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^{68}Ga-DOTA-Affibody Molecule for *In Vivo* Assessment of HER2/neu Expression with PET

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Abstract

Purpose—Overexpression of HER2/neu in breast cancer is correlated with poor prognosis. It may vary between primary tumors and metastatic lesions and change during the treatment. Therefore, there is a need for new means to assess HER2/neu expression *in vivo*. In this work, we used 68Ga-labeled DOTA-$Z_{HER2:2891}$-Affibody to monitor HER2/neu expression in a panel of breast cancer xenografts.

Methods—DOTA-$Z_{HER2:2891}$-Affibody molecules were labeled with ^{68}Ga. *In vitro* binding was characterized by a receptor saturation assay. Biodistribution and PET imaging studies were conducted in athymic nude mice bearing subcutaneous human breast cancer tumors with three different levels of HER2/neu expression. Nonspecific uptake was analyzed using non-HER2-specific Affibody molecules. Signal detected by PET was compared with *ex vivo* assessment of the tracer uptake and HER2/neu expression.

Results—^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody probe showed high binding affinity to MDA-MB-361 cells (K_D = 1.4±0.19 nM). *In vivo* biodistribution and PET imaging studies demonstrated high radioactivity uptake in HER2/neu-positive tumors. Tracer was eliminated quickly from the blood and normal tissues, resulting in high tumor-to-blood ratios. The highest normal tissue concentration of radioactivity was seen in the kidneys (227±14 %ID/g). High-contrast PET images of HER2/neu-overexpressing tumors were recorded as soon as 1 hour after tracer injection. Good correlation was observed between PET imaging, biodistribution estimates of tumor tracer concentration, and the receptor expression.

Conclusion—These results suggest that PET imaging using 68Ga-DOTA-$Z_{HER2:2891}$-Affibody is sensitive enough to detect different levels of HER2/neu expression *in vivo*.

Keywords

Affibody molecules; Molecular imaging; HER2/neu; PET; Ga-68

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Conflict of Interest The authors declare that they have no conflict of interest [16].

INTRODUCTION

The human epidermal growth factor receptor (HER2/neu; c-erbB2) is a 185 kDa glycoprotein with tyrosine kinase activity that plays an important role in regulating tumor cell survival, proliferation, maturation, and mobility [1]. It has also been shown to be involved in angiogenesis [2]. Amplification and/or overexpression of HER2/neu have been detected in approximately 20% of invasive breast carcinomas and in a significant fraction of ovary, lung, stomach, and bladder cancers. In breast cancer, HER2/neu overexpression is usually associated with increased tumor aggressiveness, resistance to therapies, high rates of recurrence, and increased mortality [3].

HER2/neu expression is routinely determined by immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH) at the time of diagnosis of the primary tumor. However, the HER2/neu status of the primary tumor does not always reflect that of metastatic lesions, and these expression levels might change during the course of the treatment [4-5]. Even though HER2/neu testing is common, there are problems associated with its sensitivity and specificity, potentially confounding the selection of appropriate patients for anti-HER2/neu treatment. Therefore, there is a need for methods that can assess global *in vivo* HER2/neu expression non-invasively and reproducibly. Molecular imaging modalities offer a strategy for determining HER2/neu expression and localizing HER2/neu positive lesions *in vivo*, including inaccessible distant metastasis [6].

Currently available HER2/neu-targeted ligands include full length monoclonal antibodies, Fab-fragments, $F(ab')_2$-fragments, diabodies, minibodies, Affibody molecules, and peptides that can be labeled with either a contrast agent for Magnetic Resonance Imaging (MRI), a fluorescent dye for Optical Imaging, positron-emitting radionuclides for Positron Emission Tomography (PET), or gamma emitting radionuclides for Single Photon Emission Computed Tomography (SPECT) [7-8]. Among these, PET is the most sensitive human-scale diagnostic imaging modality and provides the highest spatial resolution among radionuclide techniques especially when combined with Computed Tomography (CT).

Gallium-68, a metallic positron emitter, is of particular interest for PET imaging since it can be obtained from a generator, thereby, providing a convenient alternative to cyclotron-produced PET isotopes, such as ^{18}F and ^{124}I [9].

The stable oxidation state of gallium in aqueous solutions is +3 and it forms thermodynamically stable complexes with ligands containing oxygen, nitrogen, and sulfur donor atoms [9]. A variety of chelators have been developed to allow the formation of stable ^{68}Ga (+3) complexes. 1,4,7,10-tetraazacyclododecane- N,N',N'',N'''-tetraacetic acid (DOTA) is the most commonly used macrocyclic chelating agent for trivalent radiometals. Complexes of gallium with DOTA have been found to be stable *in vivo* and have been used in clinical studies [9-10].

