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Tumor immunolocalization Using ¹²⁴I-Iodine labeled JAA-F11 Antibody to Thomsen-Friedenreich alpha-linked Antigen

Richa Chaturvedi¹, Jamie Heimburg², Jun Yan¹, Stephen Koury¹, Munawwar Sajjad³, Hani H Abdel-Nabi³, and Kate Rittenhouse-Olson^{1,2,*}

¹ Department of Biotechnical and Clinical Laboratory Sciences, University at Buffalo, the State University of New York, Buffalo, New York 14214

² Department of Microbiology and Immunology, University at Buffalo, the State University of New York, Buffalo, New York 14214

³ Department of Nuclear Medicine, University at Buffalo, the State University of New York, Buffalo, New York 14214

Abstract

Clinical immunolocalization has been attempted by others with an anti- Thomsen-Friedenreich antigen (TF-Ag) mAb which bound both alpha- and beta-linked TF-Ag. In this report, ¹²⁴I labeled mAb JAA-F11 specific for alpha-linked TF-Ag showed higher tumor specificity in *in vivo* micro positron emission tomography (microPET) of the mouse mammary adenocarcinoma line, 4T1, showing no preferential uptake by the kidney. Labeled product remained localized in the tumor for at least 20 days. Glycan array analysis showed structural specificity of the antibody.

Keywords

immunolocalization; biodistribution; microPET; radiolabeled monoclonal antibody; JAA-F11; TF-Ag; breast carcinoma

Introduction

Thomsen-Friedenreich antigen (TF-Ag) is the mucin-type disaccharide Galactose β 1–3N-acetylglucosamine conjugated to proteins by an alpha O-serine or O-threonine linkage and is a tumor associated antigen which is found on human carcinomas of many types including those of the breast, colon, bladder and prostate. TF-Ag is a cell surface antigen, and cell surface carbohydrates and alterations in their expressions have been associated with malignant transformations (Dennis, 1992). TF-Ag is also expressed on the transplantable murine mammary cancer cell line 4T1. The murine monoclonal antibody (mAb) JAA-F11, an IgG₃, is highly specific in its reaction with the alpha-linked TF-Ag which is expressed on many types of human carcinomas and their metastases (Rittenhouse-Diakun et al., 1998). Most of the monoclonal antibodies which have been produced to TF-Ag have been of the IgM isotype which is less useful in radiolocalization due to its large molecular weight. JAA-

*Corresponding author: Kate Rittenhouse-Olson, 26 Cary Hall, 3435 Main St, Buffalo NY 14214, 716-829-3630 x116, fax 716-829-3601, krolson@buffalo.edu.

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F11 has been shown to block early stages of metastasis, and has been shown to have slight but significant inhibitory effects on *in vitro* tumor cell growth. In recent *in vivo* studies, prolonged survival and a reduced number of lung metastases were seen in 4T1 tumor-bearing mice treated with mAb JAA-F11 (Heimburg et al., 2006).

Since the expression of TF-Ag has been proposed to be a tumor-marker and a clinical diagnostic tool (Takanami, 1999), we hypothesize that the mAb JAA-F11 to TF-Ag, when radiolabeled with the positron emitter ^{124}I -Iodine, will improve detection of occult breast cancer metastatic tumors. JAA-F11 characterization using the structurally relevant saccharide, monosialo-GM1 ganglioside, showed a lack of reaction with JAA-F11, which is important because it shows that related carbohydrate structures on normal cells would not bind the JAA-F11 antibody. This point also differentiates JAA-F11 from the anti-TF-Ag antibody 170H.82 which previously underwent clinical trials only to be withdrawn (Longenecker et al., 1987; Dessureault et al., 1997). The 170H.82 antibody recognizes both the alpha- and beta-linked derivatives ($\text{Gal}\beta 1-3\text{GalN Aca}$ and $\text{Gal}\beta 1-3\text{GalNAc}\beta$) of TF-Ag and, therefore, could potentially interact with carbohydrate structures expressed on normal tissues such as asialo-GM1 (a natural killer cell marker), whereas JAA-F11 does not (Longenecker et al., 1987). No testing of the 170H.82 antibody in mice was reported, but the Longenecker group did perform *in vivo* tumor localization experiments with 2 other similar reagents which also bind both the TF-Ag- α and β derivatives: radiolabeled peanut lectin (PNA) (Shysh et al., 1985; Abdi et al., 1986) and radiolabeled mAb 155H.7 (Turner et al., 1988) which they reported to be similar to mAb 170H.82 (Longenecker et al., 1987). The PNA radiolocalized to the kidney both in mice (72 hours, kidney:tumor ratio of 1.3) (Shysh et al., 1985) and in humans (17 patients, only 1 in which PNA localized to a tumor) (Abdi et al., 1986). In fact in humans, the kidney localization was so dramatic, that in the discussion, the authors suggested that radiolabeled PNA might be used for renal tubular damage assessment, and found it to be concentrated in the renal tubular basement membrane (Abdi et al., 1986). In their mouse experiments with the 155H.7 antibody which has a specificity similar to 170H.82, the kidney to tumor ratio was 0.75, and the kidney was higher than any organ except the thyroid (pretreatment with cold iodine was not performed). These mouse studies were also of a limited, 72 hour, duration (Turner et al., 1988). To further understand the binding of our TF-Ag- α specific mAb JAA-F11, *in vivo* immunolocalization was performed in a system which would test antibody localization to a primary tumor in the mouse model and structural specificity analysis is presented herein using the printed glycan array of the Consortium for Functional Glycomics (<http://www.functionalglycomics.org/static/consortium/>). The glycan array tested the binding of the antibody with 200 different glycans to determine the chemical specificity of the reactivity of this mAb (Blixt et al., 2004).

Positron emission tomography (PET) is a well established modality that provides *in vivo* quantitative distribution of biomolecules. Iodine-124 is the only isotope of iodine which decays by positron emission with a half life of 4.2 days. This is ideal for the labeling of antibodies because of their long biological half life and also due to the ease of labeling. PET images can provide quantitative information of the biological processes over several weeks without sacrificing the animal. The biodistribution data obtained by using ^{124}I -labeled antibodies can be useful in determining radiation dosimetry of the ^{131}I -labeled antibodies for therapeutic use.

Materials and Methods

Monoclonal antibody

JAA-F11 is a monoclonal antibody to TF-Ag which was developed in our laboratory and is cultured in our laboratory in DMEM (Mediatech, Herndon, Va.) in 5% ultra low Ig fetal calf

serum (GIBCO, Life Technologies, Grand Island, NY) with added L-glutamine, sodium pyruvate and non-essential amino acids (Mediatech, Herndon, Va.). JAA-F11 was partially purified using the ammonium sulfate precipitation technique. The supernatant was dialyzed against water and lyophilized. IgG was purified from other contaminants and collected using a Sephadex G-200 size exclusion column. Enzyme Immunoassay (EIA) was performed on the fractions using a TF-Ag-BSA coated microtiter plate, and reactive fractions were pooled, dialyzed, and lyophilized to make a final suspension of 1.2 mg/ml in 0.1 M Phosphate Buffered Saline (PBS).

Monoclonal antibody glycan analysis

JAA-F11 (produced in our laboratory) was analyzed for reactivity with 200 glycans by the Consortium for Functional Glycomics using the printed glycan array. The method used is described on the Consortium website and is detailed in Blixt (2004). Briefly, the printed array was incubated successively with the antibody, washed, and incubated with secondary antibody labeled with FITC. After washing, the image was read in a Perkin Elmer Microscanarray XL4000 and a TIFF file of the image was stored. Image analysis was performed using the Imogene V.6 image analysis software.

Radionuclide

^{124}I was produced by the ^{124}Te (p, n) ^{124}I reaction (Sajjad et al., 2006). $^{124}\text{TeO}_2$ (tellurium oxide) target was irradiated by 14.1 MeV protons at 24 μA current for about 4 hours in a cyclotron. The radioiodine was separated by the dry distillation method. The distilled ^{124}I was trapped in 0.01 M NaOH. The ^{124}I solution was analyzed on an HPLC to determine the chemical and radiochemical purity. The separation was performed using Lichrosorb RP18 analytical column (5 μm , 250 mm \times 4.6 mm, Alltech). The mobile phase consisted of a mixture of 90% buffer (0.5 M potassium phosphate and 0.002 M tetrabutylammonium hydroxide, pH 7.0) and 10% acetonitrile. The flow rate was 1.0 ml/min and the UV detector was set at 225 nm wavelength.

