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Impact of Expression System on the Function of the C6.5 Diabody PET Radiotracer

Joshua Miller¹, Mohan Doss², Ryan McQuillen¹, Calvin C. Shaller¹, Berend Tolner³, Jian Q. Yu², Kerry Chester³, and Matthew K. Robinson¹

¹Developmental Therapeutics Program, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111

²Nuclear Medicine/PET Services, Department of Diagnostic Imaging, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111

³University College London, UCL Cancer Institute, Paul O’Gorman Building, 72 Huntley Street, London, WC1E 6BT

Abstract

The ability of engineered antibodies to rapidly and selectively target tumors that express their target antigen makes them well-suited for use as radioimaging tracers. The combination of molecular size and bivalent nature makes diabody molecules a particularly promising structure for use as radiotracers for diagnostic imaging. Previous data has demonstrated that the anti-HER2 C6.5 diabody (C6.5db) is an effective radiotracer in preclinical models of HER2-positive cancer. The aim of this study was to evaluate the impact on radiotracer performance, associated with expressing the C6.5db in the *Pichia pastoris* (P-C6.5db) system as compared to *Escherichia coli* (E. C6.5db). Glycosylation of P-C6.5db led to faster blood clearance and lower overall tumor uptake than seen with *E. coli*-produced C6.5db. However, P-C6.5db achieved high tumor:background ratios that are critical for effective imaging. Dosimetry measurements determined in this study for both ¹²⁴I-P-C6.5db and ¹²⁴I-E-C6.5db suggest that they are equivalent to other radiotracers currently being administered to patients.

Keywords

diabody; HER2; *Pichia*; *E. coli*; PET imaging

Introduction

Non-invasive imaging modalities are critical tools used in the management of cancer patients. Anatomical-based imaging modalities such as the computed tomography (CT) scan allow for detailed observation of physical structures based on differences in density. Tumors can exhibit an altered density as compared to surrounding normal tissues, thus making CT scans well suited for oncologic imaging. However, not all tumors exhibit density differences from the surrounding tissues making them refractory to imaging by CT. In addition, the goals of detecting tumors at earlier stages and differentiating between tumor and normal tissues based on cellular and molecular alterations have driven the development of molecular imaging modalities such as positron emission tomography (PET) and others. PET couples the physics of positron emitting radioisotopes with the biological properties of

radiolabeled molecules to non-invasively image tumors based on defined molecular characteristics. The most widely used PET radiotracer, 2-[¹⁸F]fluoro-2-deoxy-D-glucose (FDG), exploits the Warburg effect [1, 2], the transition seen in most tumors to a glucose-dependent phenotype, and preferentially accumulates in tumor. Therefore FDG-PET measures metabolic activity as a surrogate for malignant activity. In recent years our depth of understanding around the molecular factors that drive cancer formation and progression has increased dramatically. This, in turn, has led to the development of a host of new small molecule inhibitors and antibody therapies for treating patients with defined subtypes of cancer. The mechanisms of action by which these targeted therapies work necessitate the development of biomarker and/or imaging-based approaches to stratify patients based on predicted response.

The importance of patient stratification is exemplified by HER2-positive breast cancer (BrCa) and the clinical impact of the HER2-targeted agents. Consistent with the important role that HER2 plays in tissue development and homeostasis [3], overexpression of the receptor tyrosine kinase (RTK), which is seen in approximately 20 – 30 % of all BrCa, causes dysregulated signaling downstream of the receptor and leads to an aggressive subtype of BrCa that is associated with poor clinical outcome and high risk of relapse [4]. The anti-HER2 monoclonal antibody (mAb) trastuzumab, blocks HER2-dependent signaling [5] and its use is associated with improved patient outcome in both the metastatic and adjuvant settings [6, 7]. Eligibility for trastuzumab therapy is based on measurement of HER2 overexpression either at the protein level by immunohistochemical (IHC) staining or gene amplification via fluorescence *in situ* hybridization (FISH) techniques in biopsied tumor tissue. Despite overexpressing HER2, less than 35% of patients in metastatic setting respond to trastuzumab treatment and many that initially respond go on to develop resistance [8, 9]. In addition, preclinical data suggests that trastuzumab treatment could enhance chemosensitivity in defined patient populations, even in the absence of HER2 overexpression. Thus, the ability to either monitor for intrinsic and acquired resistance or predict response to a targeted therapy is of clinical importance. The non-invasive nature and whole-body images obtainable with PET, coupled with radiotracers capable of detecting changes at the molecular level make it well suited for this role.

PET imaging with antibody-based radiotracers represents a promising approach for detecting biomarkers and monitoring changes to biomarker expression that may correlate with response to therapy. Currently, IgG and Fab' fragments of four radiolabeled murine mAbs that are specific for tumor associated glycoprotein 72 (TAG-72), prostate-specific membrane antigen (PSMA), carcinoembryonic antigen (CEA), and epithelial cell adhesion molecule (EpCAM) have been approved by the FDA for use as imaging agents [10, 11]. Radiolabeled trastuzumab is also being evaluated as an immunoPET agent in multiple phase I trials. These agents accumulate to high levels in tumor but the prolonged serum half-life of mAbs, although well suited for use as therapeutics, limits their function as immunoPET radiotracers by increasing the time necessary to achieve sufficient image contrast and potentially leading to unacceptable levels of normal tissue irradiation. This is exemplified by the extensive blood pool levels of ⁸⁹Zr-DFO-trastuzumab seen in appropriately dosed patients at 1 – 2 days post-injection. Optimal imaging in these patients was determined to be 4 – 5 days post-injection and resulted in high spatial resolution images with good signal to background ratios [12]. Therefore, optimizing the pharmacokinetic (PK) and tumor targeting properties of engineered antibodies to obtain rapid tumor to background contrast, while maintaining sufficient levels of tumor uptake for detection, is a key step in enhancing the clinical utility of antibody-based radiotracers. We, and others, have demonstrated that the clearance properties of genetically engineered antibody fragments are well suited for use as PET radiotracers [13–16]. Our efforts to develop a HER2-targeted radiotracer have focused on the use of a genetically engineered single chain Fv (scFv)-based antibody molecule called

