Title
Development of Targeted Immuno-PET Imaging Agents for Breast Cancer Brain Metastasis (BCBM)

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Development of Targeted Immuno-PET Imaging Agents for Breast Cancer Brain Metastasis (BCBM)

by

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THESIS

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Abstract

Development of Targeted Immuno-PET Imaging Agents for Breast Cancer Brain Metastasis (BCBM)

Niecholle Roco

Transforming growth factor-β (TGF-β) is a cytokine that acts as a tumor promoter in breast cancer brain metastasis. It is tumor promoting by activating immunosuppression and enhancing breast cancer cells' ability to metastasize. Since TGF-β is overexpressed and plays a role in breast cancer brain metastasis progression, it can be exploited as a biomarker for immuno-PET imaging through radiolabeling of fresolimumab, a monoclonal antibody (mAb) that inhibits all active isoforms of TGF-β. **Methods:** An immuno-PET imaging agent was synthesized in the form of an ^{89}Zr-DFO-fresolimumab targeting vector to investigate a breast cancer brain adapted mouse model. The number of DFO chelate sites per antibody were determined. Additionally, immunoreactivity experiments were done to ensure that the integrity of the antibody was maintained after radiolabeling. Lastly, the imaging probe was tested in-vivo in a mouse model to determine characteristics of the probe's distribution. **Results:** DFO was chelated to the antibody fresolimumab. The number of DFOs per fresolimumab was determined to be 3.8 $\pm$ 0.8. The imaging probe, ^{89}Zr-DFO-fresolimumab was prepared with high radiochemical purity (> 95%) and with good radiochemical yield (~70%). Immunoreactivity was maintained, the radiolabeling process did not affect the properties of fresolimumab's ability to bind to TGF-β. μPET/CT imaging was done to determine the distribution of the radiotracer. The targeting vector was able to cross the blood brain barrier in a specialized mouse model and be localized with adequate signal in the brain tumor. **Conclusion:** Results from this study showed the targeting vector's ability to cross the compromised blood brain barrier and enough signal was seen to characterize

the brain tumor. The tumor progression was imaged with high quality using diffusion weighted MRI. This study can be used to provide the baseline untreated model to be compared against mice with brain tumors after receiving treatment in future studies. These initial studies for ^{89}Zr-DFO-Fresolimumab are promising however, further work is needed to assess the tracer characteristics.

Introduction

With the advancement of more personalized breast cancer therapies, breast cancer has become more easily detected and treated. Revolutionary treatment regimens have allowed patients diagnosed with breast cancer to have targeted therapy treatment options that will prolong life expectancy. Conversely, this increase in survivorship has been correlated with an increased prevalence of breast cancer brain metastases (BCBM). This is because with better control of systemic disease patients are living longer, allowing for the BCBM to form. Although long-term survival can be achieved for patients with breast cancer, 15-30% of patients with metastatic breast cancer develop BCBM (2). Furthermore, once diagnosed with BCBM median survival is under one year. This presents a need for BCBM detection and treatment. Molecular imaging can help aid translation of new therapies into clinical practice by providing imageable biomarkers. Imaging of mice models with BCBM can provide insight on the mechanism of disease progression and treatment response.

BCBM has limited management strategies, making it an especially deleterious disease. Currently the most utilized forms of BCBM intervention include neurosurgery or radiotherapy. Whole brain radiation therapy can also be used in conjunction with surgical resection of the tumor. However, whole brain radiotherapy has the possibility of contributing to neurocognitive decline resulting from the radiation. It is unclear whether systemic therapies for cancer are effective treatments for BCBM. This is because most cytotoxic agents do not cross the blood brain barrier (7). Gamma Knife Stereotatic Surgery (GKSRS) is a treatment option for BCBM that is becoming more widely used. GKSRS works on the principal of delivering high doses of radiation to targeted foci in the brain. Since only desired regions of the brain are receiving radiation, normal brain tissue is preserved.

TGF-β is a potential immunotherapy target of interest for BCBM therapy. TGF-β is a highly regulated and powerful cytokine with a duality and complex nature. Being highly regulated in non-cancer cells the TGF-β pathway has been shown to be a key factor for cell proliferation, differentiation, motility, adhesion, and programmed cell death. Depending on the cell type, the effect of the TGF-β pathway can conditionally be either pro-tumorigenic or tumor suppressive in function. When acting as a tumor suppressor the effects of the TGF-β pathway can limit epithelial proliferation and stop premalignant growth. As a pro-tumorigenic actor, TGF-β has opposing effects, including angiogenesis and enhanced epithelial mesenchymal transition. During cancer radiotherapy, TGF- β can act as a tumor promoter by being immunosuppressive, decreasing the effect of the radiotherapy.

One of the main mechanisms of regulation for TGF-β is that the three isoforms (TGF-β 1,2,3) of the cytokine are secreted in its latent form requiring an activation step. Activation can come in the form of furans cleaving the protein or from reactive oxygen species (ROS) interaction. The latter is especially problematic, because brain cancer treatment using gamma knife stereotactic radiosurgery creates a highly reactive environment that up-regulates TGF-β's pro-tumorigenic activity. This allows for brain metastases to evade treatment by upregulating DNA repair enzymes (2). Since TGF-β is highly variable and pathway response is context dependent, imaging of TGF-β for BCBM can provide a better understanding of morbidity and mortality associated with BCBM. The dual nature of TGF-β presents a need for TGF-β investigation as a possible marker for therapy monitoring of BCBM.