Iodination chemistry

Two different iodination strategies were examined. The antibody was radiolabeled with ^{124}I by using direct Iodogen method and indirect Bolton-Hunter method. The immunoreactivity of JAA-F11 was then determined using radioimmunoassay (RIA) and EIA described below. The Bolton-Hunter method was chosen for iodinating JAA-F11 based on the results obtained from these assays.

Iodogen method

JAA-F11 was labeled with ^{124}I by using a variation of the original Iodogen method (Fraker, 1978). MAb JAA-F11 (1.2 mg/ml in PBS) was added to sodium [^{124}I]-iodide. Iodogen coated beads (Pierce Chemical Co.) were added to the solution. The reagents were mixed in a reaction vial and incubated for 15 minutes at room temperature. [^{124}I]-JAA-F11 was purified on an HPLC (Bio-Sil SEC-125, 300 \times 7.8 mm, Bio-Rad). The mobile phase was 0.01 M PBS pH 7.4, the flow rate was 1 ml/minutes, and the UV detector was set at 254 nm wavelength.

Bolton-Hunter method

An adaptation of the Bolton-Hunter method was performed as previously described (Collingridge et al., 2002). Briefly, ^{124}I was distilled and dried in a small reaction vial (0.3 ml, Wheaton, USA). A solution of NaI carrier (5 μl , 89.2 μM), HCl (14 μl , 100 mM), and sodium phosphate buffer (10 μl , 250 mM, pH 7.4) was added to ^{124}I (14 μl , 370 MBq). The reaction was allowed to incubate at room temperature for 10 minutes. SHPP (N-

Succinimidyl-3-[4-hydroxyphenyl]propionate in 1,4-dioxane, 2 μ l, 3.8 mM) and Chloramine-T (10 μ l, 22 mM) dissolved in sodium phosphate buffer (250 mM, pH 7.4) were added with mixing and the reaction was terminated immediately (~15 seconds) with a sodium phosphate buffered solution (250 mM, pH 7.4) of Na₂S₂O₅ (10 μ l, 63 mM). The reaction product was extracted with benzene/dimethylfluoride (100 μ l, 40:1 v/v), dried with 30 mg Na₂SO₄ and transferred into another reaction vessel. The solvent was evaporated with a stream of nitrogen and JAA-F11 (10 μ g/mouse, in PBS), mixed with sodium borate buffer (5 μ l, pH 8.5, 100 mM), was added and incubated with [¹²⁵I]-SHPP at 0°C for 1 hour. The labeled antibody was separated from low molecular weight reaction side products using Sephadex G-25. The radioiodinated antibody was injected into the animals within 2 hours of labeling.

Enzyme immunoassay

The immunoreactivity of the radiolabeled antibody was determined using EIA. EIA plates (MaxiSorp™, eBioscience) previously coated with TF-Ag-BSA conjugate in coating buffer (0.1 M Na₂CO₃, pH 7.6) at a concentration of 5 μ g/mL were used for the assays. The coating solution was removed, 2% formaldehyde was added for 20 minutes, and the plate was washed 3 times with TBS. One hundred μ l of different dilutions of radiolabeled JAA-F11 were added to the wells and incubated at 37°C for 1 hour. After washing 3 times, 100 μ l of a 1:1000 dilution of anti-mouse IgG conjugated with alkaline phosphatase (Sigma) in 1% BSA-PBS buffer was added to each well for 1 hour at room temperature. After washing 3 times, 100 μ l of para-Nitrophenyl Phosphate, 1 mg/ml in diethanolamine buffer was added to each well and the plate was read at 405 nm after 1 hour.

Cell cultures

The mouse mammary adenocarcinoma cell line, 4T1, was purchased from ATCC (# CRL-2539). The 4T1 cell line is an animal model for stage IV human breast cancer (Pulaski, 1998; Pulaski et al., 2000). The murine sarcoma cell line, CMS4, which expressed low levels of TF-Ag, was kindly provided by Dr. Hideho Okada (Department of Neurosurgery, University of Pittsburgh, PA) (Tatsumi et al., 2003). Both the tumor cell lines were grown in RPMI 1640 medium supplemented with 10% FBS and adjusted to contain 2mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10mM HEPES, and 1.0mM sodium pyruvate.

Cell EIA

Whole cell EIA was performed to test the expression of TF-Ag on cancer cell lines. The cell lines tested were 4T1, CMS4, and Myeloma (ATCC Number: CRL-1580). Myeloma is the fusion partner for producing JAA-F11 hybridoma (Rittenhouse-Diakun et al., 1998), and was used as a TF-Ag negative control cell line. CMS4 was tested for use as an *in vivo* control. 4T1 and CMS4 cells were harvested by trypsinization and single-cell suspensions were made. The cells were diluted to obtain 1×10^6 cells per ml. Two hundred μ l (2×10^5 cells) of the cell suspension was placed in 5 ml tubes in 5 replicates. Two hundred μ l of 4% formaldehyde was added to each tube and allowed to stand for 20 minutes at room temperature to fix the cells. After centrifugation for 10 minutes at 1200 rpm, the supernatants were decanted carefully. Two hundred μ l of PBS-Tween-1% BSA was added to each tube for storage. The fixed cells were placed at 4 °C overnight or up to 2 weeks. A peroxidase-linked immunoassay was performed in the tubes. Two hundred μ l of 5 different JAA-F11 dilutions were added to respective tubes and incubated at 37°C for 2 hours. The tubes were washed 3 times by adding 3 ml wash buffer (1X PBS-Tween, no azide), centrifuging 10 minutes at 1200 rpm, and decanting the supernatant between each wash. Anti-mouse IgG (gamma chain specific) peroxidase conjugate was added to each tube, incubated for one hour at room temperature, and washed 3 times as before. Two hundred μ l

of o-phenylenediamine dihydrochloride substrate solution was added to each tube. After one hour, the reaction was stopped by adding 100 μ l of stop solution (1 N H₂SO₄) to each tube followed by centrifugation for 10 minutes at 1200 rpm. From each tube, 200 μ l of supernatant was transferred into respective wells in a microtiter plate and the plate was read at 490 nm.

Animals and tumor models

All animals in this study were housed and utilized in accordance with the Institutional Animal Care and Use Committees (IACUC) regulations in an Association for Assessment and Accreditation of Laboratory Animal Care International Accredited Facility. 4T1 and CMS4 cell lines were grown in female BALB/c mice, 8–10 weeks of age. 4T1 cells were implanted by injecting 1×10^6 cells subcutaneously under the right nipple, while 5×10^5 CMS4 cells were implanted subcutaneously in the left leg of the mice. Mice were injected with radiolabeled JAA-F11 by tail vein after day 10 to day 18 of tumor cell administration and were subjected to biodistribution and microPET imaging.

Study design

Each mouse was given a tail vein infusion of approximately 10 μ g JAA-F11 labeled with approximately 3.7 MBq ¹²⁴I in 0.1 ml PBS. All mice received 0.2 g/L Potassium Iodide water as a thyroid blocking regime 24 hours prior to receiving the radiotracer, and this treatment continued until the end of the study. Mice were divided in three groups. Group I (n=10) were control mice without any tumors. Group II (n=17) were mice which received 1×10^6 4T1 cells. Group III (n=3) were mice which received 1×10^6 4T1 as well as 5×10^5 CMS4 cells. Group I and group II mice received the above mentioned doses of labeled JAA-F11. Group III mice received 0.1 ml of approximately 5.55 MBq [¹⁸F]-FDG intraperitoneally 24 hours prior to receiving the above mentioned dose of JAA-F11. MicroPET imaging was performed on 3 mice each from group II and III. Biodistribution was performed on all group I, II, and III mice.