C6.5 diabody [16–18]. When radiolabeled with Iodine-124 (^{124}I) the C6.5db is capable of effectively imaging HER2-positive xenografts in our preclinical models and uptake of the radiotracer has been shown to correlate to antigen density on the tumor surface [16].

In this work we report the expression of the C6.5db in the methylotropic yeast strain *P. pastoris* (P-C6.5db) and detail how expression in *P. pastoris* impacts on the function of the antibody as a PET radiotracer in our preclinical model. In contrast to the C6.5db produced in the *E. coli* expression system (E-C6.5db) that we described previously, expression of recombinant proteins in *P. pastoris* is anticipated to result in glycosylation with branched mannose structures that are not typical of proteins produced in mammalian cells and promote rapid systemic clearance [19, 20]. In the case of antibodies this can alter their PK and tumor targeting properties [21, 22]. We report the results of studies to evaluate how PK and tumor targeting of P-C6.5db compares to that of E-C6.5db and impacts on the expected dosimetry associated with the two compounds.

Materials and Methods

Construction of a C6.5db *Pichia pastoris* expression strain

The coding region for the C6.5db was amplified from pSYN-C6.5db [18] by polymerase chain reaction with the primers GA331 (5' <GAAGCTGCAGGTCAGGTGCAGCTGTTGCAGTC>3') and GA332 (5' <ACTCGAGTTAGCTAATTAATGGTGATGATGGTGATGACCTAGGACGGTCAGCTT GGT>3'). The resulting *Pst*I/*Xho*I fragment encoded the C6.5db with a C-terminal 6X-His purification tag and stop codons in all 3 reading frames. The *Pst*I/*Xho*I fragment was cloned into *Pst*I/*Xho*I sites of pPICZαB (cat # V190-20, Invitrogen) to create a translational fusion with the vector-encoded α-factor signal sequence that is cleaved during secretion. A sequence confirmed pPICZαB::C6.5db plasmid was linearized within the AOX1 promoter by digesting with *Sac*I and transformed via electroporation into the X33 strain of *Pichia pastoris* (Invitrogen) (25 μF, 0.54 kV, 15 msec pulse). Cells were allowed to recover for 1 hr and plated on YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) plates containing either 0 or 50 μg/mL of Zeocin (cat # R25001, Invitrogen) as a selection reagent. Transformants from the 50 μg/mL selection plates were subcloned and isolated colonies were tested for expression.

P. pastoris-based fermentation expression of C6.5db

A high-expressing clone, X33[pPICZαB::C6.5db], was identified according to manufacturer's recommendation (Invitrogen). Briefly, clones were expanded overnight in YPD liquid at 30° C, diluted into 25 mL BMGY media (1% yeast extract, 2% peptone, 100mM potassium phosphate pH6.0, 1.34% yeast nitrogen base, $4 \times 10^{-5}\%$ biotin, 1% glycerol) to an $\text{OD}_{600} = 0.1 - 0.2$ and allowed to grow at 30° C, while shaking in 250 mL baffled flasks. Once $\text{OD}_{600} = 2.0 - 3.0$, 25 OD_{mL} of each clone was harvested, washed in BMMY (1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, $4 \times 10^{-5}\%$ biotin, 0.5% methanol), resuspended in 25 mL BMMY and allowed to grow for up to 68 hrs 30° C. Cells and culture supernatants were harvested at various timepoints and analyzed for C6.5db expression by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot with a penta-HIS mAb (cat # 34660, Qiagen) and anti-mouse-HRP secondary.

The identified high expressing clone was used for scale-up production in a BioFlo3000 fermentor (New Brunswick) equipped with dissolved oxygen (Metler Toledo) and pH (Metler Toledo) probes. Fermentation was carried out according to the protocol outlined by Tolner *et al.* [23]. Cells were grown in YPD + 50 μg/mL Zeocin at 30° C to an $\text{OD}_{600} =$

14.2, diluted 100-fold into 200 mL of minimal fermentation media [per L: 5% glycerol (v/v), 0.9 g CaSO₄, 14.67 g K₂SO₄, 11.67 g MgSO₄ 7H₂O, 9 g (NH₄)₂SO₄, PTM1 trace element solution] and grown for 17 hr in a 2 L shake flask. The production culture was inoculated (approx 4 L) and biomass was generated for 25 hrs followed by a limited methanol feed to induce protein expression. Media was maintained at pH 6.5 and 40% dissolved O₂ throughout the 24 hr induction phase. Cells were harvested by centrifugation and supernatants were filtered through a Buchner funnel, brought to 5 mM imidazole and purified by immobilized metal ion chromatography (IMAC) using Ni-NTA agarose (cat # 30230, Qiagen). C6.5db was eluted from the Ni-NTA agarose matrix using 200 mM imidazole agarose and subjected to high performance liquid chromatography size-exclusion chromatography over a Superdex 75 column (GE Healthcare). Protein was stored at -80° C at a concentration of 0.5 mg/mL in PBS containing 10% glycerol until ready for use.

