Quantitative Immuno-Positron Emission Tomography Imaging of HER2-Positive Tumor Xenografts with an Iodine-124 Labeled Anti-HER2 Diabody

Matthew K. Robinson, Mohan Doss, Calvin Shaller, Deepa Narayanan, James D. Marks, Lee P. Adler, Dinko E. González Trotter, and Gregory P. Adams

Abstract

Positron emission tomography (PET) provides an effective means of both diagnosing/staging several types of cancer and evaluating efficacy of treatment. To date, the only U.S. Food and Drug Administration–approved radiotracer for oncologic PET is 18F-fluoro-deoxyglucose, which measures glucose accumulation as a surrogate for malignant activity. Engineered antibody fragments have been developed with the appropriate targeting specificity and systemic elimination properties predicted to allow for effective imaging of cancer based on expression of tumor associated antigens. We evaluated a small engineered antibody fragment specific for the HER2 receptor tyrosine kinase (C6.5 diabody) for its ability to function as a PET radiotracer when labeled with iodine-124. Our studies revealed HER2-dependent imaging of mouse tumor xenografts with a time-dependent increase in tumor-to-background signal over the course of the experiments. Radioiodination via an indirect method attenuated uptake of radioiodine in tissues that express the Na/I symporter without affecting the ability to image the tumor xenografts. In addition, we validated a method for using a clinical PET/computed tomography scanner to quantify tumor uptake in small-animal model systems; quantitation of the tumor targeting by PET correlated with traditional necropsy-based analysis at all time points analyzed. Thus, diabodies may represent an effective molecular structure for development of novel PET radiotracers. (Cancer Res 2005; 65(4): 1471-8)

Introduction

The antigen-binding specificity displayed by monoclonal antibodies (mAb) is increasingly being exploited as a way to deliver radionuclides for diagnosis and treatment of cancer (1, 2). This is best exemplified by the Food and Drug Administration approval of the anti-CD20 mAbs Zevalin (ibritumomab tiuxetan) and Bexxar (tositumomab and 131I-tositumomab) for the radioimmunotherapy treatment of non-Hodgkin’s lymphoma. The requirement for accurate dosimetry predictions is inherent to the proper administration of radiopharmaceuticals (3). In the case of these agents, γ-camera imaging protocols that use the γ emissions associated with an 111In-labeled form of ibritumomab tiuxetan, or those inherent to 131I-tositumomab, are used to generate the necessary information. Although well suited for predicting dosimetry of the associated therapeutic agent, the effectiveness of these and other mAbs as true diagnostic agents is hampered by their slow clearance rate, which leads to high background levels in the diagnostic scans. Chemical cleavage of mAbs to more rapidly clearing Fab molecules has led to diagnostic agents such as the anti-carcinoembryonic antigen (CEA) arcitumomab (CEA-Scan) that exhibit higher tumor-to-background ratios than intact mAbs (4). However, these reagents are still suboptimal due in part to their monovalent association with tumor antigens.

Positron emission tomography (PET) is an imaging modality based on coincidence detection of the two opposing photons that result from annihilation of a positron and an electron (5). The use of mAbs as PET radiotracers has been investigated (6, 7) and recent work has shown promising preclinical results using the U36 chimeric mAb labeled with the positron emitting isotope 64Cu to generate a PET-based dosimetry prediction for radioimmunotherapy with an 68Y-U36 immunoconjugate (8). Despite the technical advantages of PET over γ-camera imaging, the background associated with mAb-based PET radiotracers still limits their usefulness as diagnostic agents.

Advances in antibody engineering have facilitated the development of novel antibody-based fragments with physical and pharmacokinetic properties consistent with use as dedicated diagnostic agents (9). In addition, a number of positron-emitting isotopes (e.g., 124I and 64Cu) exist that have physical half-lives that match well with the biological half-lives of smaller engineered antibody fragments such as diabodies (Fig. 1). We and others have postulated that when labeled with these PET isotopes, the tumor-targeting properties and systemic clearance rates of engineered antibody fragments would partner effectively with the imaging advantages of PET for the radioimmunodetection of cancer. Wu et al. (10) validated these predictions in preclinical imaging studies done on a dedicated small animal imager (microPET) with an anti-CEA minibody labeled with 64Cu (64Cu-DOTA-T84.66). The 64Cu-DOTA-T84.66 showed specific uptake into CEA-positive LS174T human colon carcinoma tumor xenografts compared with bilateral CEA-negative C6 glioblastoma tumor xenografts and surrounding soft tissue (average tumor/nontarget ratio was 3-4:1). Subsequent work with minibody and diabody forms of T84.66 showed the need to consider the biological properties of both the antibody molecule and the radionuclide when developing a novel radiotracer. The size of the T84.66 minibody (80 kDa) resulted in hepatic clearance and significant retention of radiometals in the liver (e.g., ~26% ID/g at 24 hours when labeled with 111In). By comparison, the 111In-T84.66 diabody (52 kDa) showed high levels of retention (>180% ID/g at 6 hours) in the kidney due to its renal clearance (11). Labeling both...
of these molecules with radiiodine decreased the nontarget tissue accumulation and resulted in higher target-to-background ratios in PET images (12).