Biodistribution and tumor uptake studies

All mice were sacrificed by injecting intraperitoneally 0.1 ml sodium pentobarbital (Fatal Plus). Group I mice were sacrificed 72, 144, and 360 hours; group II mice were sacrificed at 72, 144, 240, 360, and 480 hours; and group III mice were sacrificed at 240 and 268 hours after injection of the radiotracer. At least three mice per time point were used. Blood, muscle, liver, lungs, kidneys, spleen, heart, stomach, large and small intestines, brain, and tumor tissue were weighed and radioactivity was measured using a gamma counter. Radioactivity uptake was calculated as the percentage of the injected dose per gram of tissue (%ID/g). Group III contained a smaller number of mice, and these mice bore two tumors which caused earlier morbidity leading to early sacrifice, making biodistribution at only two time points possible.

PET Imaging

The microPET camera, Focus 120® (Siemens Concorde Microsystems previously CTI Concorde Microsystems) was used for imaging. The emission scan window was set between 350 – 750 keV. The transmission scan of some mice was also performed using a Cobalt-57 point source for 9 minutes. The transmission scan window was set between 120 – 125 keV. Analysis of the acquired data and image reconstruction with filtered back projection 2D was done using the software microPET manager 2.2.4.0.

Before scanning, the mice were anesthetized with O₂/isoflurane (1%-3% isoflurane) and imaged in the prone position in the gantry of the microPET scanner and the animal was

scanned for 30 minutes for ^{124}I and 15 minutes for ^{18}F -FDG. Three mice from group II were selected and imaged at 48, 144, 240, 360, and 480 hours after injection of the labeled tracers and were sacrificed on the last day of the study. All 3 mice of group III were imaged 90 minutes after injection of ^{18}F -FDG for 30 minutes. One day after FDG imaging, the above mentioned dose of ^{124}I -JAA-F11 was injected in 3 mice each and microPET imaging was performed. Biodistribution was performed on group III mice at 240 hours when tumor volume necessitated sacrifice.

Statistical Analysis

Comparison between data sets were determined using a two tailed unpaired t-test for two independent populations, $p \leq 0.05$ was considered significant.

Results

Antibody specificity

The glycan printed array analysis (Figure 1) illustrates the relative reactivity of JAA-F11 antibody to 200 glycan structures. It showed that the antibody bound to only 5 of the compounds: #56 **Gal β 1-3GalNAc α -Sp8** (TF-Ag), #52 **Gal β 1-3(GlcNAc β 1-6)GalNAc α -Sp8** (Core2), #54 **Gal β 1-3(Neu5Ac β 2-6)GalNAc α -Sp8** (6-Sialyl TF), #79 **Gal β 1-4GlcNAc β 1-6GalNAc α -Sp8** (6-LacNAc-Tn), and # 46 β -D-Gal-Sp8, and bound with about 1/10 the efficiency to #38 **Gal α 1-3GalNAc α -Sp8**. The binding of JAA-F11 to the first 4 of these compounds was not significantly different from each other. JAA-F11 did not bind to the 195 other saccharide structures including related structures such as: Gal β 1-3(Neu5Ac α 2-6)GalNAc α -Sp8 (6'-Sialyl TF), Gal β 1-3(Gal β 1-4GlcNAc β 1-6)GalNAc α -Sp8 (β -Galactosylated Core 2), Neu5Ac α 2-3Gal β 1-3GalNAc α -Sp8 (α 2-3Neu5Ac on Core 1), Neu5Ac α 2-3Gal β 1-3GlcNAc β -Sp0 (3'Sialyl Le^c), GalNAc α 1-3GalNAc β -Sp8 (Forsman disaccharide-3), Gal β 1-3GalNAc β -Sp8 (T_{bb}), and Fuca1-2Gal β 1-3GalNAc β 1-3Gal α -Sp3 (part of the Globo H hexasaccharide).

Radiolabeling and Reactivity

The average specific activity obtained by the Bolton-Hunter method was 57 MBq/ml (n=6) and the average radiolabeling yield obtained was 29.81% (SEM = 3.07, n=6). The radiolabeling yield for the Iodogen method was 19.7%. Immunoreactivity of the labeled antibody was determined by performing EIAs. Figure 2 shows that ^{124}I -JAA-F11 labeled with the Iodogen method is not immunoreactive with TF-Ag, whereas ^{124}I -JAA-F11 labeled with Bolton-Hunter method is reactive to TF-Ag. Indirect labeling by Bolton-Hunter method improved the immunoreactivity as seen in Figure 2a. Based on the above results, biodistribution and localization experiments were performed using JAA-F11 labeled by the Bolton-Hunter method.

Cell EIA

Whole cell EIA was performed to test the expression of TF-Ag on the cancer cell lines. The cell lines tested were 4T1, CMS4, and Myeloma (negative control). The experiment was repeated 3 times for significance, and each dilution was tested in 5 replicates. A 150 $\mu\text{g/ml}$ solution of partially purified JAA-F11 in PBS was used. Students paired *t*-test analysis was used to compare the highest concentrations of the 3 runs for each cell line. It was observed that CMS4 cells expressed significantly less TF-Ag on the cell surface than the 4T1 cells ($p = 0.019$) and thus was chosen as a control cell line for *in vivo* experiments. Although when compared to TF-Ag negative Myeloma cells, CMS4 cells did not exhibit a significant difference ($p = 0.279$), the results from all experiments showed the CMS4 results to be between the Myeloma cells and the 4T1 cells, indicating a TF-Ag low expression. Figure 2b

displays the average O.D. of the 5 replicates of the 3 runs with Standard Error bars. 4T1 cells gave 1.6 to 3 times the signal as the CMS4 cells. Myeloma cells do not form solid tumors *in vivo* so they were not selected as the *in vivo* negative control, and CMS4 was used as a cell line with 0.33 to 0.62 the TF-Ag expression of the 4T1 cells.

Biodistribution

Figures 3a and 3b show biodistribution of [¹²⁴I]-JAA-F11 in tissues of mice without any tumors and mice bearing TF-Ag positive 4T1 tumors. Care was taken to rinse the blood off the organs before counting them on the gamma counter. The biodistribution results in mice without a tumor (group I) were found to be normal, with no concentration in any organ. The biodistribution analysis of mice with TF-Ag positive tumor (group II) showed that there is preferential uptake of the radiolabeled antibody in the TF-Ag positive 4T1 tumor. As expected, activity in the blood was highest in the earliest time point and decreased overtime, and the biodistribution of group II mice showed high specific uptake in 4T1 tumors compared to other organs. The 4T1 tumor retained around 6 times more [¹²⁴I]-JAA-F11 than blood on day 20 after injection of the radiotracer (tumor = 0.19 %ID/g; Blood = 0.03 %ID/g). The biodistribution in mice with a TF-Ag positive and a TF-Ag low to negative tumor was also encouraging (Figure 3c), with 4T1 tumor retaining 1.63 times more [¹²⁴I]-JAA-F11 than CMS4 on day 10 after injection of the drug (4T1 tumor = 0.26 %ID/g; CMS4 tumor = 0.16 %ID/g). Unpaired *t*-test analysis was performed and the difference in the biodistribution of [¹²⁴I]-JAA-F11 in 4T1 and CMS4 in mice was found to be significant (*p* = 0.015). This preferential uptake *in vivo* matched well with the EIA results on these two tumor lines. In no case was preferential kidney uptake seen with JAA-F11. The higher variability in radiolabel retention in tumor at 72 hours as compared to other tissues is due to the fact that intact Ab is still in circulation, and this, combined with the fact that tumor size, vascularity, and necrosis are variable between animals, contributes to the high variability at this time point.

PET Imaging

MicroPET imaging of the mice was performed at 48, 144, 240, 360, and 480 hours. Figure 4 shows the coronal view of the serial images obtained over the 20 days of the mice bearing 4T1 tumors injected with [¹²⁴I]-JAA-F11. As seen in Figure 4, [¹²⁴I]-JAA-F11 was taken up by the 4T1 tumor as early as 48 hours. The blood pool activity remained until 144 hours (Figure 4). The radiolabeled JAA-F11 was seen to be specifically localized in the tumor until 20 days. Transmission scans were done in mice at later time points. This helped in obtaining outlines of the mice for visualization, since after 144 hours the blood pool activity is cleared and it was difficult to observe the outlines of the mouse, and only the tumor was visible. With transmission scan overlapped on the emission scan, the position of the tumor was apparent (Figure 4, 240 and 480 hours).