***E. coli*-based expression of C6.5db**

The plasmid pSYN::C6.5db was transformed into TG1 *E. coli* and protein was expressed in shake-flasks. C6.5db was isolated from the periplasmic space and purified by sequential IMAC and size exclusion chromatography as previously described [18]. Protein was stored at -80° C at 0.5 mg/mL in PBS containing 10% glycerol until ready for use.

Binding analysis of C6.5db constructs

Binding characteristics of E-C6.5db and P-C6.5db were compared by surface plasmon resonance using a BIAcore 1000 instrument. Briefly, 230 response units (RU) of HER2 extracellular domain (ECD) [24] were coupled to CM5 sensor chips (GE Life Sciences, cat # BR-1000-12) via amine-based coupling using EDC/NHS. E-C6.5db and P-C6.5db at 0nM, 100nM and 500nM were flowed over both the HER2 and a control sensor surface at a rate of 40 µL/min and regenerated with sequential pulses of 2M glycine pH2 and 1M triethanolamine pH 10. Subtracted data was evaluated using a 1:1 Langmuir binding model using the BIAevaluation 3.2 (GE Life Sciences).

Radioiodination

P. pastoris and *E. coli* produced C6.5db were radioiodinated with Na¹²⁵I (cat # NEZ033H, DuPont NEN) or Na¹²⁴I (RITVERC Isotope Products, St. Petersburg Russia) using Iodogen-coated glass beads as described in Robinson *et al.* [18]. 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 [25].

Biodistribution and Dosimetry

Blood and whole-body clearance as well as biodistribution of the C6.5db-based radiotracers in severe combined immunodeficient (SCID) mice was determined as previously described [18].

PET imaging was performed on a Siemens Biograph 16 TruePoint PET/CT scanner (Siemens Healthcare, Malvern, PA). The mice were kept anesthetized with Isoflurane in an induction chamber on the patient bed. A low dose helical CT was performed with the following parameters: 130 kV, 24 mAs, 0.6 s rotation, 16×0.6 mm collimation, pitch of 1, and reconstructed at 2 mm slice thickness and 1 mm interval. Then a PET emission scan was acquired for 10 minutes in 3D mode. The PET/CT scans were performed at 2, 24, 48, 120, and 192 hours after injection. The PET images were reconstructed using CT for attenuation correction, including corrections for random coincidences and scatter on a 336 × 336 matrix for a 63-cm diameter field view, with manufacturer supplied software using Truex algorithm with 2 iterations and 21 subsets, and 2 mm FWHM post processing filter

The PET and CT images were fused using MIM software version 5.1 (MIM Software Inc., Cleveland, OH). Uptakes were determined by drawing volumes of interest (VOIs) directly on the PET images for tumor and the following organs visualized above background activity levels: thyroid, stomach, liver, heart, and bladder. Percent Injected Dose (%ID) was calculated for tumors and the different organs at each time point using the integrated activity within the VOIs. The total measured activity in the mouse was also determined by summing all the observed activity in the mouse PET images at each time point. By fitting decay-corrected total activity in the mouse to exponential decay, the half-time for biological decay was determined for each mouse.

We used the OLINDA/EXM software (version 1.1) (Vanderbilt University, Nashville, TN) for determining the radiation doses from the imaging compounds [26]. The %ID for each organ for each time point was entered into the program, and the data were fitted to exponentials or sum of exponentials to determine the number of disintegrations per unit administered activity, usually referred to as residence time. Bladder voiding model of 1 and 4.8 hr voiding intervals were used to determine the residence time for bladder. Organ doses and the effective doses were scaled using the standard adult male model in the software.

RESULTS

Expression of C6.5db in *E.coli* and *P. pastoris* systems

The C6.5db was successfully expressed as a fully-functional, secreted protein in both the *E. coli* expression system (E-C6.5db), as previously described [27], and in the *P. pastoris* (P-C6.5db) expression system as outlined by Tolner *et al.* [23]. For this study the P-C6.5db was expressed using the plasmid pPICZaB::C6.5db and secreted into the media guided by the α -factor secretion signal that is proteolytically cleaved upon secretion. Purification of E-C6.5db and P-C6.5db by sequential Ni-affinity and size exclusion chromatography resulted in a major species that eluted in agreement with the predicted 54 kDa molecular weight of the noncovalent dimer (data not shown). SDS-PAGE analysis of the purified proteins demonstrated that both migrated at a size (~ 27 kDa) consistent with the predicted behavior of the denatured C6.5db (Figure 1A). Interestingly, P-C6.5db migration was slightly retarded as compared to E-C6.5db consistent with it being glycosylated. In addition, the P-C6.5db routinely appeared more resistant to denaturation as seen by the presence of higher molecular weight, presumed dimers on the SDA-PAGE analysis. The 28mg C6.5db/L of culture media yields obtained using the *P. pastoris* system compares very favorably to the 1 – 3 mg/L yields obtained in the *E. coli* expression [27].