We have previously shown efficient targeting of HER2-positive tumor xenografts by the anti-HER2 C6.5 diabody. As with the T84.66 diabody, the size of the C6.5 diabody (52 kDa) results in first-pass renal clearance and rapid systemic elimination (C6.5 diabody T1/2β = 6.4 hours; ref. 13). The uptake and retention of 125I-C6.5 diabody in tumor combined with the clearance kinetics of the molecule results in tumor levels (% ID/g) surpassing those in blood (% ID/mL) as early as 4 hours post-injection. These properties suggested that the C6.5 diabody could be an effective agent for the delivery of diagnostic radionuclides for the detection of HER2-positive cancer via PET. In this study, we evaluate the potential of 124I-C6.5 diabody to function as a PET radiotracer in a mouse tumor xenograft model.

Materials and Methods

Production of C6.5 Diabody. C6.5 diabody (apparent kD = 4 × 1010) was expressed in TG1 Escherichia coli and purified by immobilized metal affinity chromatography followed by high-performance liquid chromatography by size-exclusion chromatography over a Superdex 75 column (Amersham Pharmacia, Piscataway, NJ) as previously described (14). Endotoxin was removed by ion exchange chromatography over PBE94 resin (Amersham Pharmacia) and protein was stored at −70°C at 0.5 mg/mL in PBS containing 10% glycerol until ready to use.

Radiiodination of C6.5 Diabody. The anti-HER2 diabody C6.5 was radiiodinated with iodine-124 produced by RITVERC Isotope Products (St. Petersburg, Russia) and supplied by Advanced Nuclide Technologies, LLC (Indianapolis, IN) using either iodogen-coated glass beads (15) or with the water-soluble form of Bolton-Hunter reagent sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate (SHPP, Pierce Biotechnology, Rockford, IL). To radiiodinate with iodogen-coated glass beads, 0.25 mL of Na124I (1.48 GBq/mL; 2,220 GBq/mg) was neutralized with an equal volume of 2 M acetic acid precipitate as previously described (18). Beads were removed to stop the iodination reaction, percent incorporation was determined by instant TLC. Immunoreactivity of the labeled diabody was assayed by live-cell sensitometry mode. PET images were reconstructed on a 128×128×400 window of 400 to 650 keV. The retention in tumor and nontarget tissues was determined as a percentage of the injected dose localized per gram of tissue (% ID/g) as determined from decay-corrected counts as previously described (17). Values are reported as the mean and SE. Animals exhibiting tumor or organ uptake +2 SDs from the mean were considered outliers and removed from the biodistribution analysis. The percentage of protein-bound radioiodine in the blood of animals was determined by trichloroacetic acid precipitation as previously described (18).

Biodistribution. CR.17 Icr severe combined immunodeficient (SCID) mice, ages 6 to 8 weeks, were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. SK-OV-3 (3.0 × 106), MDA-361/DYT2 (3.0 × 106), or MDA-MB-468 (6.0 × 106) cells were implanted s.c. in the inguinal region of mice. At 7 weeks post-implantation, Lugol’s solution (Sigma, St. Louis, MO) was added to the drinking water at a final concentration of 0.2% to block thyroid accumulation of radioiodine, and biodistribution studies were initiated 5 days later. Cohorts of mice (n = 5) per cohort received 124I-C6.5 (labeled by either the iodogen or SHPP methods) via tail-vein injection, were euthanized at indicated times post-injection, imaged (as appropriate, see below), dissected and major organs weighed and counted in a γ well counter (Cobra Quantum, Packard Instruments, Meriden, CT) with a window of 400 to 650 keV. The retention in tumor and nontarget tissues was expressed as a percentage of the injected dose localized per gram of tissue (% ID/g) as determined from decay-corrected counts as previously described (17). Values are reported as the mean and SE. Animals exhibiting tumor or organ uptake +2 SDs from the mean were considered outliers and removed from the biodistribution analysis.