Experiments involving mice with 4T1 as well as negative control tumor CMS4 were performed. [¹⁸F]-FDG was used as a positive control and was injected in mice with 4T1 and CMS4 tumors. Localization of [¹⁸F]-FDG was observed in high metabolic areas in the body, including the two tumors. Figures 5a and 5b show microPET slices of [¹⁸F]-FDG highlighting 4T1 and CMS4 tumors in the mice, respectively. The same mice received [¹²⁴I]-JAA-F11 one day after they received [¹⁸F]-FDG. Figure 6a and 6b shows microPET slice highlighting 4T1 tumor and CMS4 respectively.

Discussion

JAA-F11 antibody bound to Galβ1–3GalNAc-alpha but not to Galβ1–3GalNAc-beta linked structures, indicating that the antibody would bind to tumor tissue and not to the central

nervous system GM1 ganglioside, asialo-GM1 of NK cells, or GD1 of glycolipids. Its lack of binding with Gal β 1–3(Neu5Ac α 2–6)GalNAc α -Sp8 indicates that this antibody would not be lost to the human plasma Gc protein which is a transporter or reservoir of vitamin D (Matsuura et al., 2004). Its lack of binding with Fuca1–2Gal β 1–3GalNAc β 1–3Gal α -Sp3 indicates lack of binding with Globo H derivatives. The binding to β -D-Gal–Sp8 was not seen in solution and may be due to the reported increase in affinity to monosaccharides seen when polyvalent solid phase presentation occurs (Mortell, 1996; Liang et al., 1997) and thus may not be biologically relevant. This type of analysis is consistent with the specificity seen in the imaging analysis, and supports the idea that this antibody will provide better imaging results than other anti-TF-Ag antibodies.

Radioiodination of JAA-F11 was first attempted using the direct iodination method since this is the most commonly used method. It was observed by EIA that the directly labeled mAb was no longer immunoreactive with TF-Ag. Analysis of the amino acid sequence of the mAb showed that there are seven tyrosine groups in the Complementarity Determining Regions (CDRs) of JAA-F11 (data not shown). Since the direct Iodogen method labels tyrosine residues in the proteins, this can alter the CDR of JAA-F11 which is the region of the antibody that binds to TF-Ag. An indirect iodination method, the Bolton-Hunter method, was utilized. This reagent reacts with lysine residues in the antibody through the formation of stable amide bonds. The radiolabeling yields obtained were of good efficiency (29.81%), and the labeled mAb was found to be immunoreactive with TF-Ag by EIA.

The biodistribution results in mice without a tumor showed no concentration to any organ, unlike the kidney localization seen by others with reagents that bound both the alpha- and beta-linked TF-Ag (Turner et al., 1988). The biodistribution results in mice with only a TF-Ag positive tumor were promising, with 4T1 tumors still retaining around 6 times more [124 I]-JAA-F11 than blood on day 20 after injection of the drug (tumor = 0.19 %ID/g; Blood = 0.03 %ID/g). The biodistribution analysis showed that there is preferential uptake of the radiolabeled antibody in the TF-Ag positive tumor and that this radioactivity remains for 20 days, a fact that bodes well for future immunotherapeutic protocols. At 20 days radioactivity remains in the tumor, and this is promising, however, radioactivity as a ratio to tumor has increased in the liver at this time point. At earlier time points much higher radioactivity is in the tumor than in the liver, at 20 days, although the liver/tumor ratio is higher, the total amount of the radioactivity that the liver retains is small compared to the tumor dose received at earlier time points, thus the dose to the tumor is much higher than the dose to the liver. In future studies liver Fc receptor blocking experiments with cold non-specific antibody will be attempted to decrease liver dose. Results by others (Yakaki et al., 2001, Ravn et al., 2007) have shown faster clearance time with fragments, but increased liver and/or kidney binding, so this may not be an appropriate change unless conjugated with Polyethylene Glycol as in the method of Li L et al., 2006. Use of a cold unrelated antibody may block Fc receptor binding sites and block some Fc mediated uptake in the liver that occurs at later time points

A TF-Ag low to negative control cell line was also investigated. To examine the expression of TF-Ag on CMS4, an indirect cellular EIA was performed, to compare to 4T1 cells. The difference was found to be significant ($p < 0.05$) according to the results of a paired Student's *t*-test. With TF-Ag signal 1.6 to 3 times higher on the 4T1 cells, CMS4 was thus chosen as a TF-Ag low cell line for the *in vivo* experiments. When injected in mice, CMS4 was observed to grow faster than 4T1. The biodistribution in mice with a 4T1 and CMS4 were also encouraging, with 4T1 tumor retaining 1.63 times more 124 I-JAA-F11 than CMS4 on day 10 after injection of the drug (4T1 tumor = 0.26 %ID/g; CMS4 tumor = 0.16 %ID/g). The difference was found to be significant ($p = 0.015$).

High uptake of [^{124}I]-JAA-F11 was observed in 4T1 tumors by microPET imaging. This proved the specificity of JAA-F11 for TF-Ag expressed on 4T1, which is in agreement with the results obtained in the *in vitro* indirect cellular EIA experiments. Although, after dissection, metastasis in the lungs of the mice were observed, radiolabeled Ab was injected at an early time point (day 10 to day 18 after tumor cell injection) to prevent excess morbidity prior to completion of the MicroPET studies. At this early time point the metastatic foci would have been very small and unable to take up enough Ab for later visualization. Future experiments will involve primary tumor removal, and longer time for metastatic focus to develop prior to radiolabeled Ab administration. Such experiments with a different Ab resulted in visualization of metastasis and it is anticipated that these experiments will also allow visualization of metastasis in our system. Immunohistochemistry on the lung metastasis (data not shown) showed reactivity with the JAA-F11 antibody.

Transmission scans were done in mice at later time points. This helped in obtaining an outline of the mouse for visualization, since after 144 hours the blood pool activity is cleared and only the tumor was visible. With transmission scan overlapped on the emission scan, the position of the tumor was apparent. The kidney uptake of JAA-F11 was less (kidney:tumor ratio of 0.48 at 72 hours) compared to the 0.75 ratio seen with the 155H.7 antibody (Turner 1988) which bound both the Gal β 1–3GalNAc- α and the Gal β 1–3GalNAc- β derivatives.

For further investigations, using Ab fragments, pretargeting, imaging in nude or SCID mice bearing TF-Ag positive and negative human breast cancer xenografts, humanization (chimerization) of JAA-F11, and ultimately radioimmunotherapy with [^{131}I]-JAA-F11 (murine JAA-F11 as well as humanized JAA-F11) should be performed.

This investigation has led to several important conclusions about the localization potential of JAA-F11 and its biodistribution in mice. High specificity and localization of the labeled mAb greater than 20 days was obtained, which is very promising from an immunotherapy point of view, the ultimate goal. These results are a step forward towards developing [^{131}I]-JAA-F11 as a potential clinically useful radioimmunotherapy drug against TF-Ag positive breast cancer metastasis. The 170H.82 antibody which bound both alpha- and beta-linked TF-Ag showed promise in breast cancer immunolocalization, withdrawn only after phase II trials (1997), and JAA-F11 shows a more restricted structural specificity and less kidney association. Further studies are warranted to determine if this finding will translate to specificity and sensitivity in human localization, and ultimately, therapeutic efficacy.

A study on utilization of multivalent single chain fragment variable (scFvs) for immunolocalization experiments was recently published (Ravn et al., 2007). These scFvs were selected from a phage library and are also TF-Ag-alpha specific, as is JAA-F11. Unfortunately, unlike our whole antibody, these fragments showed higher kidney binding than tumor binding, which the authors felt was due to filtration entrapment in the kidney of this size multimeric fragment. The future experiments with JAA-F11 will use blocking with cold unrelated Ab to block Fc binding sites, (Fab') $_2$, and humanized antibody and polyethylene glycol DOTA conjugated diabodies (Li L. 2006) rather than the scFvs multimeric fragments as the polyethylene glycol diabodies do not become entrapped in the kidney the way the scFvs do.

