uptake of ^{124}I -C6.5db is achieved to afford PET detection of tumors as small as 50 mg at 48 h p.i. in our preclinical model. In addition, we argue that ^{124}I -C6.5db, and by extension other ^{124}I -mAbs, provide a representation of the antibody bound to the tumor cell surface at the time of imaging, thus decreasing the impact of internalization rates on tumor signal and potentially providing a more quantitative approach to measuring either inherent differences in antigen expression between tumors or changes in antigen expression within a tumor in response to therapy. However, the positive impact of the rapid clearance of C6.5db is balanced by its negative effect on the limiting time available for the antibody to accumulate to high levels in the tumor (38). This rapid clearance could in principle be exploited to enable the use of residualizing isotopes with similar quantitative results. Although thyroid uptake was not quantified in this study we have demonstrated in previous work that the use of ^{124}I in conjunction with a partially residualizing labeling strategy (e.g. SHPP) does not dramatically alter the performance of the C6.5db as a radiotracer and decreases thyroid uptake (19). This suggests that such a labeling strategy could be used in conjunction with thyroid blocking to reduce thyroid exposure in patients and still provide quantitative analysis of HER2 levels.

Accumulation of antibody-based therapeutics to sufficient levels within a tumor is essential for therapeutic efficacy. The decrease in overall tumor uptake seen with the C6.5db upon trastuzumab treatment implies that trastuzumab is effectively targeting tumor in our preclinical models to induce this effect. It is intriguing to speculate that molecular imaging with agents such as the C6.5db, when used in the clinical setting, could potentially shed light on whether and how trastuzumab is targeting lesions in a patient. In addition, significant effort is ongoing in the preclinical setting to understand both how physical properties of mAbs (e.g. intrinsic affinity, molecular size, PK) dictate tumor targeting and how those properties can be modified to improve antibody targeting (for review see (39)). Imaging strategies that can function as companion diagnostics during the development process have the potential to aid in translation of new therapeutic antibodies during translation into the clinic.

Antibody-based cancer therapeutics can induce anti-tumor effects through a number of mechanisms of action including inhibiting signal transduction and/or focusing the anti-tumor effects of the immune system (40). Since its initial approval more than a decade ago trastuzumab has become standard-of-care for HER2-positive breast cancer. Despite this fact, trastuzumab's mechanism of action has yet to be definitively identified. It most likely functions through multiple processes including antibody-dependent cellular cytotoxicity (ADCC), inhibiting HER2 shedding, and blocking signaling (for review see (41)). Although somewhat controversial (42) and its relevance to the clinical setting not yet fully demonstrated (43-45), trastuzumab-induced down-regulation of HER2 has also been reported in both *in vitro* cell culture (46,47) and xenograft models (48,49). Our IHC and FACS results are in alignment with clinical findings, in that trastuzumab treatment failed to induce detectable levels of HER2 down-regulation in our model systems. Consistent with our findings, McLarty et al report that trastuzumab treatment (4 mg/kg) of athymic mice

bearing MDA-MB361 xenografts followed by SPECT imaging (3 d p.i. of trastuzumab) with ^{111}In -diethylenetriaminepenta-acetic acid-pertuzumab (^{111}In -DTPA-pertuzumab) showed a significant decrease in the tumor uptake of ^{111}In -DTPA-pertuzumab, despite no apparent decrease in HER2 levels by IHC (32). Interestingly, chronic treatment (3 weeks) induced a significant decrease in HER2 levels by IHC, and was associated with loss of HER2-positive tumor cells. In our studies, chronic treatment of mice bearing SK-OV-3 or BT-474 xenografts failed to induce an obvious change in HER2 expression (data not shown), similar to the situation seen in the clinic (43-45). It is worthy of note in this context that when McLarty et al compared trastuzumab-induced changes in HER2 density between SKBR-3 (high HER2 expression) and MDA-MB361 (moderate HER2 expression) BrCa cells in culture, they found that the effects of trastuzumab on HER2 density was more profound in MDA-MB361 cells than SKBR-3 cells (32). One possible explanation for the apparent differences in HER2 down-regulation seen in these studies may be cell line-dependent variability in receptor down-regulation.