TGF-β has pro-tumorigenic activity, making it a highly desirable target of interest for inhibition. Inhibition of the TGF-β pathway can occur in three levels: ligand level, ligand-receptor level, and the intracellular level. At the ligand level TGF-β inhibition occurs through

antisense oligonucleotides. For the inhibition at the ligand-receptor level monoclonal antibodies are used. At the intracellular level, small molecule inhibitors can be used (11).

Fresolimumab (GC1008, Genzyme) is a fully humanized monoclonal antibody (mAb) that neutralizes all active isoforms of TGF-β. Antibody inhibition for TGF-β is desirable because TGF-β is overexpressed and has pro-tumorigenic effects in breast cancer cells. Therefore, TGF-β can be used as a target for imaging BCBM. Fresolimumab was initially developed and used in a Phase I study as a therapy for patients with advanced melanoma and renal cell carcinoma (1). Furthermore, structural studies have indicated that fresolimumab shows high binding affinity and is a potent TGF-β neutralizing agent (4).

It can be argued that fresolimumab has the potential to be a valuable imaging tool for BCBM and a biomarker for breast cancer cells. Prior studies have shown low intracranial biodistribution with fresolimumab, however this study was done in models that did not have brain tumors [6]. Using fresolimumab as an immuno-PET imaging agent, therapy monitoring for a brain tumor can be quantified, and patient populations that will most likely respond to an anti-TGF-β treatment can be identified.

The long half-life (78.4 hours) of ^{89}Zr makes it a viable radionuclide for immuno-PET imaging of fresolimumab. As a result of the long half-life, imaging can be done once the background blood with radionuclide has cleared through the mouse's system. Imaging of radiolabeled fresolimumab with ^{89}Zr will provide for a robust tool in quantifying and monitoring anti-TGF-β treatment response for the animal model in use. Imaging treatment response can help streamline translation of this novel therapy of combination anti-TGF-β and gamma knife stereotactic radiosurgery into clinical practice and research. By creating a ^{89}Zr-DFO-fresolimumab conjugate, we are synthesizing an imaging tool for breast cancer brain metastases.

We propose to synthesize an ^{89}Zr-DFO-fresolimumab imaging probe that will be used for therapy monitoring of a BCBM mouse model. We hypothesize that the imaging probe will be able to cross the compromised blood brain barrier and be localized with enough PET signal to image the brain tumor. The progression of the brain tumor growth will be characterized through diffusion weighted MRI. Imaging is a powerful tool for breast cancer brain metastasis models because brain biopsies are difficult for patients who develop the disease. Through imaging we will characterize the growth and progression of the BCBM model to help streamline a novel combination immunotherapy and gamma knife stereotactic surgery regimen into clinical use.

Materials and Methods

Reagents and Instruments

Fresolimumab was purchased from Genzyme and manufactured in Cambridge Massachusetts. The bifunctional chelator, *p*–isothiocyanatobenzyldesferrioxamine (p-SCN-Bn-DFO or DFO) was purchased from Macrocyclics (Dallas, TX, USA). Zirconium-89 in 1 M oxalic acid was obtained from 3D Imaging (Little Rock, AR). TGF- β3 was purchased from PeproTech. The remaining reagents were obtained from Fisher Scientific with the highest purity available. Biological sample radioactivity was measured in a Hidex automatic γ-counter. Size-exclusion high performance liquid chromatography (SE-HPLC) was done at room temperature on an Elite LaChrom HPLC synchronized to a Shimadzu UV-Vis Detector (SPD-10A UV detector 280 nm) to quantify radioactivity using the program Peak Simple. Conjugate or radioconjugate ,20 μL, was injected through a Biosep size exclusion column (Phenomenex). The mobile phase used is 1M Phosphate Buffer Solution (PBS) at a flow rate of 1 ml/min and 800psi. PET images were obtained on an Inveon μPET/CT system and processed using Amide software.

Conjugation of Fresolimumab with DFO

Fresolimumab was modified with DFO as previously described with minor modifications [9]. Fresolimumab (GC1008, Genzyme) ,10 mg, was dissolved in 1mL of sodium carbonate (pH 9.0). The solution was mixed with 20 μL of a three fold molar excess of DFO (7.5 mg/mL) dissolved in DMSO. The reaction mixture was incubated for 30 min at 37° C. Unreacted DFO was separated from the solution using a PD-10 size exclusion column with PBS as the mobile phase. The DFO-fresolimumab product was then purified using centrifugation at 5000g and 4°C for 10 minutes. Afterward, conjugates were characterized using an HPLC with a size exclusion column. After characterization, the DFO-fresolimumab intermediate was vialed and stored at 4°C.