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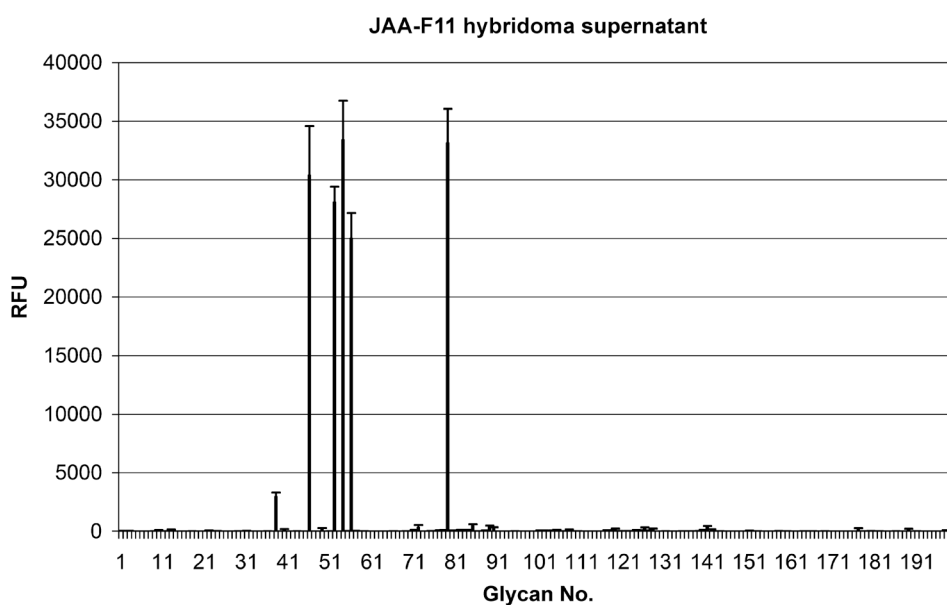


Figure 1. Analysis of JAA-F11 on 200 different glycans on a printed array indicates its specificity for the tumor antigen without much reactivity to related structures. Reactive structures are: #56 **Gal β 1-3GalNAc α -Sp8**, #52 **Gal β 1-3(GlcNAc β 1-6)GalNAc α -Sp8**, #54 **Gal β 1-3(Neu5Ac β 2-6)GalNAc α -Sp8**, #79 **Gal β 1-4GlcNAc β 1-6GalNAc α -Sp8**, and #46 β -D-Gal-Sp8, and with about 1/10 the efficiency to #38 Gal α 1-3GalNAc α -Sp8.

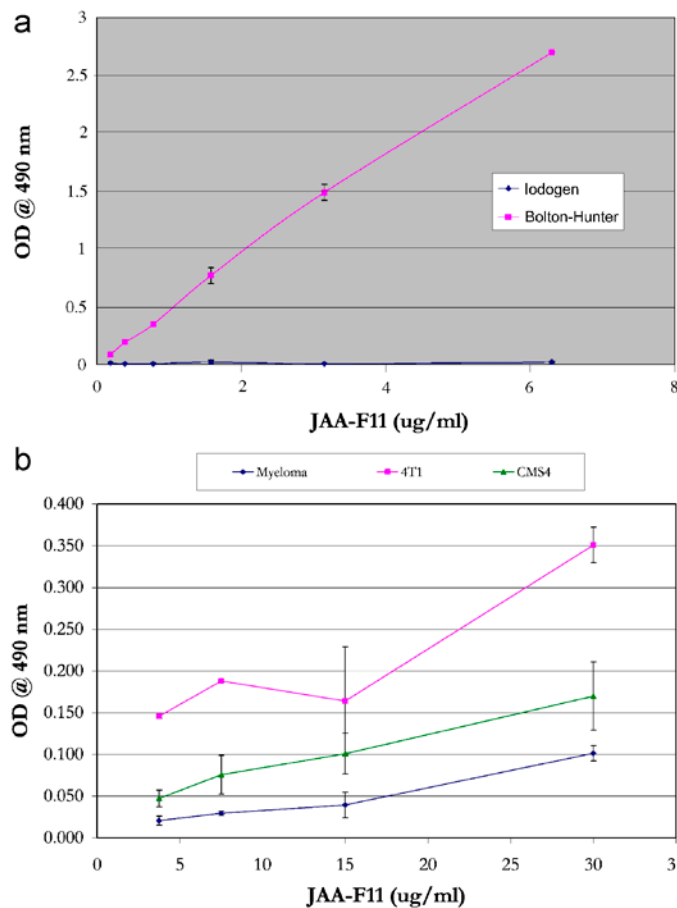


Figure 2. Antibody characterization. 2a. EIA of ^{124}I -JAA-F11 labeled by Iodogen and Bolton Hunter method showing loss of immunoreactivity of the labeled antibody with the Iodogen method and preservation of JAA-F11 binding with the Bolton Hunter method. Error bars represent ± 1 SEM. 2b. Indirect Cell EIA to test the expression of TF-Ag on cancer cell lines, showing significant JAA-F11 binding to 4T1 cells ($p = 0.019$ versus Myeloma cells) and not to CMS4 cells ($p = 0.279$ versus Myeloma cells).