The site-specific coupling of DOTA derivative during peptide synthesis provides a homogenous conjugate with well-defined chemical and biological properties. Gallium-68 also has attractive physical properties. It decays 89% of the time by positron emission and 11% by electron capture. Its half-life (68 min) matches the pharmacokinetics of radiopharmaceuticals of low molecular weight (less than 60 kDa) that are most suitable for imaging applications due to their fast blood clearance, rapid diffusion and target localization [11]. A potential disadvantage of ^{68}Ga is the relatively high energy of the emitted positrons leading to a mean range in water of 2.9 mm that can reduce spatial resolution compared to PET images obtained with other potential positron labels, e.g. ^{18}F (mean range 0.6 mm) [12].

Among all the HER2/neu-targeting agents, Affibody molecules seem to be the most promising tracers for imaging of HER2/neu receptor expression [13]. Affibody molecules were originally derived from the B-domain in the immunoglobulin-binding region of staphylococcal protein A. The B-domain is a relatively short 58-amino acid peptide (6.5 kDa) that is folded into a three-helical bundle scaffold structure [14].

Application of HER2/neu-specific Affibody molecules, labeled with a variety of radionuclides including ^{18}F, ^{64}Cu, ^{111}In, ^{90}Y, ^{177}Lu [14-15], and, lately, also with ^{68}Ga, for diagnostic and therapeutic purposes have been widely investigated [16] . These studies demonstrated the high tumor to background ratio in HER2/neu imaging due to rapid blood clearance and high-affinity, tumor-specific targeting. In addition, our recent work confirmed the potential of Affibody-based tracers for monitoring HER2/neu receptor changes in murine xenografts after therapeutic interventions [17].

Here, we report the first imaging studies with ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody probe in a panel of breast cancer xenografts with different HER2/neu expression levels as verified by flow cytometry and ELISA assay. Our results indicate that this tracer would enable non-invasive assessment of HER2/neu expression level *in vivo* by PET imaging.

MATERIAL and METHODS

General

The ^{68}Ge/^{68}Ga generator (IGG100), in which the parent isotope ^{68}Ge (1850 MBq) is adsorbed on a TiO_2 column, was procured from Eckert & Ziegler Isotope Products (Berlin, Germany). Analytical grade chemicals were used. Water was distilled and deionized (18 MΩ/cm^2) using a Milli-Q water filtration system (Millipore Corp., Milford, MA). For radiolabeling of Affibody molecules, the ^{68}Ge/^{68}Ga generator was coupled to an automated Modular Lab (Eckert & Ziegler Gmbh., Berlin, Germany). The labeling reactions were carried out in 3 mL glass vials (Wheaton Science Products, Millville, NJ). Strata–XC cartridges, 30 mg (Phenomenex, Torrance, CA), were used for pre-purification of the eluted ^{68}Ga. DOTA-$Z_{HER2:2891}$ (ABY025) and non-HER2-specific Affibody (His6-ZTaq-Cys) were kindly provided by our Cooperative Research and Development Agreement partner: Affibody, AB, Stockholm, Sweden. His6-ZTaq-Cys Affibody was conjugated with DOTA according to the method reported by Ahlgren et al. 2008 [18].

Radiolabeling

The ^{68}Ge/^{68}Ga generator was eluted with dilute hydrochloric acid (0.1 M HCl solution) to obtain ^{68}Ga in 10 mL of 0.1 M HCl solution. The ^{68}Ga solution was immediately transferred to a Strata XC cartridge (cationic polymeric sorbent), air-dried for a few seconds, and then treated with 0.7 mL of 0.02 M HCl, 98% acetone solution (a solution of 0.78 mM HCl with 97.56 mL of acetone) to release the ^{68}Ga. Pre-purification of the generator eluent was performed to reduce any trace metallic impurities and limit ^{68}Ge breakthrough. DOTA-$Z_{HER2:2891}$ (30 μg) was added to 0.5 mL of 0.25 M ammonium acetate buffer (pH 3.9). Purified ^{68}Ga eluate (400 MBq) was added to this solution and heated at 80 °C for 15 minutes.

The radiochemical purity of the labeled conjugate was determined by reverse-phase, high-performance liquid chromatography (RP-HPLC) (Beckmann-Coulter, System Gold) equipped with a UV166 detector (Beckmann Coulter Inc, Brea, CA) using a C-18 column (Agilent Extend, C-18, 4.6 ×150 mm), eluted with 0.1% TFA in water (solvent A) and 0.1 % TFA in acetonitrile (solvent B). The HPLC gradient was 20% - 40% of solvent B over 16 minutes with a flow rate of 1 mL/min. Monitoring of radioactivity during the HPLC run was performed using an inline radio-detector (Flow-Count radio-HPLC Detector System,

Bioscan, Washington DC). The reaction mixture was then applied to a NAP-10 column (5 kDa cut-off), and eluted with phosphate-buffered saline (PBS) to obtain ^{68}Ga-DOTA-$Z_{HER2:2891}$. The purity of the filtrate was determined by RP-HPLC.

Cell Lines

Human breast (BT474, MDA-MB-361, and MCF7) that express different levels of HER2/neu were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured either in RPMI 1640 (BT474) or in Dulbecco modified Eagle (MCF7, MDA-MB-361) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO) and penicillin/streptomycin (a 100 U/mL concentration of each). Cells were grown as a monolayer at 37°C in a humidified atmosphere containing 5% CO_2.