Radiolabeling of ^{89}Zr-DFO-Fresolimumab

To a reaction vial, 20 μL of water, 1.5 μL of ^{89}Zr oxalate (637 uCi) and 1.5 μL of 1M carbonate were added. The solution was mixed followed by an addition of 200 μL of HEPES 0.5M buffer (pH 7.4). Lastly, 20 μL of DFO-fresolimumab conjugate is added. Complexation occurred between the DFO and the ^{89}Zr (Fig A). The reaction was incubated at 37°C for 30 minutes, resulting in a ^{89}Zr-DFO-fresolimumab product. The solution was then purified using a PD-10 size exclusion column using normal saline in the mobile phase.

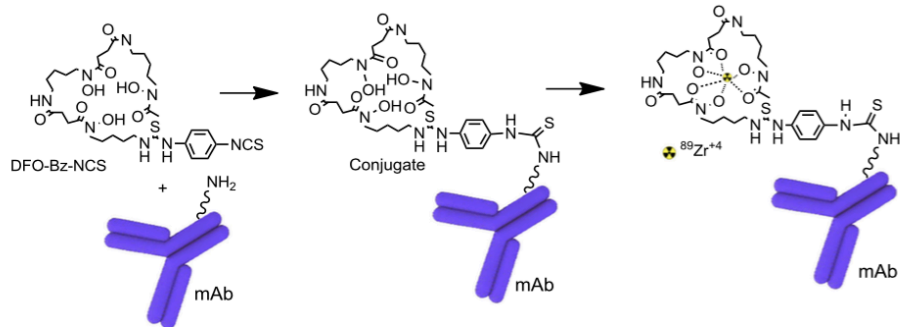


Figure A: Chemical process of conjugating and radiolabeling the antibody

HPLC/iTLC Analysis

The ratio of free ^{89}Zr to the radiolabeled ^{89}Zr -DFO- Fresolimumab product is analyzed with an instant Thin Layer Chromatography (iTLC) and SE-HPLC. The radiolabeled ^{89}Zr-DFO-Fresolimumab is spotted on a 10 cm iTLC-SG strip (Agilent Technologies- Part #: SGI0001) and developed in 10 mM EDTA (pH 4.5). The developed sample was analyzed using a Bioscan AR2000 iTLC scanner using Windows Scan Radio-TLC software version 2.2.

Conjugated products (DFO-Fresolimumab and ^{89}Zr-DFO-Fresolimumab) were also run on a SE-HPLC to determine the radiochemical purity of the final products. To determine the radioactivity peak on the HPLC, the HPLC detector's UV chromatogram is integrated with a UV chromatogram system for radioactivity (Shimadzu UV-Vis).

Determination of Average Number of Chelates linked Fresolimumab

Determination of average number of chelates linked to fresolimumab was performed following literature procedure [10]. DFO-fresolimumab, 20 μL, is reacted with 5 μL of Zr(IV) chloride in 1M oxalic acid. The radioactive Zirconium-89 is used to determine the chelate sites per

fresolimumab. Enough zirconium was used to completely saturate all the chelator binding sites. An iTLC analysis was done to determine the fraction of zirconium bound to the antibody and was used to calculate the nanomolar quantity of zirconium bound to the DFO-fresolimumab. The mean number of chelates per antibody is obtained from the calculated ratio of the nanomolar quantity of Zr bound to DFO-fresolimumab.

Binding Competition Assay

Immunoreactivity was tested in a competition assay with unlabeled fresolimumab. Recombinant human TGF-β3 was used as the target compound and diluted in PBS to a concentration of 10ug/mL and adjusted to a pH of 9.2-9.5 using PBS. TGF-β3 (50 μL of 2μg/mL) were added to the wells of an ELISA plate, incubated overnight at 4°C, and then blocked with 1% non-fat milk. ^{89}Zr –DFO-fresolimumab was then added to the wells in competition with unlabeled fresolimumab (2mg/mL) and were left to incubate for 1 hour at room temperature. The wells were then washed three times using PBS followed by an addition of 1M sodium hydroxide to detach the bound TGF-β3 from the ELISA plate. The solutions were transferred into test tubes, and radioactivity was counted with a Hydex gamma-counter.

Animal model

All animal studies were reviewed and approved by the University of California, San Francisco Animal Care and Use Committee (IUCAC). The mice were generously donated from the Department of Radiation Oncology by Mary Helen Barcellos-Hoff's lab. Mice were

inoculated with 4T1-BR5 cells in the brain. The cancer cells that thrived in the brain were selected as the subculture for mice to be surgically inoculated in the brain with the selected 4T1-BR5 cells. 4T1-BR5 cells are a highly aggressive triple breast cancer cell line that is known to overexpress TGF-β. As a negative control mice were surgically inoculated with phosphate buffer solution (PBS).

Characterizing ^{89}Zr-DFO-Fresolimumab Passage through Blood Brain Barrier

Three groups of mice were inoculated with breast cancer cells either in the brain or subcutaneously. The three groups were composed of two mice within each cohort. Within each cohort one of the mice was injected intravenously with ^{89}Zr-DFO-Fresolimumab the other mouse was injected intravenously with a mixture of ^{89}Zr-DFO-Fresolimumab and cold Fresolimumab. The mice were imaged at 1 hour, 48 hour, and 96 hour time points. The mice were imaged on a Siemens Inveon μPET/CT system.