Statistics. The Excel spreadsheet package was used to perform two-tailed t tests set up for samples with equal population variances. For each comparison, F tests were done using a web-based calculator (http://nimitz.mcs.kent.edu/~blewis/stat/TTest.html) to determine the validity of using a t test set up for samples with equal population variances. Before necropsy, individual cohorts were imaged simultaneously on a Discovery LS clinical PET/computed tomography (CT) scanner (GE Healthcare, Milwaukee, WI) using a custom-built acrylic holder mounted on the patient bed. First, a low dose CT was done, with 140 kV, 50 mA, 0.8 second per rotation, 5-mm slice thickness, and 4.25-mm slice interval. Next, a high axial resolution CT was done with 120 kV, 50 mA, 0.63-mm slice thickness, 0.5 second per rotation, with the same axial limits as initial CT. Immediately after, a PET emission scan was acquired for 10 minutes using delayed random event subtraction in two-dimensional high sensitivity mode. PET images were reconstructed on a 128×128 matrix for a 30-cm-diameter field of view, with vendor supplied software using ordered subsets expectation maximization algorithm, using the initial CT...
from the tumor xenografts. This is exemplified by the transaxial images to confirm the position of the PET signal as emanating automatically registered, allowing for fusion of the resulting PET/CT scanner is that the resulting PET and CT images were (Discovery LS PET/CT, GE Healthcare). A benefit of the clinical single bed position on a dedicated clinical PET/CT scanner designed holder (Fig. 1)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>24 h</th>
<th>48 h</th>
</tr>
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<tbody>
<tr>
<td>Tumor</td>
<td>6.34 (1)</td>
<td>2.95 (1)</td>
</tr>
<tr>
<td>Blood</td>
<td>0.95 (6.7)</td>
<td>0.22 (13.4)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.63 (9.8)</td>
<td>0.14 (21.1)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.74 (8.6)</td>
<td>0.15 (19.7)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.89 (7.1)</td>
<td>0.21 (14)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.76 (8.3)</td>
<td>0.18 (16.4)</td>
</tr>
<tr>
<td>Heart</td>
<td>0.61 (10.4)</td>
<td>0.11 (26.8)</td>
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<tr>
<td>Stomach</td>
<td>3.01 (2.1)</td>
<td>0.29 (10.2)</td>
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<tr>
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<td>0.28 (22.6)</td>
<td>0.09 (32.8)</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.39 (16.3)</td>
<td>0.07 (42)</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.21 (30.2)</td>
<td>0.05 (59)</td>
</tr>
</tbody>
</table>

Note: Cohorts of mice (n ≥ 5 per cohort) were analyzed at each time point. Average tumor and organ uptakes are expressed as percentage injected dose per gram of tissue (% ID/g).

Table 1. Biodistribution of 124I-labeled C6.5 diabody in SCID mice bearing s.c. SKOV-3 tumors

All standard errors of the means (SE) were <15% of the average unless otherwise noted. Values in parenthesis are the ratios of the average tumor to organ uptake.

*46% SE.
**26% SE.
†53% SE.

Modified from attenuation correction. The PET, CT, and fused images were visualized with the MIM software package (MIMVista Co., Cleveland, OH). Quantitation of tumor uptake by PET was done as previously described (19). Briefly, recovery coefficients were determined through use of small-animal phantoms that were positioned at various distances from the longitudinal axis of the scanner. Phantoms contained spheres of relevant volumes (0.0625–1.0 mL), activity concentrations (27 ± 2 kBq/mL), and target to background ratios (10:1 and 20:1). Tumor uptake (activity concentration in the lesions) was quantified from filtered back-projection (2-mm full-width half-maximum Gaussian filter) images reconstructed on 256 × 256 size axial slices (1.0725 × 1.0725 × 4.25 mm3 voxels) with appropriate recovery coefficients applied to the maximum intensity voxels associated with each tumor xenograft. Animals that were removed from the biodistribution analysis, described above, due either to tumor or nontarget organ uptake that differed by >2 SDs were not removed from this analysis.