Fluorescence-Activated Cell Sorter (FACS) Analysis

For HER2/neu detection, cells were cultured overnight in T75 flasks (Sarstedt). The following day cells were rinsed twice with (PBS) and detached with cell stripper (Cellgro). For every sample, 1×10^6 cells were collected, and incubated for 45 min on ice with anti-mouse Neu(24D2)-fluorescein isothiocyanate monoclonal antibody or with IgG_1-fluorescein isothiocyanate mouse isotypic control (Santa Cruz Biotechnology, Inc.). After antibody staining, all cells were washed with PBS and resuspended in 0.3 mL of PBS for data acquisition.

All solutions were kept on ice. Flow cytometry was performed using a LSRII (BD Biosciences, San Jose, CA). FACSDiVa software (BD Biosciences, San Jose, CA) was used for data acquisition and FlowJo software for analysis (Tree Star Inc.). For each sample, 20,000 events were recorded, and the population corresponding to single cells was gated and analyzed as a histogram plot. Results are reported as mean fluorescence intensity.

Enzyme-Linked Immunosorbent Assay (ELISA)

The HER2/neu protein level in tissue lysates was measured according to the procedure reported previously [17]. Briefly, the tumor tissue was homogenized on ice, using a Polytron homogenizer, and lysed in resuspension buffer provided with the ELISA kit (Calbiochem). Cell debris was removed through centrifugation of the suspensions at 13,000 rpm (4°C) for 15 min. The supernatants were collected and stored at -80°C. Before use, the protein concentration was estimated by the bicinchoninic acid assay kit (Pierce), according to the manufacturer's protocol. Then the HER2/neu protein level was measured by an ELISA kit, according to the manufacturer's recommended procedure. Results were expressed in nanograms of HER2/neu per milligram of protein. The data are presented as the mean of 2 replicates, with each experiment repeated 3 times.

In Vitro Cell-Binding Assays

The *in vitro* binding characteristics of the ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody was evaluated using saturation cell-binding assays. The day before the experiments, MDA-MB-361 cells were seeded in six-well plates at concentration of 5×10^5 cells per ml. For receptor saturation analysis, the cells were incubated (4°C, 2 h) with increasing concentrations (0.06–220 nM) of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody, either alone or with an additional 50-fold excess of non-labeled $Z_{HER2:2891}$-Affibody molecules. Then, the cells were washed with cold phosphate-buffered saline (PBS, pH 7.0), trypsinized, and the cell-associated radioactivity was measured using a γ-counter (1480 Wizard 3, Automatic Gamma Counter, Perkin-Elmer, Waltham, MA). Specific binding was plotted against the total molar concentration of added ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody. The K_D value and the concentration of the tracer required to saturate the receptors (B_{max}) were analyzed by non-linear regression using

GraphPad Prism5 (GraphPad Software, San Diego, CA). The B_{max} value (nM) was converted to the number of HER2/neu receptors per cell. The assay was performed in duplicate and repeated in 2 separate experiments.

Tumor Model

All animal studies were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the National Institutes of Health. Cells suspended in Matrigel (BD Bioscience) were implanted subcutaneously into the shoulder region of 5- to 7-week old female athymic nude mice: 5×10^6 MDA-MB-361, MCF7 or 8×10^6 BT474 cells. For MCF7 and BT474 cells estrogen pellets (1.72 mg; Innovative Research of America) were implanted 24 hours before breast cancer cells inoculation and remained in place until the end of the study. Tumors (150–200 cm^3) developed after 3–5 weeks.

Biodistribution

^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody (3.7–6.7 MBq, 10-13 μg, 100 μL) was injected into the tail vein of mice bearing BT474 tumors. Mice were euthanized, and their major organs were dissected and weighed at 1 and 3 hours after injection. Four mice were used for each data point. Radioactivity uptake in the tissues was measured, along with a standard of the injected dose, using a γ-counter. The results were expressed as percentage injected dose per gram of tissue (%ID/g).

To study the blood kinetics and the stability of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody *in vivo*, the blood was collected in vials containing 30 μl of heparin at 15, 30, 45, 60, 120, and 180 minutes post-injection. The samples were weighed, and the radioactivity was measured by γ-counter. Then, the blood was centrifuged (10 min at 10,000×g) and fractions of plasma (200–300 μl) were separated on NAP-10 columns. The mean concentrations of radioactivity in the blood (% ID/g) were plotted against time after injection, and the curve was fitted by single exponential curve.

To confirm the specificity of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody binding *in vivo*, one group of mice bearing BT474 (very high HER2/neu expression) was injected with the non-HER2-specific Affibody molecules, His6-ZTaq-Cys-DOTA-^{68}Ga. One hour later, mice (n = 4) were euthanized and their organs analyzed as described above. This time point was chosen since it showed the optimal tumor-to-background ratio for the binding of the labeled HER2-specific Affibody molecules.