Obtaining Ideal Concentration for Fresolimumab

Mice (n=11) were injected intravenously with either a low amount of cold concentration (specific activity 252 μCi/mg) or a high amount of cold fresolimumab (specific activity 89 μCi/mg). The mice were then imaged at a 24 hour and 96 hour time point with a μPET/CT scan using a Siemens Inveon μPET/CT system.

Determining Specificity of the ^{89}Zr-DFO-Fresolimumab Using ^{89}Zr-Isotype

Mice were injected with an isotype (BioXCell) for fresolimumab. The isotype is an IgG similar to Fresolimumab, except the isotype does not have the regions of specific binding to TGF- β. The mice were injected with the isotype having an activity of 715 μCi and imaged using a Siemens Inveon μPET/CT system at a 24 hour and 96 hour time point.

Imaging Tumor Progression for Breast Cancer Brain Metastasis Mice

To image the progression of the brain tumors in the mice inoculated with the 4T1-BR5 breast cancer cells. Serial diffusion weighted MRI images were taken until two weeks after inoculation with the breast cancer cells. A diffusion weighted MRI imaging sequence was used with a b-value of 119.38 s/mm^2. The TR was 1000 ms, TE was 21.48 ms. The matrix was 256 x 192 and the slice thickness was 0.75 mm with a gap of 0.25mm. The MRI imaging system used was a 600MHz Varian MRI.

Results and Discussion

The primary aim is to provide evidence that ^{89}Zr-DFO-fresolimumab can surpass the compromised blood brain barrier and be localized to image a breast cancer brain tumor. fresolimumab is a fully humanized, monoclonal antibody that neutralizes all active forms of TGF-β, a cytokine that activates a known tumor promoting signaling pathway when overexpressed in breast cancer cells. Development of ^{89}Zr-DFO-fresolimumab will provide an imaging tool that can aid in monitoring treatment progression of BCBM when treated with

fresolimumab, an inhibitor for TGF-β. By understanding more about TGF- β localization, information can be learned about TGF-β pathway in regards to breast cancer brain metastasis.

Isotopic dilution assays revealed an average of 3.8 ± 0.8 DFOs per fresolimumab (Equ.1).

$$\frac{\frac{\text{Bound } ^{89}\text{Zr}}{\text{Unbound } ^{89}\text{Zr}} \times \text{moles of } Zr^{4}}{\text{moles of antibody}}$$

Equation 1. Determining Chelate-to-mAb Ratio.

DFO was used as a bifuctional chelator to conjugate Fresolimumab to ^{89}Zr . The process of labeling of the Fresolimumab with ^{89}Zr was reproducible. The radiochemical yield was 76% based on HPLC, which is comparable to the radiochemical yield (Figure 2) of different antibodies bound to^{89}Zr found in the literature where the radiochemical yield is (>85%) [8, 9]. While the HPLC and iTLC showed high radiochemical purity (> 97%) (Figure 1). The specific activities of the ^{89}Zr-DFO-Fresolimumab used throughout the study ranged from 252 to 19,800 μCi/mg.

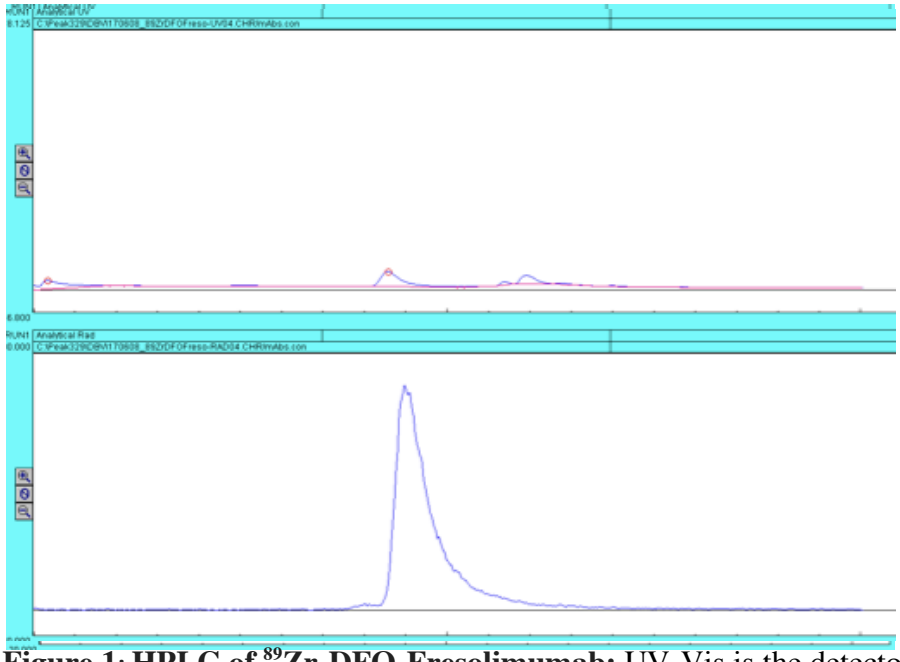


Figure 1: HPLC of ^{89}Zr-DFO-Fresolimumab: UV-Vis is the detector for the HPLC, while RAD indicates radioactivity detection. Both the peaks for radioactivity and HPLC UV-Vis were detected at the same time with high fidelity. This indicates successful binding between the DFO-Fresolimumab and ^{89}Zr.