Results

124I-C6.5 Diabody Efficiently Targets to HER2-Positive Tumor Xenografts. To test the hypothesis that the C6.5 diabody would function as an effective PET radiotracer for the detection of HER2-positive tumors, C6.5 diabody was radioiodinated with the positron emitting isotope 124I and given via tail-vein injection to SCID mice bearing established s.c. HER2-positive human ovarian carcinoma (SK-OV-3) xenografts. Tumor xenografts used in this study ranged in size from 0.11 to 0.86 gram (mean tumor size = 0.43 g), all of which were detectable by CT scan (see below). Cohorts of animals (n = 7 per cohort) were euthanized at the appropriate time points, positioned within a custom-designed holder (Fig. 1B) and imaged simultaneously using a single bed position on a dedicated clinical PET/CT scanner (Discovery LS PET/CT, GE Healthcare). A benefit of the clinical PET/CT scanner is that the resulting PET and CT images were automatically registered, allowing for fusion of the resulting images to confirm the position of the PET signal as emanating from the tumor xenografts. This is exemplified by the transaxial slice of a PET/CT fusion shown in Fig. 1C. Subsequent to imaging, animals were dissected and biodistribution of the radioiodinated diabody was determined (Table 1). Targeting kinetics of the 124I-C6.5 diabody was consistent with that previously seen with both the 125I-C6.5 diabody and the anti-CEA diabody T84.66 (13, 20). Uptake, as measured as percent of injected dose per gram of tissue (% ID/g), peaked by 4 hours post-injection (9.8% ID/g) and cleared ~3-fold from the tumor over the remainder of the study (6.3% ID/g at 24 hours and 3% ID/g at 48 hours). Coincident with the drop in % ID/g in tumor was a more rapid clearance from nontarget tissue (0.95% ID/mL of blood at 24 hours and 0.22% ID/mL at 48 hours), leading to increased tumor-to-background ratios at later time points (Table 1). The clearance rate of the C6.5 diabody pairs well with the physical half-life of iodine-124 (4.18 days), allowing for significant retention of radioactivity in the tumor at 48 hours. This time-dependent increase in tumor-to-background signal is displayed very dramatically in coronal sections through the level of the tumor xenografts (Fig. 2). However, tumor uptake was detectable and quantifiable (see below) by PET for all tumor sizes studied and by the earliest time point (4 hours) analyzed in this study.

Modification of Labeling Strategy. The divalent nature of the C6.5 diabody increases tumor retention due to avid binding but also results in cross-linking and internalization of the HER2 target antigen. A byproduct of internalization is catabolism of the diabody with subsequent release of 124I-tyrosine and free 124I into circulation (21). This process is evident at later time points in Fig. 2 as uptake of 124I by both the stomach and the thyroid, natural sites of iodine metabolism. Dehalogenation may impair radiotracer function due to both durable retention of 124I in these normal tissues and loss of internalized tracer from tumor. Multiple...
and SHPP-labeled C6.5 exhibited similar levels of tumor targeting in nature of the SHPP reagent, necropsy analysis revealed that iodogen SK-OV-3 cells (data not shown). Despite the partially residualizing internalization exhibited by MDA-361/DYT2 cells compared with strategies by taking advantage of the faster rate of HER2 in an effort to enhance any differences between the labeling this labeling procedure.

The immediately visible by PET imaging 48 hours post-injection (Fig. 3). The diabody labeled with either method exhibited tumor targeting that did not seem to greatly affect its function as a PET radiotracer; C6.5 diabody was radioiodinated with 124I either (A) directly with iodogen, given to SCID mice bearing MDA-MB-361 tumor xenografts, and animals were imaged 48 hours post-injection.

Labeling strategies have been developed to block the dehalogeneration process (22–24). For our purposes, we selected a water-soluble form of Bolton-Hunter reagent, SHPP, to indirectly label lysine residues in the C6.5 diabody.

To compare the function of 124I-SHPP-labeled C6.5 diabody with directly labeled diabody, aliquots of C6.5 were labeled simultaneously with either the iodogen or SHPP methodologies and injected into tumor bearing mice. Both methodologies resulted in radiolabeled diabody that was >97% radiochemically pure by instant TLC but the SHPP methodology decreased the immunoreactive fraction of the diabody ~2-fold compared with the iodogen labeling methodology. This decrease in immunoreactivity did not seem to greatly affect its function as a PET radiotracer; C6.5 diabody labeled with either method exhibited tumor targeting that was readily visible by PET imaging 48 hours post-injection (Fig. 3). The ~40% decrease in the % ID/g of indirectly labeled diabody targeted to the MDA-361/DYT2 tumors (0.9% ID/g for SHPP compared with 1.5% ID/g for iodogen at 48 hours, Table 2) is most likely explained by the decrease in immunoreactivity resulting from this labeling procedure.

MDA-361/DYT2 tumor xenografts were selected for these studies in an effort to enhance any differences between the labeling strategies by taking advantage of the faster rate of HER2 internalization exhibited by MDA-361/DYT2 cells compared with SK-OV-3 cells (data not shown). Despite the partially residualizing nature of the SHPP reagent, necropsy analysis revealed that iodogen and SHPP-labeled C6.5 exhibited similar levels of tumor targeting in the MDA-361/DYT2 tumors suggesting that internalization did not significantly impact the outcome of the experiment. It should be noted that the quantity of iodogen-labeled C6.5 diabody retained in the MDA-361/DYT2 tumors was ~2-fold lower than that normally seen for SK-OV-3 tumors at 48 hours post-injection. This may be explained by the lower level of HER2 expression on MDA-361/DYT2 as compared with SK-OV-3 cells (see below). Consistent with the thyroid and stomach uptake seen in the PET images (Fig. 3), animals receiving SHPP-labeled diabody contained lower levels of free iodine in their blood than animals receiving iodogen-labeled diabody (P < 0.005); 97.9 ± 1.39% of all iodine in the blood of the SHPP cohort (n = 5) was trichloroacetic acid precipitable at 48 hours post-injection compared with 88.9 ± 1.33% for the iodogen cohort (n = 5).