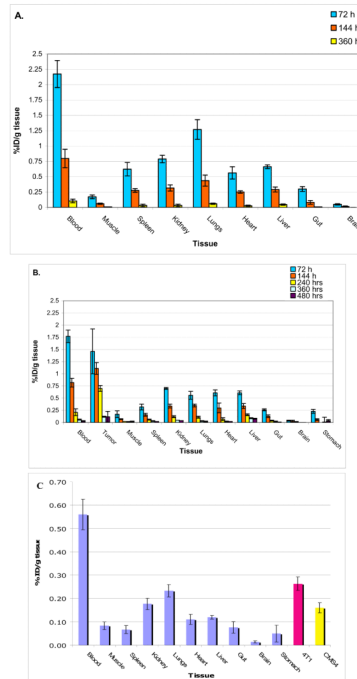
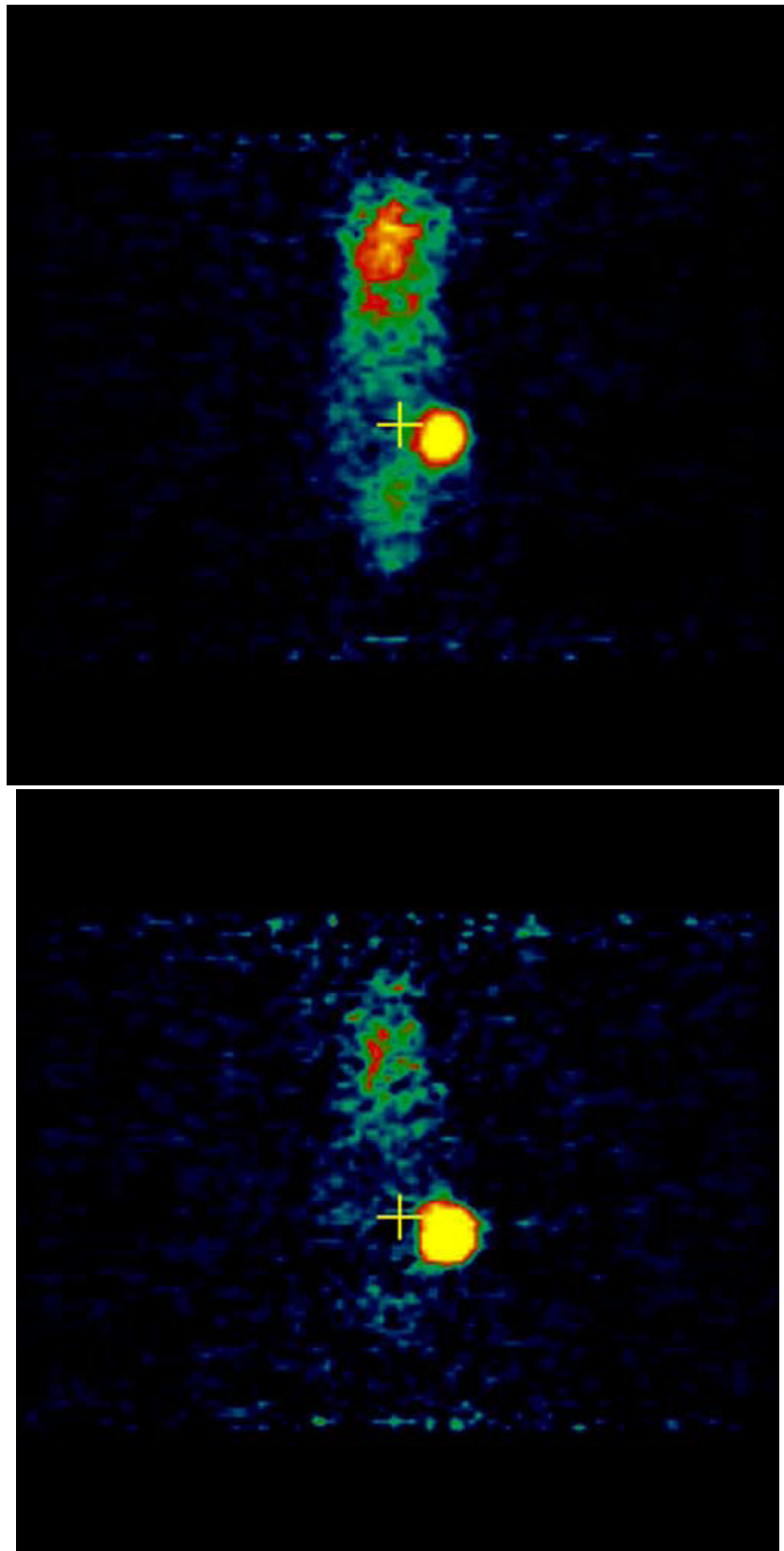
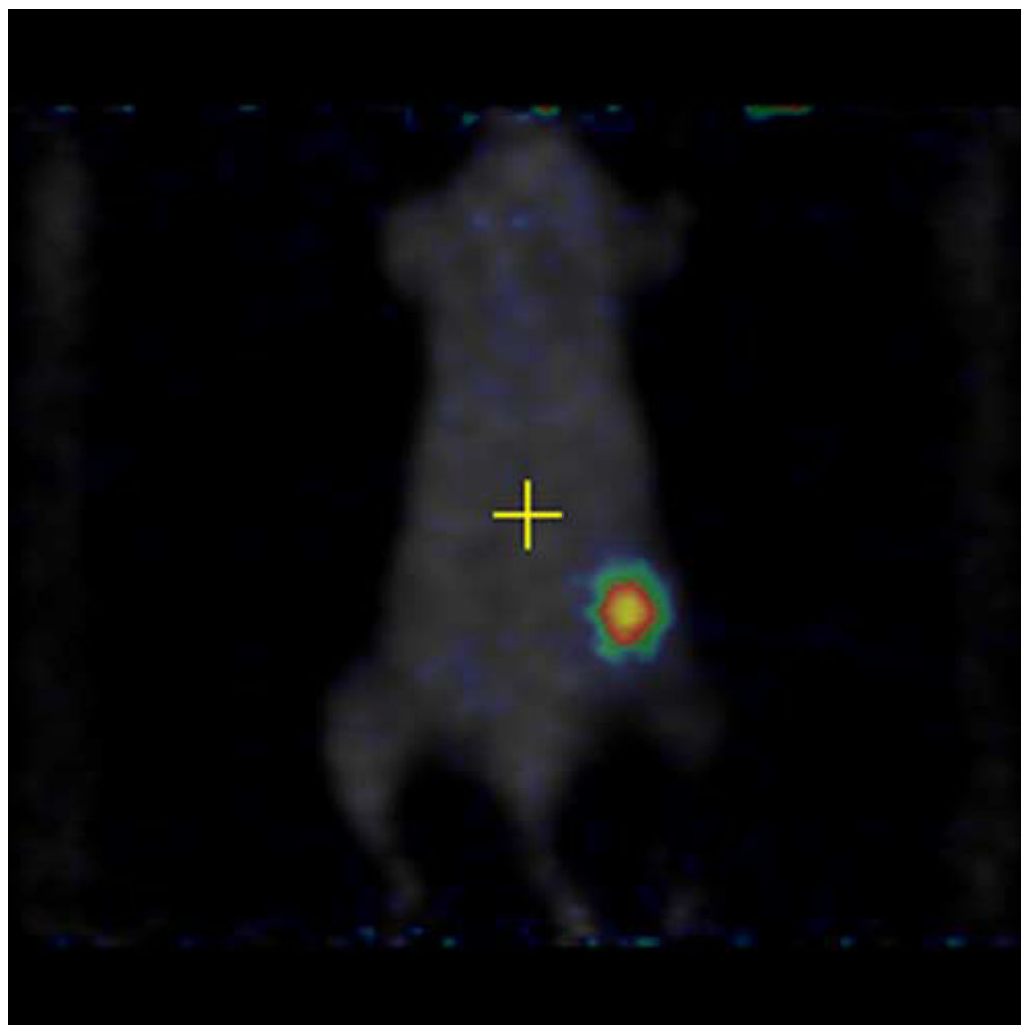
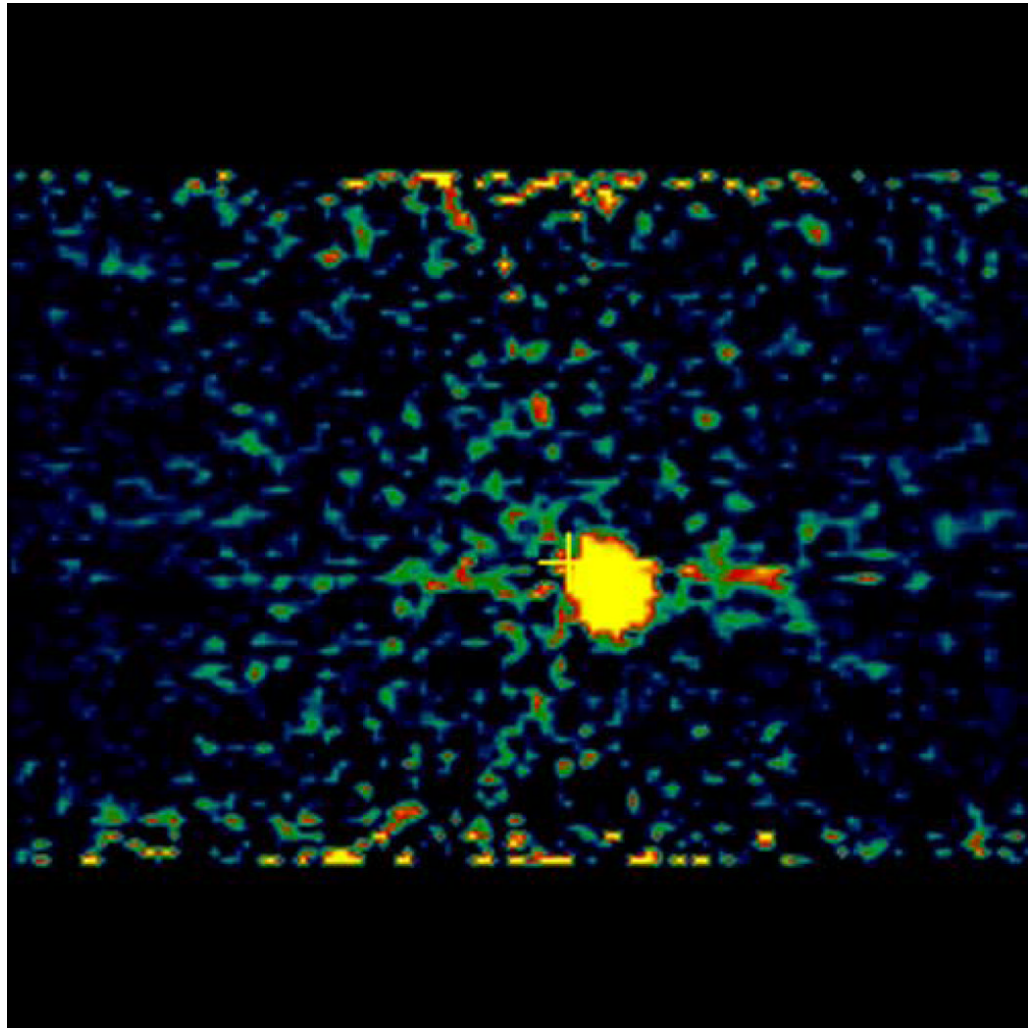


Figure 3. Biodistribution of ^{124}I -JAA-F11. 3a. mice without tumor, 3b. mice bearing TF-Ag positive 4T1 tumor, 3c. mice bearing 4T1 and CMS4 tumors imaged at 240 hours.