Radiochemical purity: 97.38%

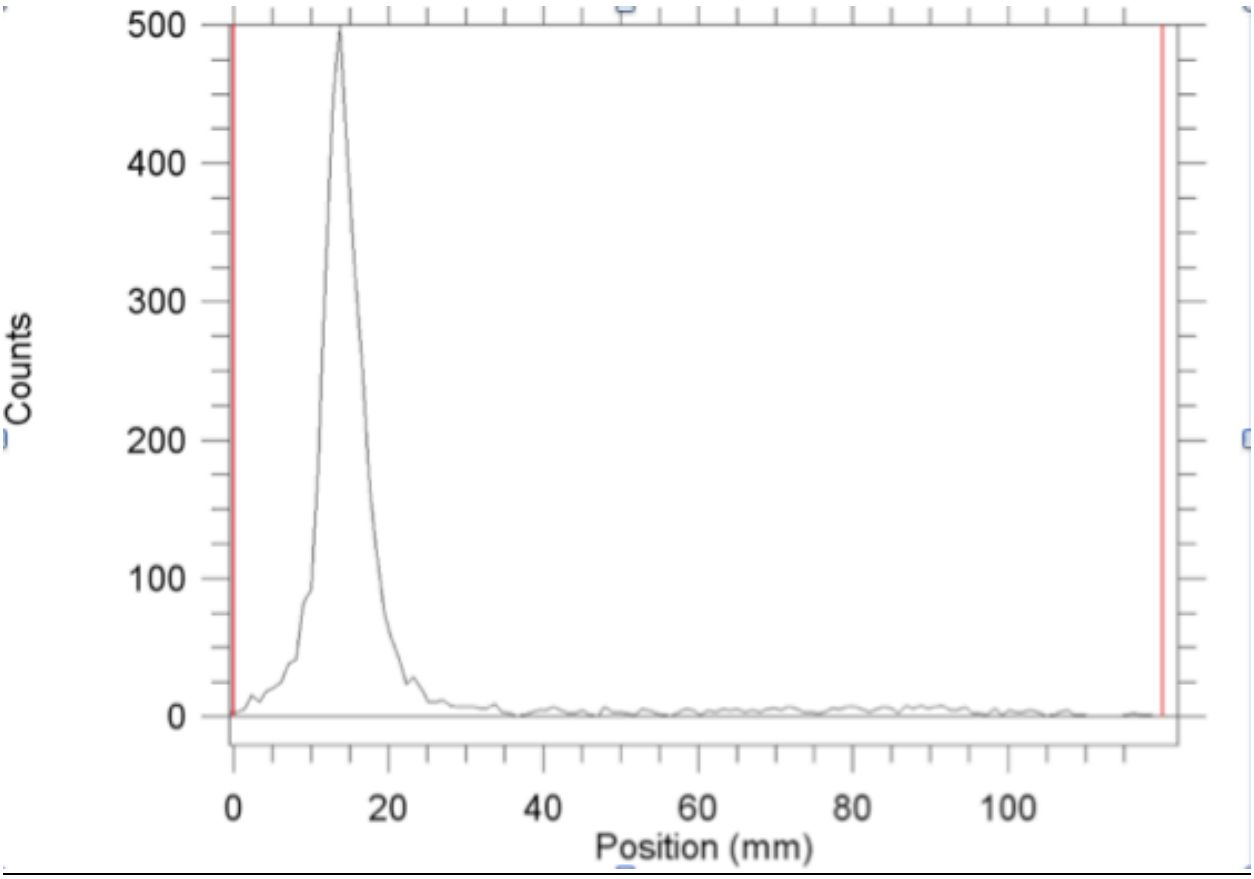


Figure 2: iTLC of ^{89}Zr-DFO-Fresolimumab. Radio-iTLC were run on silica strips using an eluent of 10 mM EDTA.

To prove that labeling fresolimumab did not alter the activity of fresolimumab a competitive binding experiment was done using unlabeled fresolimumab and ^{89}Zr-DFO-Fresolimumab competing to bind to TGF-β3. Out of the three isoforms of TGF-β, TGF-β3 was used because it has the highest affinity for fresolimumab (dissociation constant, 1.4 nM). Therefore, it serves as a sensitive indicator for immunoreactivity. This experiment resulted in an average 50% inhibition of maximum binding of 16 nM fresolimumab (Figure 3), indicating preserved function for fresolimumab when radiolabeled with ^{89}Zr. Within the literature, prior values for an IC_{50} include 18nM for competing unlabeled Fresolimumab and ^{89}Zr-DFO-

fresolimumab to TGF-β3 [3]. The values for IC_{50} obtained (16 nM) is comparable to the IC_{50} value obtained from a study investigating ^{89}Zr-DFO-fresolimumab done previously, reporting an IC_{50} of 18 nM [3].