PET images of animals injected with 124I-SHPP-C6.5 diabody exhibited an overall increase in background signal compared with scans of animals injected with iodogen-labeled diabody (Fig. 3). The increase in background was consistent with biodistribution results (Table 2) that showed statistically significant differences in retention in nontarget tissues such as muscle (0.12 versus 0.06% ID/g, P = 0.005), intestine (0.15 versus 0.09% ID/g, P = 0.01), lung (0.31 versus 0.21% ID/g, P = 0.02) and heart (0.6 versus 0.11% ID/g, P < 0.0001). The increase in retention of radioiodine in these tissues, presumably due to the residualizing nature of the SHPP moiety, could account for the broad diffuse increase in PET signal. In addition, the decrease in the signal to noise ratio observed in scans of the 124I-SHPP-C6.5–injected animals was most likely compounded by the adverse effect of the labeling method on the immunoreactivity of the radiotracer.

Tumor Targeting Is HER2 Dependent. We did PET and biodistribution studies in mice bearing either HER2-positive (SK-OV-3) or HER2-negative (MDA-MB-468) tumor xenografts to confirm that targeting of radioiodinated C6.5 diabody to the tumor xenografts was due to antigen-dependent recognition of tumor cells rather than nonspecific trapping of diabody within tumors due to differences in the physiology of tumor versus normal tissue (e.g., lack of draining lymphatics, leaky vasculature, etc.).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SHPP</th>
<th>Iodogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Blood</td>
<td>0.29</td>
<td>0.25</td>
</tr>
<tr>
<td>Liver</td>
<td>0.16</td>
<td>0.19</td>
</tr>
<tr>
<td>Lung</td>
<td>0.31</td>
<td>0.21</td>
</tr>
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<td>Spleen</td>
<td>0.14</td>
<td>0.21</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.43</td>
<td>0.42</td>
</tr>
<tr>
<td>Heart</td>
<td>0.6</td>
<td>0.11</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.18</td>
<td>0.23</td>
</tr>
<tr>
<td>Bone</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.12</td>
<td>0.06</td>
</tr>
</tbody>
</table>

NOTE: Cohorts of SCID mice (n = 8 per cohort) bearing s.c. HER2-positive (MDA-361/DYT2) tumor xenografts were injected with C6.5 diabody labeled with 124I either indirectly (SHPP) or directly (iodogen) and biodistribution was analyzed 48 hours post-injection. Average tumor and organ uptakes are expressed as percentage injected dose per gram of tissue (% ID/g). All standard errors of the means (SE) were <15% of the average. Values in parenthesis are the ratios of the average tumor to organ uptake.
MDA-MB-468 cells were selected as the HER2-negative tumorigenic cell line based on reports in the literature (25, 26) and were confirmed to be HER2-negative by flow cytometry (data not shown). Mice bearing MDA-MB-468 (mean tumor size = 0.21 g) or SK-OV-3 (mean tumor size = 0.51 g) tumor xenografts were injected with \(^{124}\text{I}-\text{SHPP}-\text{C6.5} \) diabody and tumor targeting was analyzed 48 hours post-injection. PET imaging showed antigen dependent targeting of the C6.5 diabody (Fig. 4). C6.5 diabody analyzed 48 hours post-injection. PET imaging showed antigen post-injection.

**Quantitative PET imaging** can be a powerful component of the development and *in vivo* evaluation of novel radiotracers in murine model systems. The bore size of clinical PET scanners allow for simultaneous imaging of multiple mice (see above) but the large intrinsic resolution (~ 5 mm full-width half-maximum) compared with the size of murine tumor xenografts force the use of correction methods to obtain meaningful radiotracer concentration data from PET images. In the case of I-124–labeled antibodies, the resulting blurring is also somewhat exacerbated by the long positron range of \(^{124}\text{I}\). We have recently developed a recovery-coefficient correction method to obtain meaningful tracer concentration data from murine PET images produced with a clinical scanner (19). To validate this correction method, we applied it to mice imaged in this study. The tumor uptake (% ID/g) of C6.5 and background signal at the 4, 8, 24, and 48 hour time points represent a large range of the values expected to be encountered during development of novel small antibody-based radiotracers. Activity concentrations in the tumor xenografts of all 28 mice were determined and converted to % ID/g tumor derived by PET differed from the necropsy-based value on average by 0.69 versus 6.07 \(F = 0.14\) for HER2-positive tumors and 0.41 versus 3.16 \(F = 0.59\) for HER2-negative tumors.