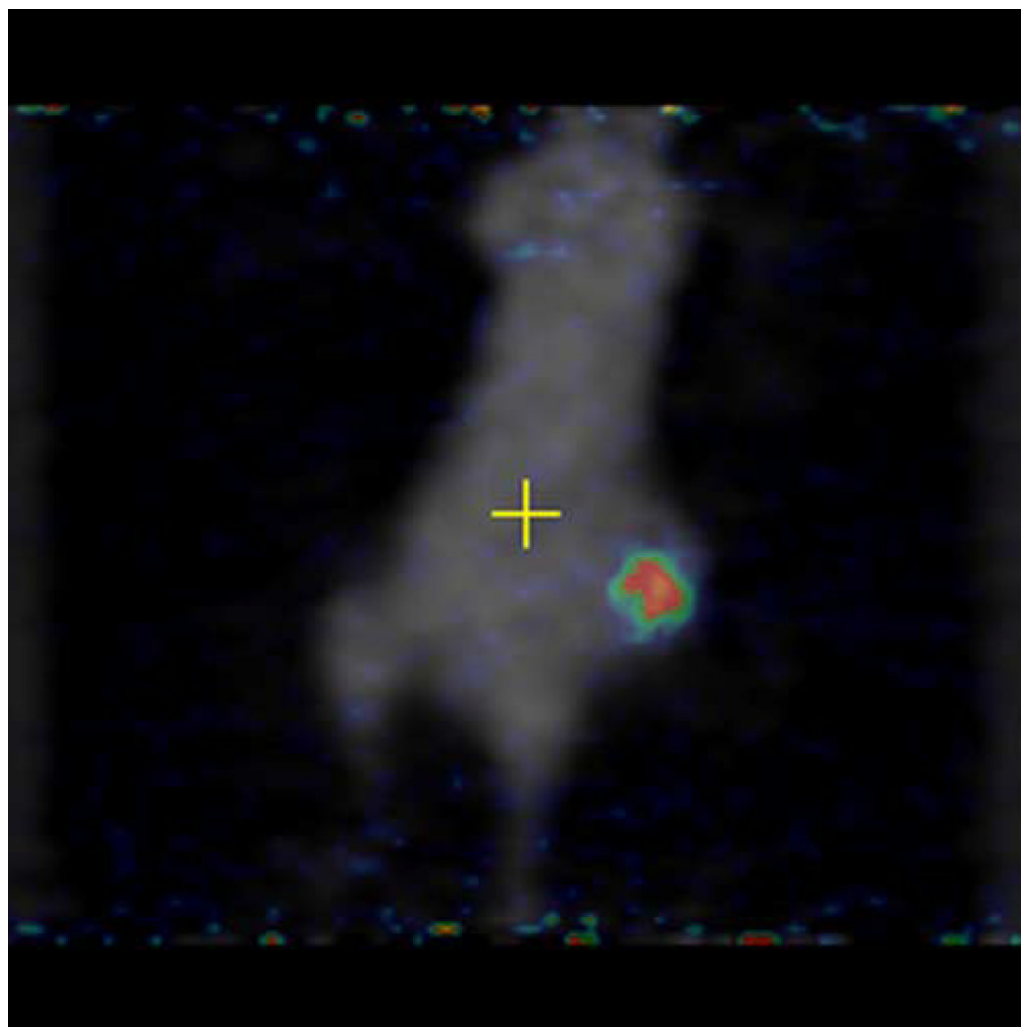
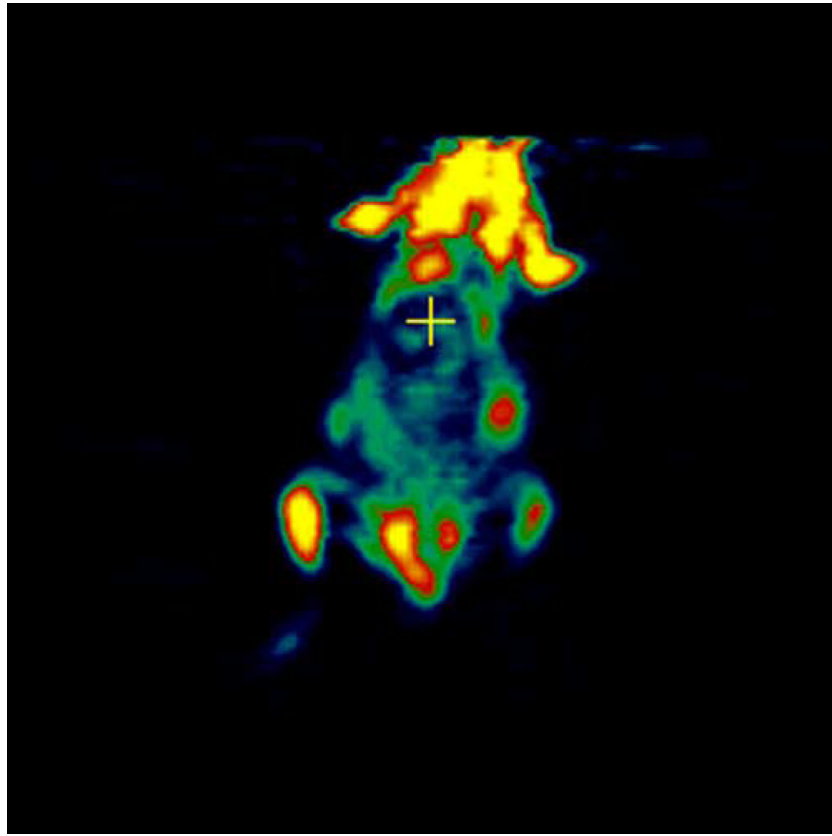


Figure 4. microPET images showing immunolocalization of ^{124}I -JAA-F11 in mice with 4T1 tumors imaged for 48, 144, 240, 360, and 480 hours. Before scanning, the mice were anesthetized with O_2 /isoflurane (1%–3% isoflurane) and imaged in the prone position in the gantry of the microPET scanner and the animal was scanned for 30 minutes for ^{124}I .