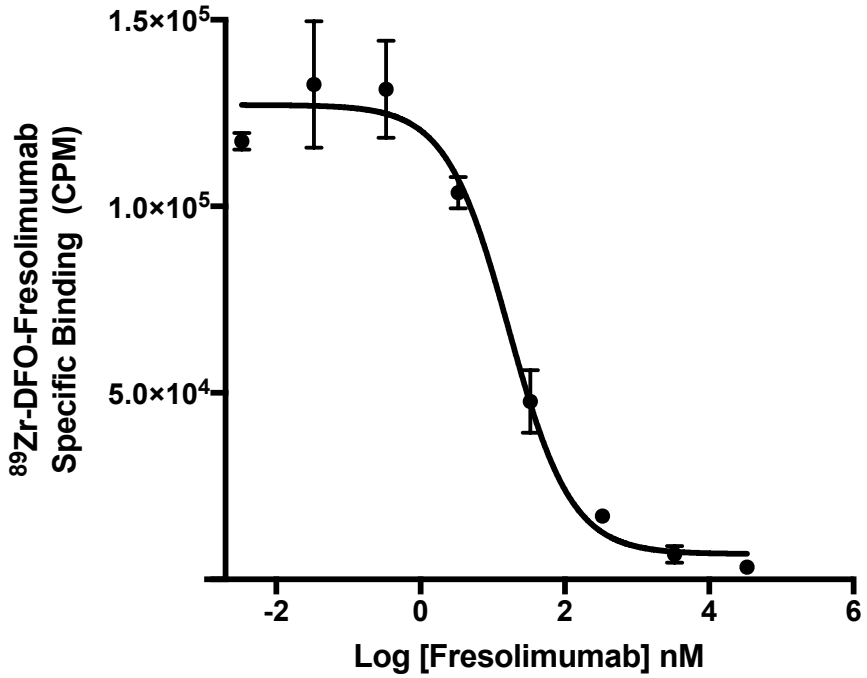
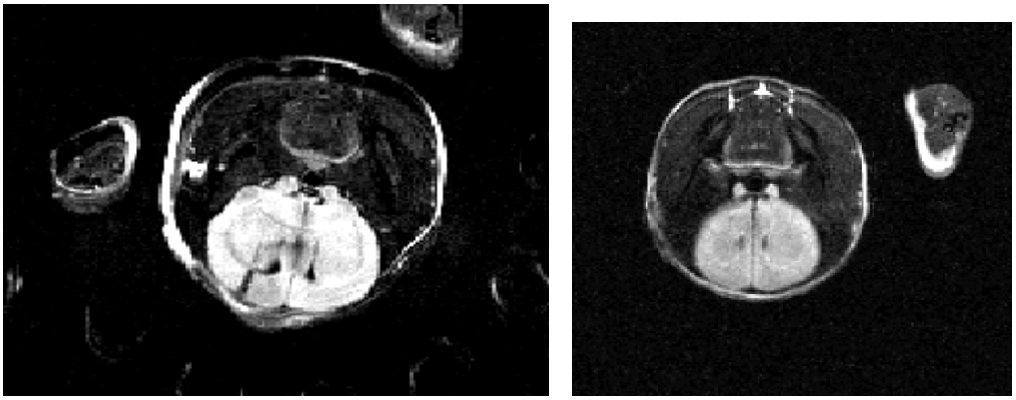


Figure 3: Competitive binding assay using cold fresolimumab and ^{89}Zr-DFO-Fresolimumab, IC_{50} =16 nM

After investigating the properties of TGF-β in vitro, studies were done to investigate the mouse model inoculated with 4T1-BR5 cells, a cell line that is known to overexpress TGF-β. In an effort to characterize the brain tumor growth and progression, serial MRI images were taken of the mice inoculated with either PBS or 4T1-BR5 cells. On the first day of imaging for both the control mouse which is the mouse inoculated with PBS mouse and the experimental mouse inoculated with 4T1-BR5 there is some regions of scarring seen in the MRI. The regions of scarring and inflammation can be identified through the regions of hypo-intensity in the brains of

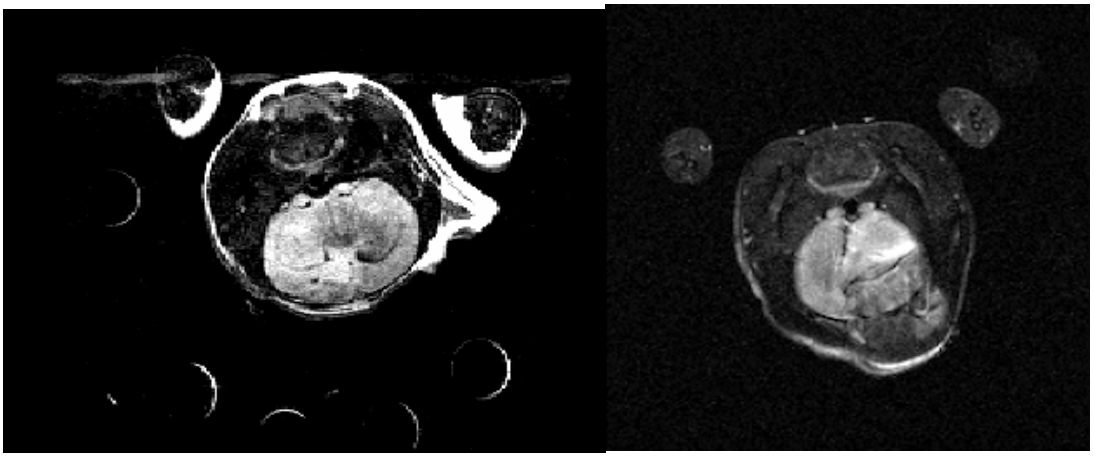
the two mice. After two weeks, the mouse inoculated with PBS has no remaining scarring and the brain seems healthy. To contrast, the mouse that is inoculated with 4T1-BR5 has grown a large tumor. The tumor cells are highly aggressive and begin to digest the skull, and the tumor begins to protrude outside of the skull region (Figure 4).