Despite an inability to detect down-regulation of HER2 by FACS and IHC, our results are consistent with those of other groups (42,50) and suggest that *in vivo* targeting of ^{124}I -C6.5db is perturbed as an early response to trastuzumab-based therapy. This is particularly true in the context of the results obtained with ^{111}In -DTPA-pertuzumab and our data demonstrating that the C6.5db binds to HER2 near the epitope bound by pertuzumab. The mechanism by which trastuzumab treatment inhibits the targeting of both pertuzumab and C6.5db-based PET radiotracers is unclear. The epitopes for C6.5db and pertuzumab are located in domain II of the HER2 extracellular domain, distinct from the domain IV epitope bound by trastuzumab (51). This, coupled with the inability of trastuzumab to compete with C6.5db for HER2 binding, suggested that the therapeutic levels of trastuzumab circulating in the animals, or by extension patients, should not compete for HER2 binding and therefore should not have resulted in the decreased *in vivo* targeting, nor the time-dependent decrease in binding to cells treated with trastuzumab in culture. In light of trastuzumab's complicated mechanism of action, it is interesting to speculate that trastuzumab treatment results in a physical change to the receptor, such as altered clustering or dimerization patterns. Data from Kani et al (52) demonstrating that binding of antibodies to HER2 alters its partitioning in the membrane, particularly with regards to localization with HER3, coupled with recent data from Junttila et al (53) demonstrating that trastuzumab inhibits ligand-independent signaling through the HER2/HER3 heterodimer supports this hypothesis. The manner in which this would result in decreased binding of C6.5db is not yet clear. Altered packing of the receptor may result in steric inhibition of C6.5db binding to domain II. Alternatively, binding of trastuzumab to HER2 may prevent cross-linking of two HER2 molecules by C6.5db, forcing monovalent association of the radiotracer. Monovalent binding of the C6.5db is predicted to lower its functional affinity 40-fold, decrease its cell surface residence from 5 hr to 5 min, and significantly lower tumor uptake *in vivo* (54). Efforts to more fully address the basis for the decreased uptake and determine whether this decreased binding can function as a measure of therapeutic response are underway.

Beyond treatment with trastuzumab, agents such as the hsp-90 inhibitors 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-demethoxygeldanamycin (17-DMAG) have been reported to induce rapid and transient degradation of HER2 as part of their proposed mechanisms of action and this down-regulation has been imaged with trastuzumab-based radiotracers (20,55,56). The ability to monitor the efficacy of this type of agent in a robust manner, with dedicated radiotracers such as the C6.5db, has the potential to improve the development and clinical outcome associated with its use. Smith-Jones (20) demonstrated that ^{68}Ga -trastuzumab F(ab')₂ can detect a 50% decrease in HER2 expression in BT-474 xenografts treated with Hsp-90 inhibitors and that the change in HER2 density can be detected before subsequent tumor inhibition is apparent by FDG-based imaging (57).

Interestingly, and consistent with our inability to observe down-regulation of HER2 upon trastuzumab treatment, IHC was unable to detect less than a 70% reduction in HER2 expression in BT-474 BrCa xenografts in athymic mice treated with the heat shock protein-90 (Hsp-90) inhibitor 17-demethoxygeldanamycin (17-DMAG) (55). Thus, differences in trastuzumab-based HER2 down-regulation seen between preclinical and clinical studies may be due, at least in part, to the inability of IHC to sensitively detect those changes.