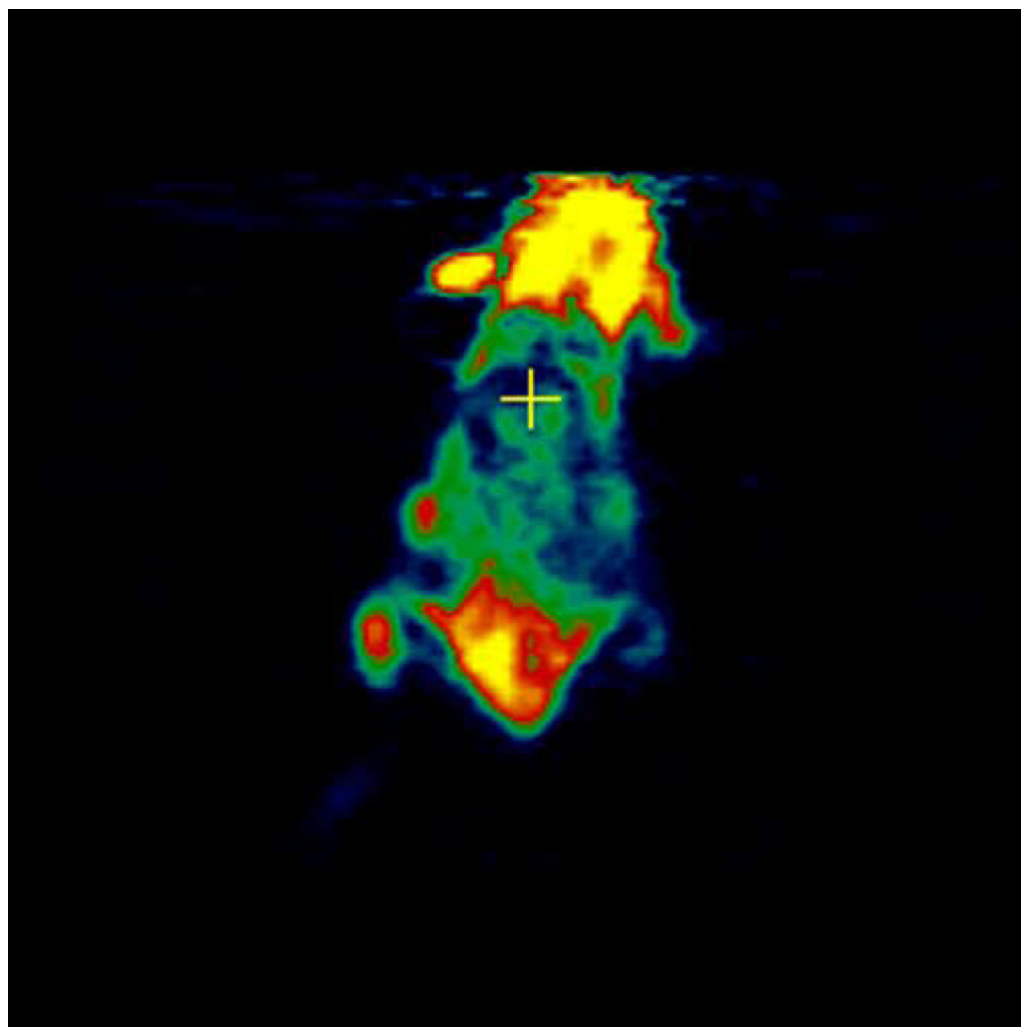
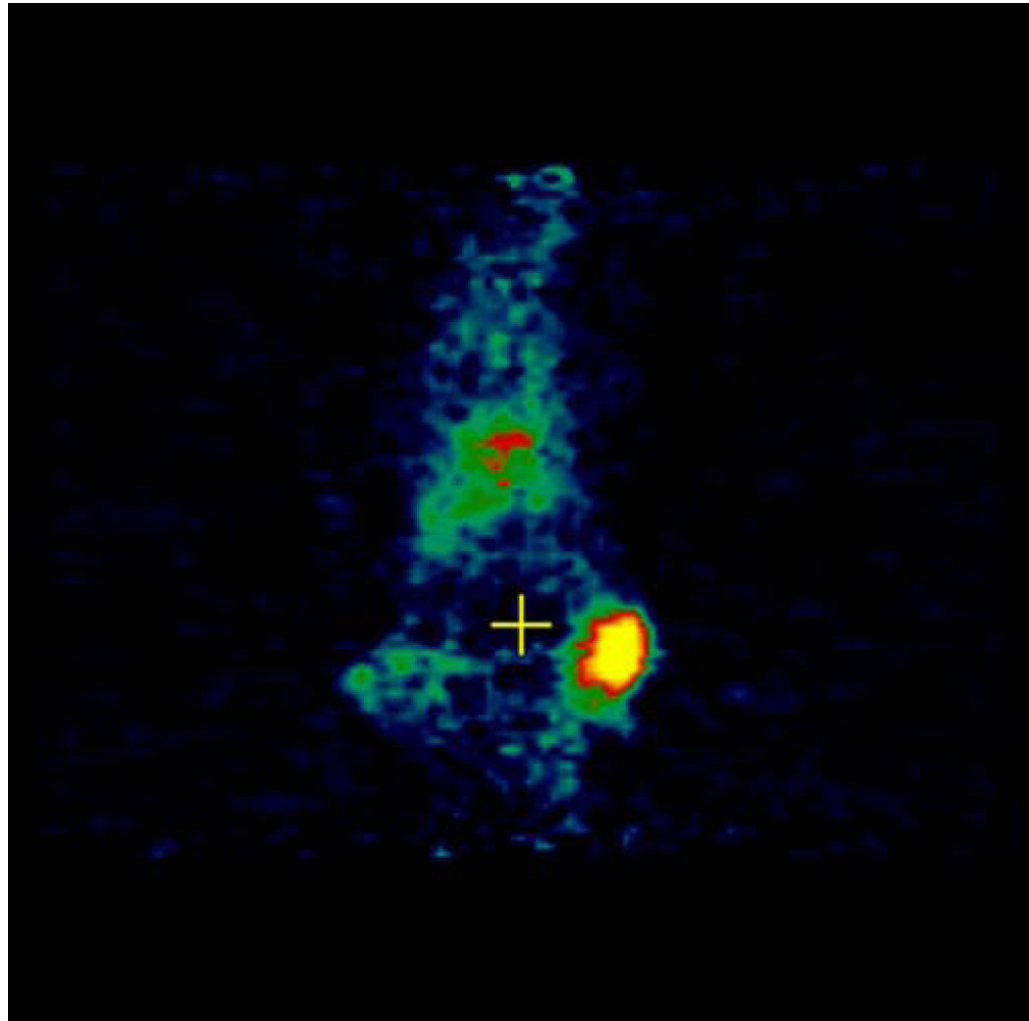


Figure 5. microPET images showing immunolocalization of ^{18}F -FDG in mice with 4T1 and CMS4 tumors. 5a. the planar slice which showed maximum 4T1 localization is shown. 5b. the planar slice which showed maximum CMS4 localization is shown. Tumors were not on the same plane, therefore 2 images were used. Before scanning, the mice were anesthetized with O_2 /isoflurane (1%-3% isoflurane) and imaged in the prone position in the gantry of the microPET scanner and the animal was scanned for 15 minutes for [^{18}F]-FDG.



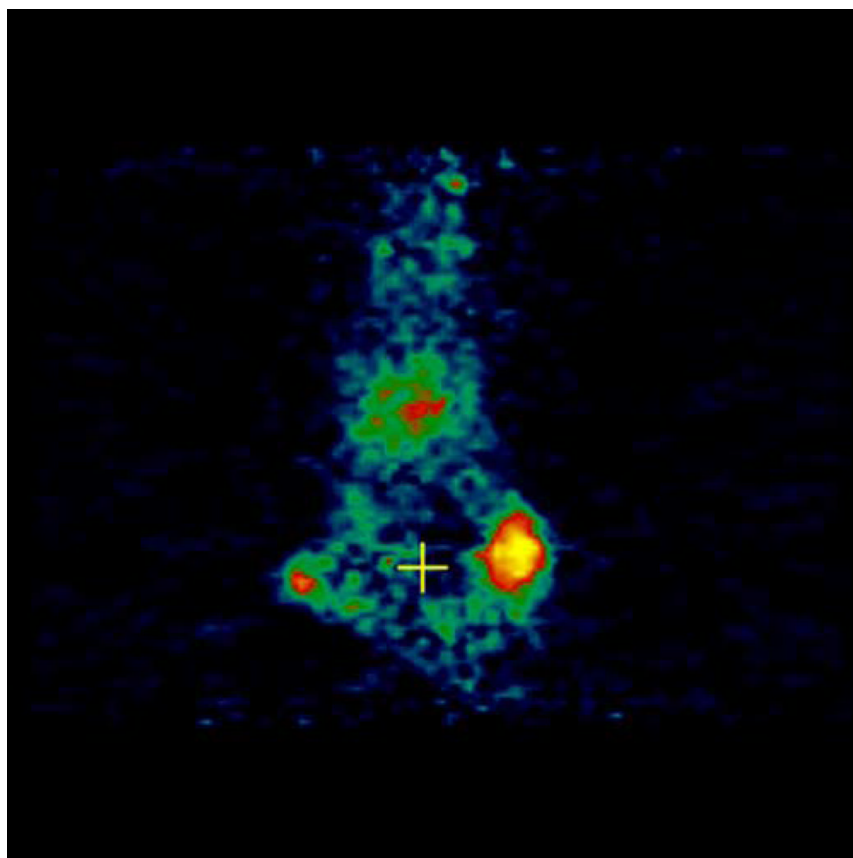


Figure 6. microPET images showing immunolocalization of [^{124}I]-JAA-F11 in mice with 4T1 and CMS4 tumors. 6a. image with maximum 4T1 tumor localization, 6b. image with maximum CMS4 tumor localization. Tumors were not on same plane, therefore 2 images were used. Before scanning, the mice were anesthetized with O_2 /isoflurane (1%–3% isoflurane) and imaged in the prone position in the gantry of the microPET scanner and the animal was scanned for 30 minutes for ^{124}I .