Consistent with the prediction that P-C6.5db would be glycosylated, Western blot analysis of purified P-C6.5db with Concanavalin A, a lectin with specificity for mainly internal and non-reducing terminal α -mannosyl groups, showed a defined band at the appropriate molecular weight for C6.5db (Figure 1B). To determine if glycosylation negatively impacted on the ability of P-C6.5db to bind HER2 its binding was compared to E-C6.5db. As depicted in Figure 1C, purified E-C6.5db and P-C6.5db exhibited similar binding to HER2 ECD as measured by SPR on a low density HER2 sensor surface ($R_{max} = 150$ RU). Within the limits of this experiment, analysis of the binding data generated with E-C6.5db and P-C6.5db using a 1:1 Langmuir mode suggests that E-C6.5db and P-C6.5db have similar binding affinities. The binding affinity of P-C6.5db ($K_D = 1.1 \times 10^{-8}$ M, $k_a = 1.74 \times 10^5$ M⁻¹ s⁻¹, $k_d = 1.95 \times 10^{-3}$ s⁻¹) was equivalent to that of E-C6.5db ($K_D = 1.4 \times 10^{-8}$ M, $k_a = 1.54 \times 10^5$ M⁻¹ s⁻¹, $k_d = 2.21 \times 10^{-3}$ s⁻¹). Importantly, these values are consistent with that of the parental C6.5 scFv ($K_D = 1.6 \times 10^{-8}$ M, $k_a = 4.0 \times 10^5$ M⁻¹ s⁻¹, $k_d = 6.3 \times 10^{-3}$ s⁻¹) when expressed in *E. coli* [28]. This data supports the idea that glycosylation associated with expression in the *P. pastoris* system (Figure 1B) did not impact negatively on the ability of the diabody to bind its target antigen.

Biodistribution and pharmacokinetics of *E. coli*- and *P. pastoris*-produced C6.5db

Biodistribution and PET imaging studies were carried out with both E-C6.5db and P-C6.5db antibody that had been radiolabeled with either ^{124}I or ^{125}I as appropriate. In all cases radiotracers were at specific activities of 37 kBq/ μg and radiochemical purity of 95–99%. Immunoreactivity of radioiodinated proteins was approximately 85% for ^{125}I -labeled proteins with ^{124}I -labeled proteins ranging from 31 – 77% for *E. coli* and 50 – 60% for *P. pastoris*. Although a single preparation of ^{124}I -E-C6.5db had low immunoreactivity (31%) as measured by *in vitro* binding studies, possibly due to radiolysis, the *in vivo* behavior of the radiotracer was consistent with that of higher activity preps and therefore considered representative in this analysis.

Consistent with the predicted impact of glycosylation in the *P. pastoris* expression system, P-C6.5db demonstrated a more rapid systemic clearance than E-C6.5db in SCID mice bearing HER2-positive SK-OV-3 tumor xenografts (Figure 2). At 4 hours post-injection a significantly lower ($P < 0.001$) level of P-C6.5db ($11.3 \pm 0.4\%$ of initial dose) remained in circulation as compared to E-C6.5db ($69.9 \pm 7\%$). This is despite a similar level of whole-body retention seen with both radiotracers at 4 hours post-injection ($85.7 \pm 2\%$ P-C6.5db vs. $89.7 \pm 1.8\%$ E-C6.5db; $P = 0.18$). By 24 hours both blood levels (0.6 ± 0.1 vs. 1.3 ± 0.2 , $P = 0.01$) and whole-body levels (5.0 ± 0.3 vs. 10.2 ± 1.9 , $P = 0.03$) of P-C6.5db were approximately half that seen with E-C6.5db. Although absolute values were even lower at 48 hours the trends for both blood and whole-body levels continued and maintained statistical significance ($P < 0.01$). The more rapid clearance of P-C6.5db from circulation correlated with a statistically significant decrease in overall tumor uptake at 4 hours post-injection (Table 1). At 4 hours post-injection P-C6.5db was retained in tumors at only $5.08 \pm 0.6\%$ ID/g as compared to E-C6.5db that was present at $7.8 \pm 0.5\%$ ID/g ($P = 0.01$). Statistically significant differences ($P = 0.03$) in tumor targeting levels were maintained between P-C6.5db and E-C6.5db at each time point analyzed with a $1.9 \pm 0.2\%$ ID/g vs. $3.8 \pm 0.7\%$ ID/g respectively at 24 hours and $1.1 \pm 0.1\%$ ID/g vs. $1.8 \pm 0.1\%$ ID/g respectively at 48 hours. The rapid systemic clearance of P-C6.5db is predicted to occur because of contributions of hepatic clearance routes. Interestingly, liver activity levels seen with P-C6.5db were lower than observed with E-C6.5db at both 4 and 24 hours post-injection ($P < 0.03$).

Despite the lower overall tumor uptake seen with P-C6.5db, which could have a deleterious impact on its efficacy as a radiotracer, the more rapid blood clearance resulted in a trend toward higher tumor:blood values for P-C6.5db than E-C6.5db at all time points analyzed (Table 2). Although this trend failed to reach significance ($P = 0.18$) the high tumor:blood levels achievable with P-C6.5db, it reached approximately 12:1 at 24 hours and 36:1 at 48 hours, provided the rationale for evaluating P-C6.5db as a PET radiotracer. As seen in Figure 3, P-C6.5db was capable of effectively imaging HER2-positive tumors in our preclinical model.