**Table 3. Biodistribution of \(^{124}\text{I}\)-labeled C6.5 diabody in SCID mice bearing either HER2-positive or HER2-negative tumors**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>HER2 positive</th>
<th>HER2 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>1.1 (1)</td>
<td>0.32 (1)</td>
</tr>
<tr>
<td>Blood</td>
<td>0.26 (4.2)</td>
<td>0.33 (1)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.26 (4.2)</td>
<td>0.29 (1.1)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.22 (5)</td>
<td>0.25 (1.3)</td>
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<tr>
<td>Spleen</td>
<td>0.14* (7.9)</td>
<td>0.18 (1.8)</td>
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<tr>
<td>Kidney</td>
<td>0.4 (2.8)</td>
<td>0.44 (0.7)</td>
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<tr>
<td>Heart</td>
<td>0.26 (4.2)</td>
<td>0.27 (1.2)</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.09* (12.2)</td>
<td>0.12* (2.7)</td>
</tr>
<tr>
<td>Bone</td>
<td>0.06 (18.3)</td>
<td>0.06 (5.3)</td>
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<tr>
<td>Intestine</td>
<td>0.13 (8.5)</td>
<td>0.12* (2.7)</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.05 (22)</td>
<td>0.05 (6.4)</td>
</tr>
</tbody>
</table>

NOTE: Cohorts of mice (\(n = 5\) per cohort) bearing s.c. tumor xenografts derived from either HER2-positive SK-OV-3 or HER2-negative MDA-MB-468 cells were analyzed at 48 hours post-injection. Average tumor and organ uptakes are expressed as percentage injected dose per gram of tissue (% ID/g). All standard errors of the means (SE) were <15% of the average unless otherwise noted. Values in parenthesis are the ratios of the average tumor to organ uptake. *19.4% SE.

**Results.**

**Quantitative PET imaging** can be a powerful component of the development and *in vivo* evaluation of novel radiotracers in murine model systems. The bore size of clinical PET scanners allow for simultaneous imaging of multiple mice (see above) but the large intrinsic resolution (~ 5 mm full-width half-maximum) compared with the size of murine tumor xenografts force the use of correction methods to obtain meaningful radiotracer concentration data from PET images. In the case of I-124–labeled antibodies, the resulting blurring is also somewhat exacerbated by the long positron range of \(^{124}\text{I}\). We have recently developed a recovery-coefficient correction method to obtain meaningful tracer concentration data from murine PET images produced with a clinical scanner (19). To validate this correction method, we applied it to mice imaged in this study. The tumor uptake (% ID/g) of C6.5 and background signal at the 4, 8, 24, and 48 hour time points represent a large range of the values expected to be encountered during development of novel small antibody-based radiotracers. Activity concentrations in the tumor xenografts of all 28 mice were determined and converted to % ID/g using the correction methodology and the tumor weights obtained during necropsy. Those values were then compared the % ID/g values derived from necropsy-based analysis (Fig. 5). Over the entire range of % ID/g present in the tumors, the PET-based quantitation method showed a strong correlation with the necropsy-based analysis \((R^2 = 0.96291)\). This was also true when comparing the average % ID/g for a given time point. At all four time points, the % ID/g tumor derived by PET differed from the necropsy-based value by less than the experimental error (4 hours = 9.76 ± 0.41 versus 11.26 ± 1.41; 8 hours = 9.39 ± 0.9 versus 10.30 ± 2.25; 24 hours = 5.71 ± 0.69 versus 6.07 ± 2.12; 48 hours = 2.99 ± 0.14 versus 3.16 ± 0.59).

Although the resolution of the clinical scanner that we employed for these studies decreases from 5-mm full-width half-maximum at the center of the field-of-view to 7-mm full-width half-maximum 10 cm from the center, this had no noticeable effect on the quality of the images we were able to obtain. Additionally, this decrease in resolution was incorporated into the described quantitation method.