PET/CT Imaging

PET scans were performed using the NIH Advanced Technology Laboratory Animal Scanner (ATLAS). CT images were obtained with the CT component of the NanoSPECT/CT scanner from Bioscan (Bioscan, Inc., Washington, DC). Spatially registered and fused PET and CT images were obtained by using a custom-designed adapter to the ATLAS bed support that allowed the Bioscan imaging bed (and the animals) to be reproducibly transferred between machines that had been previously calibrated for accurate spatial registration. The mice were anesthetized using isoflurane/O_2 (1.5-2.0 % v/v), placed prone in the center of the field of view of the scanner, and given an injection of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody (3.7–6.7 MBq, 10-13 μg, 100 μL) via the lateral tail vein. Whole-body scanning was performed with a 2-cm axial field of view (3 bed positions each of 10 min) at 1, 2, 3 hours after radiotracer injection and/or a 10-min emission scan (single field of view) was recorded 1 hour post injection, both with a 100- to 700-keV energy window. The images were reconstructed with a 2-dimensional ordered-subsets expectation maximum

algorithm (2D-OSEM). No correction was applied for attenuation or scatter. For each scan, regions of interest (ROI) were manually drawn over the tumor and normal tissue. The maximum counts per pixel within the tumor or normal tissues were obtained from multiple regions of interest (counts/s/cm^3). The results were calculated as %ID/g using a calibration constant obtained from scanning the ^{68}Ga source, assuming a tissue density of 1 g/ml, and dividing by the injected dose, decay-corrected to the time of scanning.

The CT component of the nanoSPECT/CT was configured to acquire image data with the X-Ray tube high voltage set to 65 keV, a sampling time of 1500 ms, and pixel size of 200 microns. The CT images were reconstructed using the Fast Cone Beam FBP algorithm with a Shepp Logan and a frequency cut off at 100% of the Nyquist frequency. ATLAS and CT images were spatially registered and fused as described above.

Statistics

Statistical analysis was performed using GraphPad Prism5. Correlations between PET quantification and biodistribution data were made using Pearson correlation test ($p < 0.05$). A p-value of less than 0.05 was considered as significant.

RESULTS

HER2/neu expression

Table 1 presents relation of HER2/neu expression in the tumor xenografts used in this study to *in vitro* binding of fluorescence-labeled Affibody molecules to the corresponding cell-lines *in vitro*. There is a statically significant linear correlation (R^2>0.99) between the obtained values.

Radiolabeling of ^{68}Ga-DOTA- $Z_{HER2:2891}$ Affibody

The elution profile of the ^{68}Ga-DOTA- $Z_{HER2:2891}$-Affibody reaction mixture showed RCP exceeding 90% with the product eluting at a retention time of 7.2 minutes. Specific activity of the radiolabeled conjugate was in the range of 2.8 – 5.2 GBq/nmol. Uncomplexed ^{68}Ga eluted at 1.8 minutes. The elution profile of the purified ^{68}Ga-DOTA-$Z_{HER2:2891}$ Affibody showed the presence of a single peak (retention time of 7.2 minutes) corresponding to the isolated product (Fig.1.).

HER2/neu Binding Saturation

In vitro binding of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody to HER2/new on MDA-MB-361 cells was investigated using saturation binding assay. In a control experiment, a large excess of non-labeled Affibody molecules was added in order to saturate HER2/neu receptors and demonstrate binding specificity. A representative saturation binding curve is shown in Fig.2. The data indicated a single class of high-affinity binding sites that had a mean K_D value of 1.4±0.19 nM. The B_{max} value, expressed as the number of HER2/neu receptors per cell, was calculated to be (9.7±0.02)×10^5.

Biodistribution

Tumor and normal tissue uptake of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody in mice bearing tumors with very high HER2/neu expression (BT474) at the time points 1 and 3 hours post injection is shown in Table 2. There was very high (31±2.1 %ID/g) accumulation of the radioactivity in the tumors as early as 1 hour post injection. At that time, tumor-to-blood activity ratios were 27±4.4 and increased to 91.8±13.7 three hours later.

Notably, the highest normal tissue concentration of radioactivity was seen in the kidneys. The uptake ranged from 223±26 %ID/g to 227%±14.2 ID/g. The tracer uptake in all other organs was low over the entire time course of the experiment.

The *in vivo* HER2/neu binding specificity was evaluated using non-HER2-specific Affibody molecules. One hour post injection the tumor-associated radioactivity in mice bearing BT474 tumors was comparable to that of surrounding tissues: 1.1±0.4 %ID/g in the tumor, 1.3±0.2 %ID/g in the liver and 0.2±0.1%ID/g in the muscles. Notably, the tumor uptake was 31-fold lower than observed in mice receiving the HER2/neu-specific ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody. This difference confirmed that the tracer accumulation in these tumors was HER2/neu-dependent.