In conclusion, we hypothesize that molecular imaging with antibody-based radiotracers has the potential to make a positive impact in both guiding the development and use of targeted therapies that inhibit either the activity or expression of cell-surface proteins. The targeting properties of engineered antibody fragments, such as the C6.5db, are well suited for PET imaging and can provide specific information regarding the expression and modulation of targets in a non-invasive manner, regardless of their location. One important future goal is to test C6.5db in transgenic mouse models that express HER2 antigen on normal tissues and shed those antigens into the bloodstream, similar to the clinical setting (58). The development of trastuzumab has revolutionized the treatment of both early and advanced stage HER2-positive BrCa, but acquired resistance to treatment is frequently encountered in advanced disease, and in a small proportion of early stage patients after adjuvant therapy. It is interesting to speculate that molecular imaging, as with the C6.5db or similar antibody-based agents, may serve as an effective method to monitor patients for initial response as well as for development of resistance to trastuzumab.

Statement of Translational Relevance

Strategies to both predict and monitor patient response are critical for the effective development and clinical implementation of targeted therapies. Molecular imaging strategies, such as positron emission tomography (PET) are well suited to this role. We have previously described an antibody-based PET radiotracer, C6.5 diabody (C6.5db), which selectively binds to HER2. Here we demonstrate that imaging with the C6.5db has the potential to quantify HER2 levels in vivo thus predicting response to trastuzumab therapy. We also provide data to suggest that C6.5db-based PET imaging may be an effective strategy for monitoring patient response to trastuzumab or other HER2-directed therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Tumor size and impact on imaging with the C6.5db

SCID mice bearing SK-OV-3 tumor xenografts of varying sizes in four discrete anatomical locations were treated with ^{124}I -C6.5db via tail-vein injection and imaged 48 h p.i. on a clinical PET/CT scanner. C6.5db was labeled with ^{124}I using SHPP. The representative images shown here are from a mouse bearing xenografts that were 25 mg (left chest wall; chevron), 50 mg (right chest wall; arrow), 95 mg (left inguinal space; notched arrow), and 362 mg (right inguinal space; arrowhead) in size. PET signal is detectable in the 50 mg, 95 mg and 362 mg tumors but not in the 25 mg tumor.

Fig. 2. C6.5db does not compete with trastuzumab for binding to HER2 *in vitro*

A) Flow cytometry shows that pretreatment of SK-OV-3 and BT-474 cells with the anti-HER2 mAb trastuzumab does not block binding of the C6.5db to cells as compared to those pretreated with the anti-CD20 mAb rituximab. B) Pretreatment of SK-OV-3 and BT-474 cells with C6.5db also does not block trastuzumab binding to cells C) HER2 ECD-coupled agarose beads pretreated with saturating amount of trastuzumab precipitate C6.5db to equal levels as beads pretreated with either rituximab or PBS. D and E) Pretreating SK-OV-3 and BT-474 cells with either pertuzumab (D) or C6.5db (E) blocks the ability of the other antibody to bind to the cells. Shaded histogram- no primary antibody, DB-C6.5 db, R- Rituximab, T-trastuzumab, P-pertuzumab.

Fig. 3. Trastuzumab blocks tumor targeting of C6.5db

ImmunopET imaging of SK-OV-3 tumors shows a decrease in tumor uptake of ^{124}I -C6.5db after trastuzumab treatment for 3 d as compared to uptake in tumors treated with rituximab.

Fig. 4. Decrease in tumor uptake is not associated with decrease in HER2 levels on tumors

A) Mice bearing SK-OV-3 or BT-474 tumor xenografts were treated with either trastuzumab or rituximab, and subjected to IHC with the anti-HER2 mAb, CB11 (Biogenex), which does not cross-react with trastuzumab. Trastuzumab treatments lasting out to 7 days caused no significant change in HER2 levels on the tumor cell surface as compared to rituximab-treated controls. B) SK-OV-3 and BT-474 cells treated with trastuzumab over a time course of 72 h at 37°C showed a decrease in C6.5db binding (left panel), but no decrease in the level of cell surface HER2 (right panel) as compared to untreated controls. Cell surface HER2 was measured with a Phycoerythrin-conjugated anti-HER2 mAb (Becton Dickinson Biosciences, San Jose, CA) that does not compete with trastuzumab.