Dosimetry time course of *E. coli*- and *P. pastoris*-produced C6.5db measured by PET

The ability of P-C6.5db to effectively image HER2-positive xenografts coupled with the faster clearance suggested that its use may lead to lower patient exposures than E-C6.5db while providing equivalent, or better, image contrast. SCID mice bearing SK-OV-3 tumor xenografts were injected with either ^{124}I -P-C6.5db or ^{124}I -E-C6.5db and imaged serially over a 192 hour time course to perform dosimetry calculations. Selected slices of coronal views of PET/CT fusion image are shown for one representative mouse from each group at each time point analyzed (Figure 4). The five time points over 192 hours showed typical uptake patterns associated with the targeting and clearance of the C6.5db. In addition to tumor, high uptake was observed in the bladder, stomach, and thyroid, consistent with renal

clearance and release of free iodine upon catabolism of the antibody [27]. Uptake of E-C6.5db and P-C6.5db in the liver, based on dosimetry studies (Table 3), demonstrated that the percentage of injected dose (%ID) of both radiotracers present in the liver at 2 hours post-injection was equivalent (4.27 ± 2.66 vs. 3.30 ± 1.17 , $P = 0.73$). At subsequent time points the level of P-C6.5db in liver trended higher than levels observed with E-C6.5db, reaching the level of statistical significance by 120 hours ($P = 0.05$). Immediately following the final imaging time-point, the mice were euthanized and dissected so that physical measurements of activity in their organs could be taken. As depicted in Table 4, uptake in tumor and liver as measured directly by both imaging and biodistribution correlated well. The correlation was less robust in stomach and heart where uptake calculated by imaging was higher, and presumably due to the process of emptying the stomach contents and blood from the heart prior to counting during the biodistribution. Likewise, conversion of standardized tumor uptake data of ^{125}I -labeled compounds from %ID/g (Table 1) to %ID by taking into account average tumor volume demonstrates that 1.3 %ID and 0.8%ID of ^{125}I -E-C6.5db was retained in tumors at 24 and 48 hours post-injection, respectively. At the same time points 0.8%ID and 0.6%ID of ^{125}I -P-C6.5db were retained in tumor. These values correspond well to the levels of ^{124}I -labeled antibodies that were measured to be in tumor during the dosimetry studies (Table 3) and point to the reproducible targeting observed between different experiments with both P-C6.5db and E-C6.5db. In addition, activity levels measured by imaging in a microsphere phantom study, performed at lesion to background levels that bracket those seen in our animal model, demonstrate a good correlation with known activity levels in the spheres (See Supplemental Table 1).

The average %ID data for a series of 24 tissues for each group of mice was extrapolated to the human equivalent %ID to calculate the total effective dose of an adult male using the OLINDA/EXM software assuming both a 4.8 hour and 1.0 hour bladder voiding model. The five organs with the highest average effective dose equivalents in each model are displayed in Table 5. In all cases the highest dose was to the thyroid, consistent with the use of the iodogenbased method for labeling of the radiotracers and release of free radioiodine upon internalization and degradation of the radiotracer. Use of the 4.8 hour bladder voiding model results in an average effective dose of 0.36 ± 0.13 and 0.35 ± 0.08 mSv/MBq for E-C6.5db and P-C6.5db, respectively. Use of a 1 hour bladder voiding model lowered the average effective doses to 0.31 ± 0.13 mSv/MBq and 0.30 ± 0.08 mSv/MBq, respectively. This data suggests that radiation dose from P-C6.5db and E-C6.5db are similar, and can potentially be reduced slightly by frequent voiding.

DISCUSSION

The need for methods to quantify therapeutic biomarkers has increased concomitantly with the development and use of targeted therapies to treat cancer. PET is a quantitative imaging modality that exploits biochemical processes to image tumors. Thus it provides the potential to gain more information related to therapeutic efficacy than standard anatomical-based modalities, which rely on structural changes assessed in response to standards such as Response Evaluation Criteria In Solid Tumors (RECIST). In addition, biochemical changes in response to therapy are often manifested well in advance of structural changes providing a more rapid evaluation of therapeutic efficacy. This is exemplified by the use of FDG-PET vs. CT scans for monitoring the response of Gastrointestinal Stromal Tumors (GIST) to imatinib mesylate. A decrease in the metabolic activity measured by Standard Uptake Value (SUV) post-treatment, as compared to pre-treatment levels, was superior to CT at detecting early response to therapy and was also associated with a longer progression-free survival [29, 30]. A large number of radiotracers capable of measuring different aspects of tumor biology, which are applicable to a wide variety of cancers, are currently at various stages of clinical development [31, 32]. Included among these radiotracers are the compounds

fluorothymidine (FLT) to measure proliferation rate [33] and fluoromisonidazole (FMISO) to measure hypoxia [34]. Fluorinated versions of the annexin V protein are also being developed to measure apoptosis [35]. Other compounds, such as the fluorinated analog of the androgen receptor-binding ligand (fluorodihydrotestosterone, FDHT) [36–38] are being developed to monitor characteristics specific to defined subtypes of tumors.