Blood Kinetics

The mean radioactivity in the blood expressed as %ID/g is shown in Fig.3 as a function of time after administration of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody. The serum clearance was rapid with an elimination half-life of 20.8 min. The blood plasma was also evaluated for metabolic stability of ^{68}Ga-labeled Affibody. To distinguish radiolabeled Affibody (high molecular weight) from small-molecule metabolites (low molecular weight), plasma samples were eluted through NAP-10 columns (5 kDa cut-off) with PBS. Reference analysis of a solution containing ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody molecules on the NAP10 column resulted in 97% of the radioactivity eluted in the high molecular weight (HMW) fractions. The majority of radioactivity from plasma samples was also associated with the HMW fractions, indicating that the tracer was still present in the blood at 2 hours post injection (Fig.3. - Table insert).

PET/CT Imaging

A representative PET/CT whole-body image of a mouse bearing BT474 tumor, obtained 2 hours post tracer injection, is shown in Fig.4. The axial sections comparing mice with different levels of HER2/neu expression are shown in Fig.5.

High contrast images were recorded 1 hour post injection, indicating high tracer uptake in tumors expressing high (BT474) and medium (MDA-MB-361) levels of HER2/neu as compared with the low-expressing tumors (MCF7). The radioactivity uptake in the tumors was significantly higher than in normal tissues except for the kidneys. The ROI values ranged from 31±7.0 %ID/g for BT474 to 18.8±2.5 %ID/g for MDA-MB-361. We observed poor, but still detectable signal from MCF7 tumors, the ROI value was 3.2±0.4 %ID/g. Notably, we found a strong and significant correlation between %ID/g values obtained from biodistribution and PET quantification studies ($R^2 = 0.94$, $p < 0.0001$) for all tumor bearing mice (Fig.6A). Both, tracer accumulation and the signal detected in tumor by PET depended on the receptor expression, as assessed by ELISA (Fig.6B).

Discussion

Molecular imaging has great potential for characterization of therapeutically important targets in malignancies [19], including HER2/neu [6, 20]. Because HER2/neu expression levels in metastases may be quite different from the primary tumor [21], assessment of the specific expression of these receptors on cancer cells in various locations may be useful in selecting appropriate therapy [22]. However, the development of HER2/neu-specific imaging probes for routine clinical use has not yet been successful.

In our previous work [15], we labeled Affibody molecules with ^{18}F - widely available and a nearly pure (97%) positron emitter with a relatively short, but clinically useful, half-life of 110 minutes. However, ^{18}F production requires a cyclotron, and the radiolabeling

procedures involving this radionuclide require several low-yielding steps that are often time consuming. Therefore, in this study, we evaluated the potential of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody [23] as an imaging agent in xenografts with different HER2/neu expression levels.

Gallium-68 presents an attractive alternative as a radionuclide for PET imaging. It can be obtained from commercially available ^{68}Ge-^{68}Ga generators, which have a half-life of 271 days and, thus, can provide an almost constant supply of ^{68}Ga for over one year. The labeling procedures are well established and relatively straightforward. Also, when prorated, the unit cost of ^{68}Ga from generators is reasonable when amortized over a year, which makes it even more attractive for busy nuclear medicine departments, particularly those with limited or no access to cyclotrons.

Most of the efforts in developing tracers for HER2/neu focused on using anti-HER2/neu antibodies. However, antibodies are rather bulky proteins typically characterized by limited tumor penetration and slow clearance from the circulation. A successful molecular imaging probe requires high affinity and specificity binding to tumor-associated structures with minimal non-specific accumulation in normal tissues and rapid clearance from the background. Optimally, it should easily penetrate the tumor parenchyma and rapidly clear from the blood to increase the tumor to background ratio. Recently, significant progress has been made in protein display technology that has led to the discovery of many small proteins and peptides [24-25]. Among them, Affibody molecules have been shown to meet most of the aforementioned requirements providing a suitable platform for development of new imaging probes [14, 26].

Our current results demonstrated the high affinity of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody for HER2/neu receptors on MDAMB-361 cells *in vitro.* The K_D was similar to those found with other Affibody radioconjugates and was comparable to antibodies used clinically for imaging and therapy (e.g., the reported affinity of trastuzumab to HER2/neu is 5 nM) [27].

The *in vivo* pharmacokinetics data were also promising. The elimination half-life estimated by measuring radioactivity in blood samples at several time points after injection was approximately 21 min. This relatively fast clearance resulted in high tumor-to-blood ratios as well as high-contrast PET images of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody molecules as early as 1 hour post tracer injection. It is noteworthy that the obtained tumor-to-blood ratios were higher than for other imaging probes including ^{68}Ga-DOTA-MUT-DS, ^{68}Ga-ABY-002, ^{68}Ga-labeled DOTA-F(ab')$_2$ fragment of Herceptin, as well as full length antibodies and antibody fragments labeled with ^{124}I, ^{89}Zr and, ^{86}Y [28-31].