Mice Injected With PBS



Day 1 **Day 14**

Mice Injected With 4T1-BR5



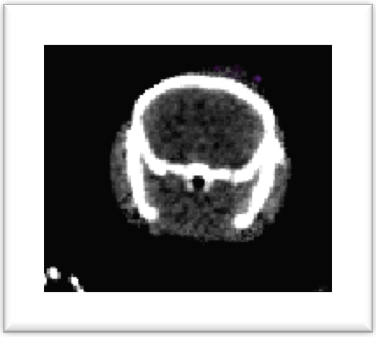
Day 1 **Day 14**

Figure 4: Characterizing the tumor burden for mice using diffusion weighted MRI

Once the tumor burden was characterized, the next step was to test the radiolabelled probe, ^{89}Zr-DFO-fresolimumab, in vivo. The goal was to see whether the imaging probe could reach the target site of the brain tumor. Two mice were imaged using μPET/CT 4 days post injection. One mouse was injected with ^{89}Zr-DFO-Fresolimumab and the other was injected with a combination of ^{89}Zr-DFO-fresolimumab and cold fresolimumab. The two mice showed no PET signal in the brain when imaged 1 hour post injection and 24 hours post injection. Four days after injection, signal was seen localized in the brain in the mouse imaged with radiolabeled product and cold fresolimumab combined (Figure 5). Cold fresolimumab may block sites of low-affinity, non-specific binding throughout the mouse's body. By blocking sites of non-specific binding with cold fresolimumab, the targeting vector labeled with ^{89}Zr can reach the brain tumor and bind with high affinity to TGF-β. Therefore, the targeting vector can be localized with enough signal to be detected in a PET scan.

A ^{89}Zr-DFO-Fresolimumab+ Cold Fresolimumab
Specific Activity: 19,800μCi/mg

96 Hours post injection; signal is seen localized in the brain tumor



B) ^{89}Zr-DFO-Fresolimumab
Specific Activity: 252 μCi/mg

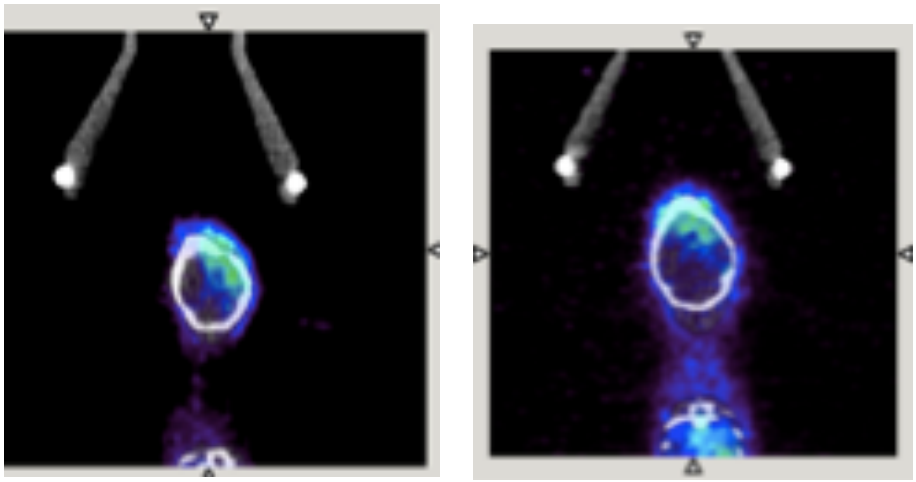
96 Hours post injection, no signal is seen coming from the brain tumor.

Figure 5: microPet/CT Imaged 4 days post injection

Next, an animal study was done to find the ideal concentration of cold fresolimumab. Two formulations were made one with a specific activity of 252 μCi/mg, and another with a specific activity of 89 μCi/mg. Between the two formulations, there was uptake of the radiotracer in the brain tumor. However, there was no difference seen in tumor uptake between the two specific activities (Figure 6). By the 96 hour time point both formulations achieved high uptake in the tumor with comparable Standard Uptake Values (SUV). The SUV for the mice injected with higher specific activity was 0.573, while the SUV for the mice injected with lower specific activity was 0.550. The comparable uptake could be a result of enhanced permeability and retention effect, or through binding of the fresolimumab to TGF-β. With no difference between the low and the high concentration of cold fresolimumab, the low concentration of cold fresolimumab, and therefore higher specific activity was used for further experiments.