We, and others, have hypothesized that antibody-based radiotracers can be used to effectively image and phenotype tumors based on antigen expression and thereby help guide therapy decisions. In previous work we demonstrated that the anti-HER2 engineered antibody fragment C6.5db is capable of accumulating in tumors at sufficiently high levels with appropriate contrast to normal tissue to be an effective PET radiotracer for the detection of HER2-positive tumors in preclinical models [16, 27]. Image quality, in terms of absolute tumor uptake and tumor to background signal, need to be balanced with other considerations, such as the total radiation exposure received by the patient during the scan, when designing novel radiotracers. In an effort to further optimize the C6.5db for translation toward clinical evaluation we hypothesized that accelerating the systemic clearance of the C6.5db may increase its utility as a radiotracer by improving the tumor to background ratios achievable with the radiotracer while retaining sufficient absolute uptake of the radiotracer in tumor to allow for efficient tumor detection. This in turn would have the potential to decrease the total radiation exposure received by the patient. In this work we examined the impact associated with altering the PK of the radiotracer by producing the C6.5db in two distinct expression systems. Modification of the PK was evaluated in the context of overall tumor targeting and the internal radiation dosimetry, as calculated through use of the OLINDA software package.

Here, PK modulation was achieved by fermentation-based expression of C6.5db in *P. pastoris*, a system that has been used for production of a number of therapeutic proteins, including antibodies [23, 39–41]). *P. pastoris* modify proteins with both N- and O-linked glycosylation [20, 40] that are non-mammalian in structure, resulting in rapid systemic clearance via pattern recognition mannose receptors (MR) that are present on a number of cell types, including liver endothelial cells [42]. This is exemplified by the rapid, MR-based clearance of glycosylated MFECPI, an antibody-enzyme fusion, as compared to non-glycosylated MFECPI that leads to a 375-times lower level of enzyme activity in serum 6 hours after injection [19, 39, 43]. Although not formally tested, we hypothesize that the clearance difference seen between E-C6.5db and P-C6.5db was due to rapid MR-based clearance through the liver, which is likely taking place even before the first time points analyzed in this study. Consistent with this hypothesis, the C6.5db has the potential for being glycosylated. It contains five Asn residues, one within the V_h and four within the V_l domains. However, none of these are found within the context of the Asn-X-S/T consensus motif for N-linked glycosylation [44] and are not predicted to be glycosylated by the web-based analysis software NetNGly1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc>). Likewise C6.5db contains a total of 53 Ser and Thr residues spread across both domains of the protein. NetOGly3.1 predicts that four of these residues, Thr141, Ser145, Thr155, and Thr206 are potential sites for glycosylation (<http://www.cbs.dtu.dk/services/NetNGlyc>). Although we did not identify defined sites of glycosylation on P-C6.5db, binding by Concanavalin A and the rapid clearance, together point to the antibody being glycosylated in this system. Importantly, *in vitro* binding activities suggest that glycosylation does not impact on the antigen-binding properties of the antibody. Further analysis to map the sites of modification is warranted.

Imaging with both E-C6.5db and P-C6.5db resulted in clear visualization of the tumor xenografts by at least 24 hours post-injection in our preclinical model of HER2-positive cancer. Despite the fact that P-C6.5db cleared more rapidly from the circulation and

exhibited lower overall tumor uptake it displayed a trend toward higher tumor to background ratios than those observed with E-C6.5db. By 24 hours both radiotracers compared favorably to the tumor to background ratios achieved with ^{89}Zr -trastuzumab at 144 hours in preclinical imaging studies [45]. When translated into patients ^{89}Zr -DFO-trastuzumab reached a maximum tumor to background ratio of 7.6 at 144 hours [12]. Rapid generation of high tumor to background ratios suggest that both P-C6.5db and E-C6.5db could function as effective radiotracers in contrast to radiolabelled trastuzumab while potentially limiting patient exposure to radiation as the radioisotope is rapidly removed from the body. Radiolabeled trastuzumab suffers from the additional complication that it binds to the same epitope as trastuzumab and therefore has different targeting properties in the setting of trastuzumab therapy [12] due to receptor saturation. In contrast, it has been previously demonstrated that C6.5db does not compete at the binding site occupied by trastuzumab, making it a potential candidate for concurrent use in monitoring trastuzumab therapy [16].

Imaging studies carried out under Radioactive Drug Research Committee (RDRC) approval limit patient exposure to 30 mSv. This is important in the context of ^{124}I -based PET imaging. The positron emission and multiple gamma emissions associated with decay of ^{124}I results in an effective dose that is approximately 20% higher than that associated with the therapeutic isotope ^{131}I [46]. Based on our estimation, the average effective dose for C6.5db-based radiotracers would be ~ 0.36 mSv/MBq for a 4.8 hr bladder voiding model. More frequent voiding reduces the effective dose slightly, as the 1 hour bladder voiding model reduces the effective dose to ~ 0.31 mSv/MBq. This suggests that the estimated effective dose for both E-C6.5db and P-C6.5db is comparable with other ^{124}I -based PET radiotracers, such as the IgG-based radiotracer ^{124}I -HuMV833 (0.3 mSv/MBq) [47], ^{124}I -MIBG (0.25 mSv/MBq) and ^{124}I -NaI (6.50 mSv/MBq) [48]. Our image-based quantification is also supported by the correlation between the 192 hr image and biodistribution uptake data obtained in this study (Table 4). In biodistribution studies such as ours, volume integrated activity in organs are used to estimate the uptake in the organs. Phantom studies have shown this method results in a much smaller partial volume effect (Supplemental Table 1) in comparison to using peak activity per unit volume to estimate the activity in lesions [49]. Another source of error in estimating human doses is the scaling processes used to estimate human dosimetry from murine data. However, we hypothesize that both of these effects would impact both radiotracers equivalently, thus allowing for a direct comparison between the two. Absolute determination of dosimetry will require clinical translation of these agents.