Because of the fast blood clearance, the images showed high tumor accumulation as compared with other tissues (except the kidneys) in the high- and intermediate-expressing tumors. In highly HER2/neu-expressing BT474 tumors the uptake reached 31±7.0 %ID/g as soon as 1 hour post tracer injection, and is was higher than the accumulation levels recently described by Ren et al. [30] and Tolmachev et a [16] for SKOV3 tumors. The absolute tumor accumulation reported by these authors was only 4.12±2.2 %ID/g (2 hours post injection of an Affibody-based, 2-helix ^{68}Ga-DOTA-MUT-DS molecule) and 8.9±1.0 %ID/g (45 min post injection of ^{68}Ga-ABY-002). It should be noted that SKOV3 cells express even more HER2/neu receptors than BT474 (ELISA results from our laboratory: 335 ng/mg for SKOV3 *versus* 260 ng/mg for BT474). These results are in line with our previous data obtained with radio-fluorinated probe [17]. It should be stressed that correction for the uptake for nonspecific binding of His6-ZTaq-Cys-DOTA-68Ga was not necessary since the accumulation of this non-HER2-specific tracer was approximately at the same level as surrounding tissues, and tumors were not detectable. We have previously shown a strong association between the uptake of ^{18}F-FBEM– ZHER2:342-Affibody tracer in the panel of

breast cancer tumor xenografts with different HER2/neu expression [17]. Also, McLarty et al., using mice bearing tumors with a wide range of receptor levels, showed a correlation between 111In-DTPA-trastuzumab accumulation and HER2/neu density in individual tumors [32].

In this work, we investigated the correlation between HER2/neu expression determined by PET imaging *in vivo* and the biodistribution data for each particular tumor model following injection of ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody and by comparison to HER2/neu protein concentration measured in the same tumors *ex vivo* by ELISA. Based on our experience with ^{18}F-labeled Affibody molecules and considering the short half-life of gallium, the biodistribution and imaging studies were done at 1 and 3 hours post tracer injection. There was no indication of label instability since the tracer was rapidly cleared from the blood and liver. By analyzing the biodistribution and imaging data, we were also able to correlate the tracer uptake with number of HER2/neu receptors. Unlike results reported for antibodies by McLarty et al. [32], who had to apply appropriate corrections for non-specific tumor uptake and blood flow, we found a strong linear correlation between PET values [%ID/g] and biodistribution data that also corresponded to HER2/neu level in each tumor. It is important to note that using gallium-labeled agents the estimation of receptor expression may be limited for the very low receptor expressing tumors. This is due to the greater positron range of ^{68}Ga (1.9 MeV) compared for example to ^{18}F that could lead to poorer spatial resolution and erroneous assignment of the ROI. The intrinsic resolution of small-animal PET scanners has improved to a level where the positron range of the radionuclide eventually could influence the effective spatial resolution. Jorn van Dalen *et al.* showed that the image resolution of a PET scanner with a 1.5 mm intrinsic system resolution is decreased by using PET emitters with large positron ranges such as ^{68}Ga and ^{124}I [33]. This could impact: (i) the detection of small lesions, (ii) assessment of low receptor expression, (iii) the ability to distinguish lesions that are close together, as well as (iv) quantification of tumor uptake. Therefore, the positron energy and range must be considered when planning small-animal imaging. On the other hand from clinical point of view, Yang *et al.* demonstrated that the conventional FWHM of ^{18}F and that of ^{68}Ga are indistinguishable in soft tissues (3.01 mm vs. 3.09 mm) [34]. This implies that with the spatial resolution of 5-7 mm for current clinical scanners, image quality using ^{68}Ga-labeled tracers should be comparable to ^{18}F-labeled agents.

We observed very high radioactivity accumulation in the kidney. These values are comparable to those previously reported for gallium-labeled Affibody molecules [16]. Use of ^{68}Ga as well as other metal positron emitters including ^{64}Cu and ^{89}Zr, leads to residualization that results in trapping of radionuclides in lysosomes after internalization and degradation of the tracer [35-36]. Because of this phenomenon, the kidney is the major dose-limiting organ for this tracer. However, this is not a major diagnostic limitation since renal lesions are uncommon in breast cancer.

Positively charged amino acids, such as lysine and arginine are now commonly used for renal protection in clinical trials of peptide receptor-targeted radionuclide therapy and may be useful in this setting as well [37]. Studies of the possible effect of positively charged amino acids on accumulation of radioactivity in the kidney following ^{68}Ga-DOTA-$Z_{HER2:2891}$ Affibody administration are, however, outside the scope of this paper. In conclusion, our results indicate that differences in HER2/neu expression levels can be detected *in vivo* by PET imaging with ^{68}Ga-DOTA-$Z_{HER2:2891}$-Affibody. This generator-produced agent may, therefore, be useful in the characterization HER2/neu-positive tumors as an alternative to ^{18}F-labeled compounds especially where access to a cyclotron is limited.