Table 1
Biodistribution of ^{125}I -C6.5 diabody in SCID mice bearing s.c. xenografts of tumor cells with varying HER2 density

Tissue	24 h post-injection			48 h post-injection		
	SK-OV-3	MDA-MB361	MDA-MB231	SK-OV-3	MDA-MB361	MDA-MB231
Tumor	1.91	1.11	0.31	0.76 [†]	0.41	0.10
Blood	0.53	0.32	0.49	0.16	0.10	0.13
Liver	0.32	0.20	0.24	0.11	0.09	0.09
Lung	0.45	0.30	0.37	0.12	0.09	0.12
Spleen	0.20	0.13	0.17	0.08	0.05	0.07
Kidney	0.50	0.36	0.44	0.17	0.17	0.16
Heart	0.35	0.27	0.31	0.10	0.09	0.08
Stomach	0.24	0.18	0.11 [*]	0.07	0.05	0.05
Intestine	0.27	0.23	0.27	0.06	0.04	0.05
Muscle	0.06	0.10	0.07	0.02	0.02	0.02

Cohorts of 4-5 mice were analyzed 24 and 48 h post-injection of ^{125}I -C6.5 diabody. Average tumor and organ uptake are expressed as percentage of injected dose per gram of tissue (%ID/g). All standard errors of the mean (SEM) were $\leq 20\%$ of the average unless otherwise indicated.

^{*} 28% SEM.

[†] 33% SEM.

24 h: SK-OV-3 vs. MDA-MB361 $p=0.002$; SK-OV-3 vs. MDA-MB231 $p<0.0001$; MDA-MB361 vs. MDA-MB231 $p=0.021$; SK-OV-3 vs. MDA-MB361 $p=0.021$; MDA-MB361 vs. MDA-MB231 $p<0.0001$.

Table 2
Impact of antibody pre-treatment on biodistribution of ^{125}I -C6.5 diabody in SCID mice bearing s.c. SK-OV-3 tumor xenografts

Tissue	Organ Uptake	
	trastuzumab	rituximab
Tumor	1.58	2.71 [*]
Blood	0.51	0.56
Liver	0.34	0.34
Lung	0.46	0.42
Spleen	0.42	0.40
Kidney	0.59	0.58
Heart	0.35	0.31
Stomach	1.46 [†]	1.06
Intestine	0.22	0.21
Muscle	0.10	0.10

Cohorts of 5 mice were pretreated with trastuzumab or rituximab for 3 d, and analyzed 24 h post-injection of ^{125}I -C6.5db. Average tumor and organ uptake are expressed as percentage injected dose per gram of tissue (%ID/g). All standard errors of the means (SEM) are < 15% of the average unless otherwise indicated.

[†] 23% SEM,

* 24% SEM

Table 3
Impact of antibody pre-treatment on biodistribution of ^{125}I -C6.5 diabody in SCID mice bearing s.c. BT-474 tumor xenografts

Tissue	Organ Uptake	
	trastuzumab	rituximab
Tumor	1.42	3.55**
Blood	0.20	0.14
Liver	0.20	0.16
Lung	0.18	0.20
Spleen	0.15	0.10
Kidney	0.27	0.22
Heart	0.14	0.10
Stomach	0.50	0.27
Intestine	0.27	0.07
Muscle	0.08	0.03

Cohorts of 4 mice were pre-treated with trastuzumab or rituximab for 7 d, and analyzed 24 h post-injection of ^{125}I -C6.5db. Average tumor and organ uptake are expressed as percentage injected dose per gram of tissue (%ID/g). All standard errors of the means (SEM) are < 20% of the average unless otherwise indicated.

**
 39% SEM