In conclusion, the anti-HER2 engineered antibody fragment C6.5db produced in either the *P. pastoris* or *E. coli* expression systems can function effectively as a PET radiotracer when radiolabelled with ^{124}I . Selection of expression system for translation of this radiotracer into the clinic will require an evaluation of the benefits and drawbacks of each system. Presumed differences in clearance mechanisms (liver vs kidney) and relative radiosensitivity of those organs may dictate expression system use. In addition, altered clearance routes may result in unsatisfactory background signal in organs that are known sites of metastasis for certain diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Production of C6.5db in *P. pastoris*. A) SDS-PAGE analysis of purified E-C6.5db and P-C6.5db. B) Western blot analysis of P-C6.5db with concanavalin A demonstrating mannosylation of C6.5db and a high degree of mannosylation in crude yeast lysate. C) SPR analysis of E-C6.5db and P-C6.5db at 100nM and 500nM demonstrates equivalent binding of the two antibodies.

Figure 2.

Blood clearance of *P. pastoris*- vs. *E. coli*-produced ^{125}I -C6.5 diabody. ^{125}I -C6.5db was administered to SCID mice bearing SK-OV-3 tumor xenografts via tail-vein injection. Levels of ^{125}I -C6.5db in blood, as a percentage of initial levels, were measured at 4, 24, and 48 hours p.i.

Figure 3.

PET fusion images of SCID mouse with ^{124}I -labeled P-C6.5db. A single SCID mouse bearing an SK-OV-3 tumor xenograft was treated with ^{124}I -C6.5db via tail-vein injection and imaged at 24 hours p.i. on a clinical PET/CT scanner. (1&2) Coronal and axial sections from CT scan. (3&4) Coronal and axial sections from PET scan. (5&6) Coronal and axial sections showing fusion of CT and PET images.

*E. coli**P. pastoris* **Figure 4.**

PET fusion images of ^{124}I -C6.5db. Cohorts ($n = 5$) of SCID mice bearing SK-OV-3 tumor xenografts were treated with ^{124}I -C6.5db via tail-vein injection and imaged at 2, 24, 48, 120, and 192 hours p.i. on a clinical PET/CT scanner. Images of a representative from each cohort at each time are shown. The radiotracer signal is visible in the tumor, bladder, stomach, liver and thyroid. *P. pastoris* C6.5db shows greater presence in the liver by 24 hours, as expected for a mannosylated protein. Image contrast was adjusted on an individual basis for ideal organ differentiation; brightness is not directly associated with organ activity. 1. Stomach, 2. Urinary bladder, 3. Tumor, 4. Liver, 5. Thyroid, 6. Heart.

Table 1

Biodistribution of *P. pastoris*- vs. *E. coli*-produced ¹²⁵I-C6.5 diabody in SCID mice bearing s.c. SK-OV-3 xenografts

	4 h post-injection		24 h post-injection		48 h post-injection	
	<i>E. coli</i>	<i>P. pastoris</i>	<i>E. coli</i>	<i>P. pastoris</i>	<i>E. coli</i>	<i>P. pastoris</i>
Tumor	7.75 *	5.08 *	3.84 *	1.92 *	1.77 **	1.11 **
Blood	69.98 **	11.35 **	1.32 **	0.61 **	0.20 **	0.117 **
Liver	2.73	1.53	0.18	0.10	0.05	0.04
Lung	5.45	2.46	0.35	0.16	0.07	0.04
Spleen	2.88	2.41	0.20	0.12	0.06	0.04 (35%)
Kidney	5.43	4.11	0.38	0.23	0.12	0.08
Heart	4.38	2.36	0.26	0.14	0.04	0.03
Stomach	8.53	7.96	0.59 (36%)	0.27	0.07	0.08 (44%)
Intestine	2.22	1.63	0.18	0.08	0.02	0.03
Muscle	1.14	0.97	0.08	0.03 (23%)	0.01	0.01 (40%)

Cohorts of 4 –5 mice were analyzed 4, 24 and 48 h post-injection of ¹²⁵I-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 20% of the average unless otherwise indicated. P-values were calculated for the Tumor and Blood groups

* P 0.05

** P 0.01.

Table 2

Biodistribution of *P. pastoris* vs. *E. coli*-produced ^{125}I -C6.5 diabody in SCID mice bearing s.c. SK-OV-3 xenografts

	<i>E. coli</i>		<i>P. pastoris</i>	
	%ID/g tumor	Tumor : Blood ratio	%ID/g tumor	Tumor : Blood ratio
4 hrs	7.75*	1.2	5.08*	1.6
24 hrs	3.84*	9.5	1.92*	11.9
48 hrs	1.77**	29.5	1.11**	36.7

Pharmacokinetics of *P. pastoris* vs. *E. coli*-produced ^{125}I -C6.5 diabody. ^{125}I -C6.5db was administered to SCID mice bearing SK-OV-3 tumor xenografts via tail-vein injection and values were measured 4, 24, and 48 hours p.i. Tumor: blood ratios increased for both cohorts through 48 Hrs.