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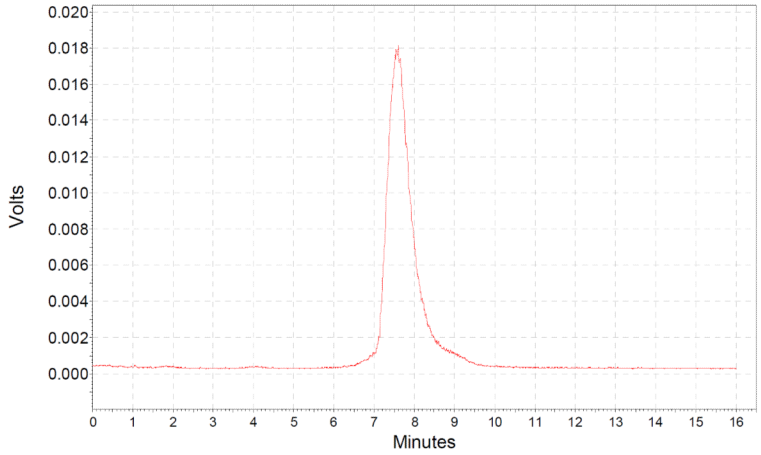


Fig.1.
HPLC radiochromatogram of purified ^{68}Ga-DOTA-$Z_{HER2:2891}$ Affibody.

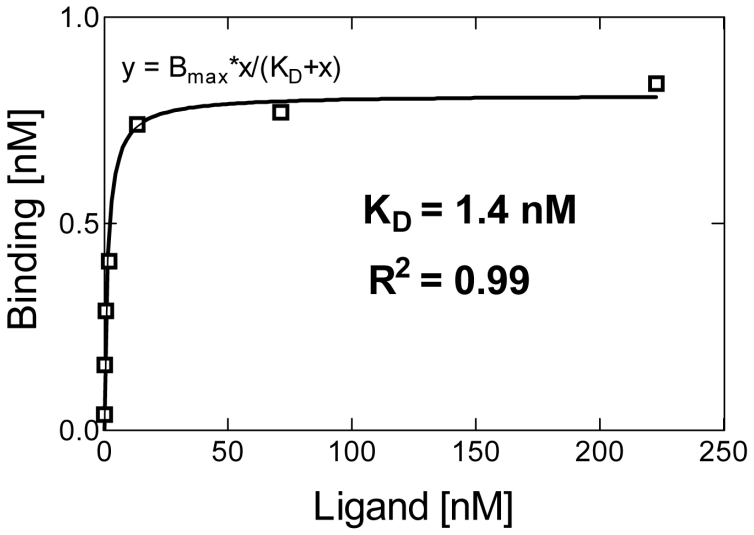


Fig.2.

Representative results of saturation assay using MDA-MB-361 cells (concentration of radiolabeled Affibody molecule versus concentration bound). The K_D of ^{68}Ga-DOTA-$Z_{HER2:2891}$ Affibody was determined to be 1.4 nM.

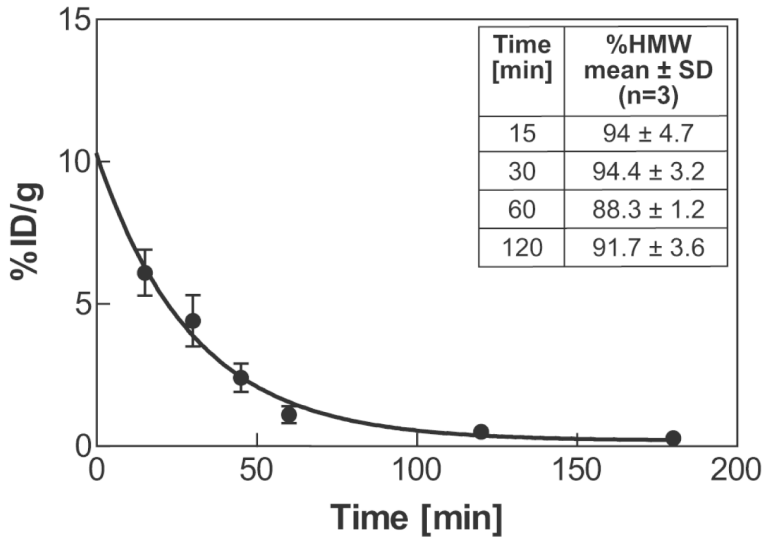


Fig.3.

Blood kinetics. The squares represent % ID/g in the blood with an exponential curve fit. *Insert Table*: average HMW fractions of the plasma-associated radioactivity. Each point represents mean ± SD (n = 3).

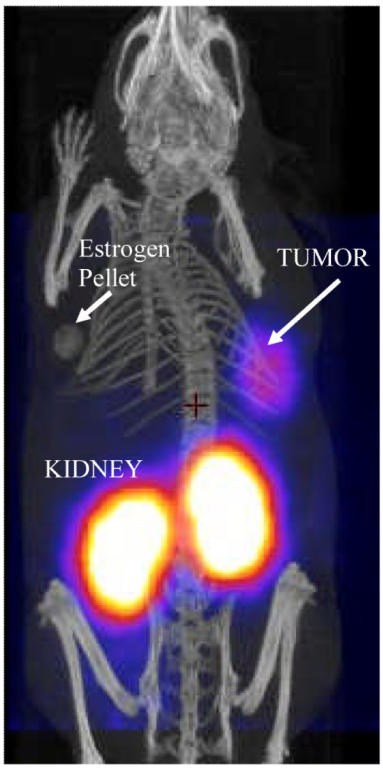


Fig.4.
Posterior whole-body fusion PET/CT image of representative athymic mouse implanted subcutaneously in the right shoulder with BT474 breast cancer cells two hours post tracer injection.