**Figure 4. Imaging with anti-HER2 diabody is antigen dependent. C6.5 diabody was radiolabeled with \(^{124}\text{I}\) using SHPP. Radiolabeled diabody was given to SCID mice bearing (A) HER2-positive SK-OV-3 or (B) HER2-negative MDA-MB-468 tumor xenografts, and animals were imaged 48 hours post-injection.**
Furthermore, 18F-fluoro-deoxyglucose imaging make it an effective method for both detection and staging of HER2-positive disease, these experiments show that a clinical PET/CT scanner can be used during preclinical development to rapidly and quantitate the performance of novel radiotracers in multiple mice. In addition to demonstrating the performance of the anti-HER2 C6.5 diabody as a radiotracer for the detection of HER2-positive tumors with PET. Using 124I-labeled C6.5 diabody, we successfully imaged and quantitated radiotracer uptake in tumors of clinically relevant size (0.11–0.86 g) on a dedicated clinical scanner. In addition to demonstrating the performance of the anti-HER2 C6.5 diabody as a radiotracer for the detection of HER2-positive disease, these experiments show that a clinical PET/CT scanner can be used during preclinical development to rapidly quantify the performance of novel radiotracers in multiple mice in a single scan.

The high resolution, sensitivity, and quantitative nature of PET make it an effective method for both detection and staging of cancerous lesions. The only approved radiotracer for PET-based detection of cancer is 18F-fluoro-2-deoxy-glucose (T1/2β = 110 minutes). This glucose derivative measures increased glucose accumulation as a surrogate for malignant activity and has proven effective in the diagnosis and staging of a large variety of tumors (e.g., lung and colon; ref. 27). However, there are a number of tumors (e.g., prostate, bronchoalveolar carcinoma, and neuroendocrine tumors) that do not readily incorporate 18F-fluoro-deoxyglucose (28). Furthermore, 18F-fluoro-deoxyglucose imaging can be complicated by significant uptake into metabolically active tissues (e.g., brain and heart) and at sites of infection (29). Thus, imaging compounds targeted at tumor-associated antigens, like the 124I-conjugated anti-HER2 diabody described here, may provide a means for overcoming the difficulties associated with 18F-fluoro-deoxyglucose PET.

In this study, we showed that the C6.5 diabody and the clinical scanner could be used to effectively image and quantify uptake in tumor xenografts at least as small as 0.1 g in size when derived from cells that express high (SK-OV-3) levels of HER2 and 0.3 g when the tumors expressed moderate levels of HER2 (MDA-361/DYT2). The tumor targeting we achieved with the C6.5 diabody against the MDA-361/DYT2 and SK-OV-3 tumors allowed for comparable tumor-background ratios to those seen with the anti-CEA diabody and minibody constructs against LS174T tumors in studies with a dedicated animal scanner (12). Based on quantitative flow cytometry and Skatchard analysis, the SK-OV-3 and MDA-361/DYT2 cells that were used to generate the tumor xenografts for these experiments were found to express ~1 × 10⁶ and 3.75 × 10⁵ copies of HER2 per cell, respectively, when cultured in vitro. By comparison, LS174T cells can bind 2.7 × 10⁶ copies of the anti-CEA mAb COL-1 implying a minimum of 2.7 × 10⁶ copies of CEA per cell (30). Our results suggest that immunoPET imaging can be effectively used to detect tumors with significantly lower quantity of antigen.

A number of additional factors need to be considered in selecting an antigen target and antibody construct for immunoPET imaging. For example, the presence of shed antigen can decrease the sensitivity of the imaging system and the size of the antibody-based construct will dictate its rate of systemic clearance and its ability to extravasate and penetrate into tumor masses. Whereas CEA is commonly shed from tumors, HER2 can also be shed at significant levels and its impact will need to be addressed. In terms of size of the antibody construct, increased tumor targeting can be achieved with intact mAbs or larger fragments, but it comes at a cost of increased time between injections and imaging, increased dose to patients, and higher background levels. This is evident when our results are compared with those reported by others using intact mAb. HuA33 is a humanized mAb that binds the A33 antigen expressed on >95% of colorectal cancers. A33 is expressed by SW1222 colorectal cancer cells in vitro at ~3.7 × 10⁵ copies per cell (31). A comparison of the tumor-targeting specificity between 124I-HuA33 mAb in BALB/c nude mice bearing SW1222 tumors compared with the results reported here for C6.5 show that directly labeled C6.5 reaches tumor/blood ratios (1.3:1) by 48 hours that are not reached until after 96 hours post-injection with the mAb. Consistent with the increased blood retention of the 124I-HuA33mAb (T1/2β = 38.2 hours) % ID/g in tumor peaks at much higher levels of % ID/g than that seen with the C6.5 diabody (50 % ID/g at 96 hours post-injection compared with 10% ID/g at 4 hours post-injection for the C6.5 diabody). This increased tumor uptake may come at the expense of increased patient dose; in addition to positron emission (Eaverage = 819 keV), 124I decays via a number of high-energy γ emissions.