* P 0.05

** P 0.01.

Table 3

Average organ activity (% ID) for *P. pastoris* vs. *E. coli*-produced ^{124}I -C6.5 diabody in SCID mice bearing s.c. SK-OV-3 xenografts

<i>E. coli</i>						
Time (hr)	Tumor	Thyroid	Heart	Liver	Stomach	Bladder
2	1.61 ± 0.80	2.51 ± 0.43	3.46 ± 0.20	4.27 ± 2.66	4.88 ± 2.10	3.48 ± 1.31
24	1.09 ± 0.47	1.16 ± 0.88	0.34 ± 0.32	0.46 ± 0.26	1.18 ± 1.38	0.82 ± 0.54
48	0.63 ± 0.13	0.22 ± 0.10	0.09 ± 0.05	0.19 ± 0.07	0.26 ± 0.18	0.17 ± 0.08
120	0.18 ± 0.16	0.18 ± 0.21	0.02 ± 0.01	0.05 ± 0.02	0.03 ± 0.01	0.03 ± 0.02
192	0.08 ± 0.10	0.06 ± 0.02	0.01 ± 0.00	0.04 ± 0.00	0.01 ± 0.01	0.01 ± 0.01

<i>P. pastoris</i>						
Time (hr)	Tumor	Thyroid	Heart	Liver	Stomach	Bladder
2	1.5 ± 0.60	1.7 ± 1.4	0.82 ± 0.22	3.3 ± 1.17	5.7 ± 1.8	17 ± 8.7
24	0.72 ± 0.47	1.2 ± 0.49	0.15 ± 0.04	0.76 ± 0.15	0.65 ± 0.32	1.09 ± 0.43
48	0.59 ± 0.46	0.55 ± 0.14	0.10 ± 0.04	0.39 ± 0.10	0.34 ± 0.22	0.15 ± 0.01
120	0.12 ± 0.10	0.30 ± 0.14	0.02 ± 0.01	0.19 ± 0.05	0.07 ± 0.07	0.04 ± 0.01
192	0.05 ± 0.04	0.20 ± 0.07	0.02 ± 0.01	0.12 ± 0.02	0.04 ± 0.06	0.01 ± 0.01

Average organ activity for mice injected with *P. pastoris*- vs. *E. coli*-produced ^{124}I -C6.5 diabody. ^{124}I -C6.5db was administered to SCID mice bearing SK-OV-3 tumor xenografts via tail-vein injection and mice were imaged at 2, 24, 48, 120, and 192 hours p.i. on a clinical PET/CT scanner. All values are decay corrected. Standard error (+/- SEM) is given following each average organ activity.

Table 4

Comparison of organ uptake as measured by PET and biodistribution

<i>E. coli</i> C6.5db			
	Biodistribution	PET-based Measurement	
Tumor	8.52E-02 +/-	4.66E-02	8.89E-02 +/- 6.03E-02
Liver	5.60E-02 +/-	1.88E-03	4.25E-02 +/- 2.50E-03
Heart	2.79E-03 +/-	6.05E-04	1.25E-02 +/- 2.50E-03
Stomach	2.05E-03 +/-	2.60E-04	1.50E-02 +/- 5.00E-03

<i>P. pastoris</i> C6.5db			
	Biodistribution	PET-based Measurement	
Tumor	5.16E-02 +/-	1.31E-02	4.97E-02 +/- 1.65E-02
Liver	1.18E-01 +/-	8.07E-03	1.42E-01 +/- 1.16E-02
Heart	5.51E-03 +/-	1.01E-03	1.80E-02 +/- 4.90E-03
Stomach	4.78E-03 +/-	4.68E-04	5.20E-02 +/- 2.97E-02

Organ activity for mice injected with either *P. pastoris*- vs. *E. coli*-produced ¹²⁴I-C6.5 diiodotyrosine (C6.5db) tumor xenografts were treated with ¹²⁴I-C6.5db via tail-vein injection, and uptake was quantified at 192 hours p.i. by biodistribution and from data obtained through PET imaging. Average values ± standard error (SEM) are reported.

Table 5

Highest average organ doses (mGy/MBq) and Effective Dose (mSv/MBq) by Group

Target Organ	Average Dose (mGy/MBq)	SEM	Target Organ	Average Dose (mGy/MBq)	SEM
1.0 hour void interval					
<i>E. coli</i>					
Thyroid	4.1E+00	2.1E+00	Thyroid	4.1E+00	2.1E+00
Stomach Wall	3.4E-01	2.0E-01	Urinary Bladder Wall	1.0E+00	1.3E-02
Urinary Bladder Wall	2.6E-01	1.2E-02	Stomach Wall	3.4E-01	2.0E-01
Heart Wall	1.6E-01	3.4E-02	Heart Wall	1.6E-01	3.4E-02
Pancreas	1.0E-01	2.6E-02	Uterus	1.4E-01	1.3E-02
Effective Dose (mSv/MBq)	3.1E-01	1.3E-01	Effective Dose (mSv/MBq)	3.6E-01	1.3E-01
<i>P. pastoris</i>					
Thyroid	4.2E+00	8.3E-01	Thyroid	4.2E+00	8.3E-01
Stomach Wall	2.8E-01	6.0E-02	Urinary Bladder Wall	1.0E+00	5.8E-03
Urinary Bladder Wall	2.5E-01	5.0E-03	Stomach Wall	2.8E-01	6.0E-02
Liver	9.9E-02	5.3E-03	Uterus	1.3E-01	6.0E-03
Pancreas	8.7E-02	8.2E-03	Liver	1.0E-01	5.3E-03
Effective Dose (mSv/MBq)	3.0E-01	7.8E-02	Effective Dose (mSv/MBq)	3.5E-01	7.8E-02

Five highest ranking organ doses (mGy/MBq) and Effective Dose (mSv/MBq) by group for *E. coli*- vs. *P. pastoris*-produced protein. Values were calculated based on bladder voiding intervals of 1.0 and 4.8 hours.