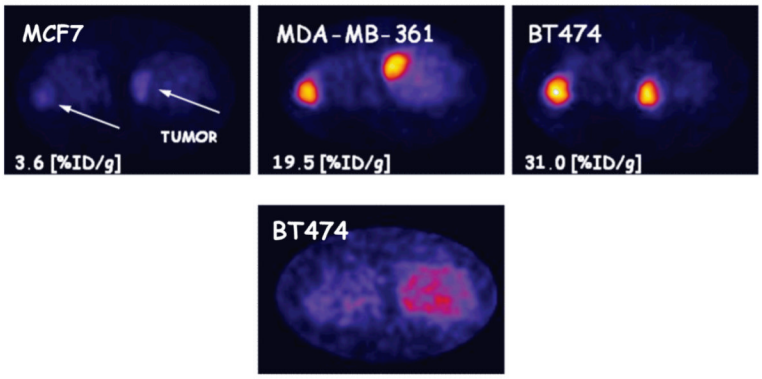
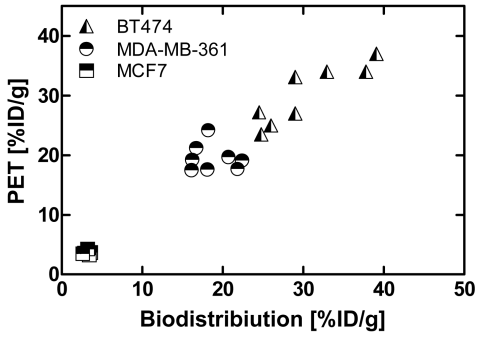


Fig.5.

Axial sections one hour post ^{68}Ga-DOTA-$Z_{HER2:2891}$ Affibody injection in mice bearing tumors with different HER2/neu expression Mcf7< MDA-MB-361 < BT474 (upper panel). Non-specific binding of His6-ZTaq-Cys-DOTA-68Ga in nude mice bearing BT474 (lower panel).

A.



B.

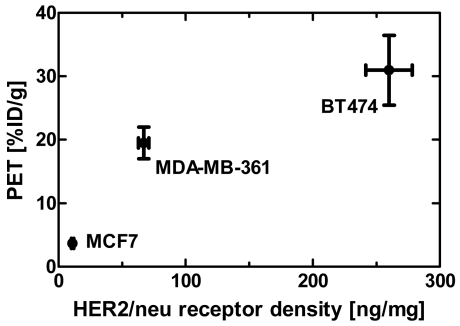


Fig.6.

Correlation between: A. PET quantification and biodistribution data ($p < 0.0001$; $R^2=0.95$) B. HER2/neu expression measured *ex vivo* and 68Ga-DOTA-$Z_{HER2:2891}$ Affibody uptake *in vivo* ($R^2 = 0.84$); in mice bearing tumors with different HER2/neu expression (n = 5-8).

Table 1

Expression of HER2/neu in breast carcinoma cells and tissue lysates measured by flow cytometry and ELISA assay respectively.

	FACS/ cell lysates			ELISA/ tissue lysates	
	Cells only	**IgG_1**	**Neu(24D2)**	**Neu(24D2) - background**	**ng/mg**
BT474	113	104	9 599	9 486	260 ± 18.2
MDA-MB-361	113	114	2 993	2 879	67 ± 4.1
MCF7	60	61	283	222	11 ± 0.9

Table 2

Biodistribution results for ^{68}Ga- DOTA-$Z_{HER2:2891}$ and His6-ZTaq-Cys-DOTA-68Ga Affibody injection in nude mice bearing BT474, mean ± SD (n = 4).

	^{68}Ga- DOTA-$Z_{HER2:2891}$		His6-ZTaq-Cys-DOTA-68Ga
	1 h after injection	**3 h after injection**	**1 h after injection**
Blood	1.13±0.39	0.28±0.0235	1.2±0.36
Heart	0.87±0.32	0.3±0.04	
Lungs	1.9±0.49	0.56±0.07	
Kidney	223±26	227±14.2	172.2±12.5
Spleen	0.9±0.22	0.49±0.03	
Liver	2.04±0.25	1.7±0.49	1.3±0.2
Pancreas	0.6±0.05	0.38±0.031	
Tumor	31±2.1	25.7±2.2	1.1±0.4
Bone	1.2±0.5	0.47±0.09	
Small intestine	0.6±0.2	0.43±0.09	
Muscle	0.4±0.09	0.14±0.02	0.2±0.1
Ratios:			
T/Blood	*27± 4.4*	*91.8± 13.5*	
T/Muscle	*91.8± 13.7*	*128± 41*	