Unlike the CEA or A33 antigens mentioned above, the HER2 receptor is readily internalized. Cross-linking of two HER2 molecules by the C6.5 diabody is thought to induce internalization of HER2 into the lysosomal compartment. Once internalized, radioiodinated antibodies are particularly susceptible to deiodination when the iodine is directly conjugated to tyrosine residues (21). In vitro this leads to active retention of radioiodine in tissues that express the NA-1 symporter (e.g., thyroid, stomach, and salivary glands). Signal associated with such radioiodine uptake may obscure signal from tumor-targeted diabody, especially in areas surrounding those sites of normal iodine uptake. To address this, we examined the utility of using a modified version of the Bolton-Hunter conjugation method that indirectly attaches the 125I to lysine residues (32). When radiolabeled, a significant portion of the indirectly labeled diabody was capable of binding to HER2-positive cells. However, the immunoreactive fraction of

**Discussion**

We have developed a divalent antibody fragment that functions effectively in our preclinical model as a radiotracer for the imaging of HER2-positive tumors with PET. Using 124I-labeled C6.5 diabody, we successfully imaged and quantitated radiotracer uptake in tumors of clinically relevant size (0.11–0.86 g) on a dedicated clinical scanner. In addition to demonstrating the performance of the anti-HER2 C6.5 diabody as a radiotracer for the detection of HER2-positive disease, these experiments show that a clinical PET/CT scanner can be used during preclinical development to rapidly quantify the performance of novel radiotracers in multiple mice in a single scan.

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the SHPP-conjugated C6.5 diabody was still markedly decreased compared with the directly labeled preparation (36% versus 72%) indicating that modifications to this diabody will be necessary to take full advantage of this labeling strategy. For example, a potential conjugation site for SHPP exists within the heavy chain CDR3 of the C6.5 scFv. This lysine residue (100 g, Kabat nomenclature; ref. 33) can be substituted to an alanine residue without affecting affinity of the molecule for HER2 (34). Alternatively, an approach similar to the one described by Olafsen et al. (35) could be used to modify the COOH termini of the diabody with free cysteine residues for site-specific conjugation of the SHPP moiety. Despite this decrease in immunoreactivity, imaging studies done with the 124I-SHPP-labeled diabody revealed sufficient tumor-targeting specificity to allow for high resolution imaging of the tumor xenografts. Significant decreases in radioiodine accumulation in the thyroid and stomach were also observed. This occurred without increasing renal retention, a possible side effect due to the renal clearance of this molecule combined with the residualizing nature of the radiolabeled SHPP. These results indicate that active uptake by normal tissues can be moderated by altering the labeling strategy, leading to significant improvements in the specificity of PET/CT imaging.

Necropsy-based biodistribution analysis has historically been the accepted method for determining the tumor-targeting of novel agents during preclinical development. We have recently described a PET-based quantitation method which we developed using phantoms to generate recovery coefficients that could be applied to small animal models for the purpose of determining tumor targeting (19). This method provided an accurate account of tumor uptake in a small pilot study done in tumor bearing mice. A major goal of the current study was to validate the results of that pilot study in larger cohorts of mice bearing tumors over a range of sizes. In the studies reported here, we obtained close agreement with the tumor uptake we measured by traditional necropsy-based methods thereby validating this PET-based methodology. Our method of PET quantitation differs from other methodologies (8, 36) in that it does not rely on direct measurement of the complete activity in the target (which would require segmentation of the PET target image), but on the application of recovery coefficients estimated a priori to the maximum intensity voxel of the PET image of the target. The recovery coefficient depends on the target mass (obtained at necropsy), background activity concentration (measured directly from the PET image) and position of the target in the bore of the scanner (measured directly from the PET image). By removing the need for segmentation, we have eliminated an important potential source of uncertainty in the quantitation procedure. Thus, our method may prove more accurate in the estimation of target activity concentration, especially when the target activity concentration is fairly homogenous and its size is comparable to the resolution full-width half-maximum of the scanner. Our results indicate that these assumptions are well-founded for tumors of the type and size used in this study and validates the method for acquiring tumor-targeting data in the context of an in vivo model system. In addition, the method may prove useful for monitoring the impact of therapeutic strategies, particularly those directed at the same target antigen. However, although we achieved close agreement between the two quantitation methods, the PET-based quantitation method reported here depended upon tumor volumes determined by necropsy. Therefore, it will be necessary to develop reliable CT-based tumor-volume estimates before the utility of this method can be fully realized.

As cancer therapeutics become increasingly targeted, an understanding of the molecular underpinnings driving the metastatic progression of an individual’s disease will be invaluable for both guiding therapy and determining its effectiveness. This is exemplified by the successful use of the HER2-directed antibody, Trastuzumab, in women with HER2 overexpressing breast cancer (37, 38). Development of diagnostic radiotracers, such as the anti-HER2 diabody described here, may ultimately provide us with the ability to predict which patients may benefit from these therapeutic strategies.

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