4T1-BR5 Mouse

A. 24 Hours **96 Hours**



4T1-BR5 Mouse

B. 24 Hours **96 Hours**

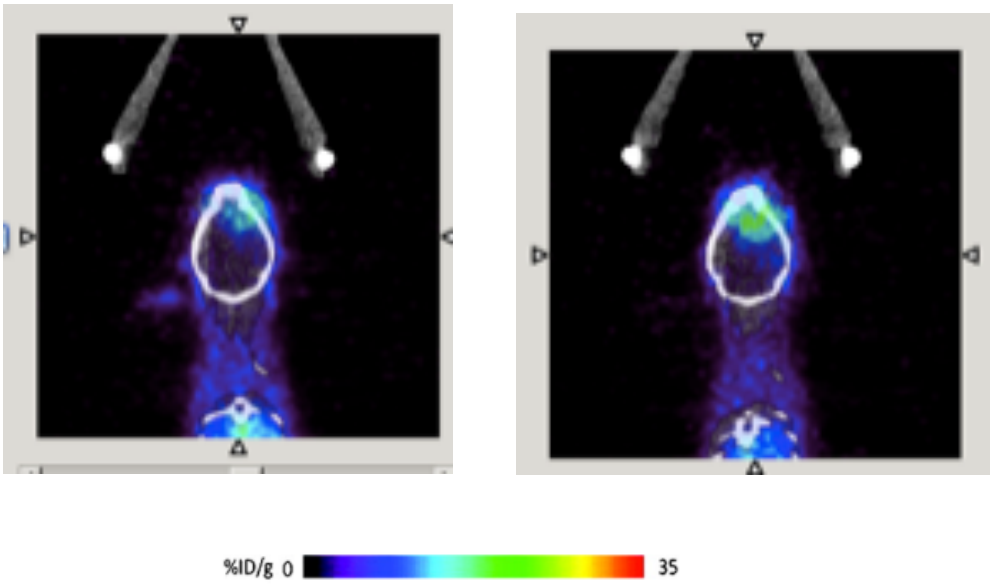


Figure 6: A) High Specific Activity: (Specific Activity: 252 uCi/mg, SUV 0.573)
B) Low Specific Activity: (Specific Activity: 89 uCi/mg, SUV 0.550)

Once the passage of the imaging probe into the brain was characterized a control experiment was done using mice that were inoculated with PBS. The mouse was injected with ^{89}Zr-DFO-fresolimumab and imaged serial μPET/CT images at 24 hour and 96 hour were obtained. As expected, there was no radiotracer uptake seen in the brain for mice inoculated with PBS (Fig 7).

Mice injected with PBS

24 Hours **96 Hours**

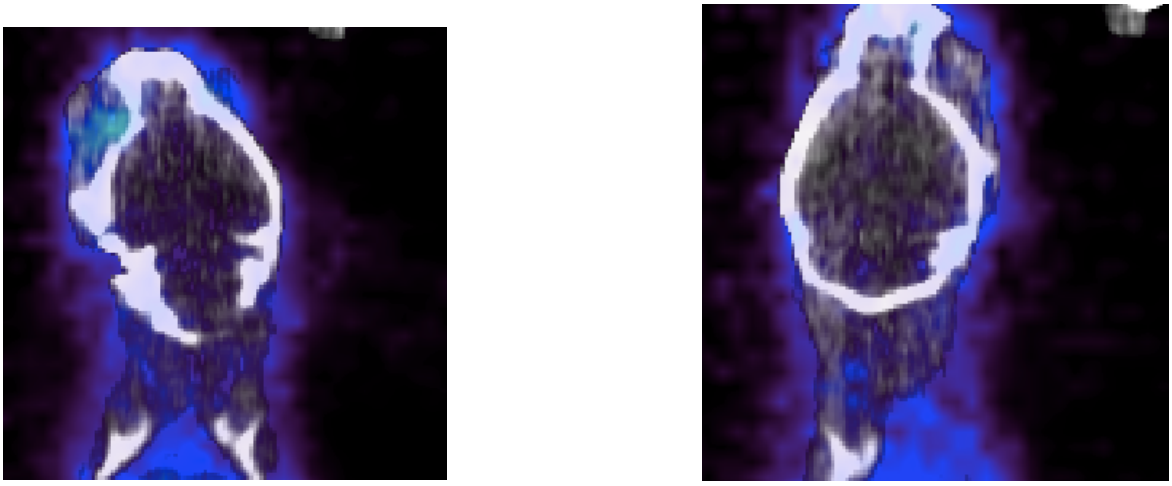


Figure 7) Mice inoculated with PBS and injected with ^{89}Zr-DFO-Fresolimumab

To investigate the specificity of the ^{89}Zr-DFO-fresolimumab imaging probe, tumor bearing mice were injected with an isotype for fresolimumab. The isotype was an antibody very similar to fresolimumab, however the isotype lacked the site of specific binding to TGF-β. While the expectation was to see no uptake in the brain, what was seen was highly variable uptake in the brain (Figure 8). A possible explanation could be that the FC region of the antibody binds non-specifically to macrophages and other immune cells with FC receptors. The brain tumor

could have sites of inflammation resulting in signal from the macrophages and immune cells linked to the FC chain of the radiolabelled isotype. Other explanations could be that through enhanced permeability and retention effect, accumulation of the radiotracer is seen in the brain.

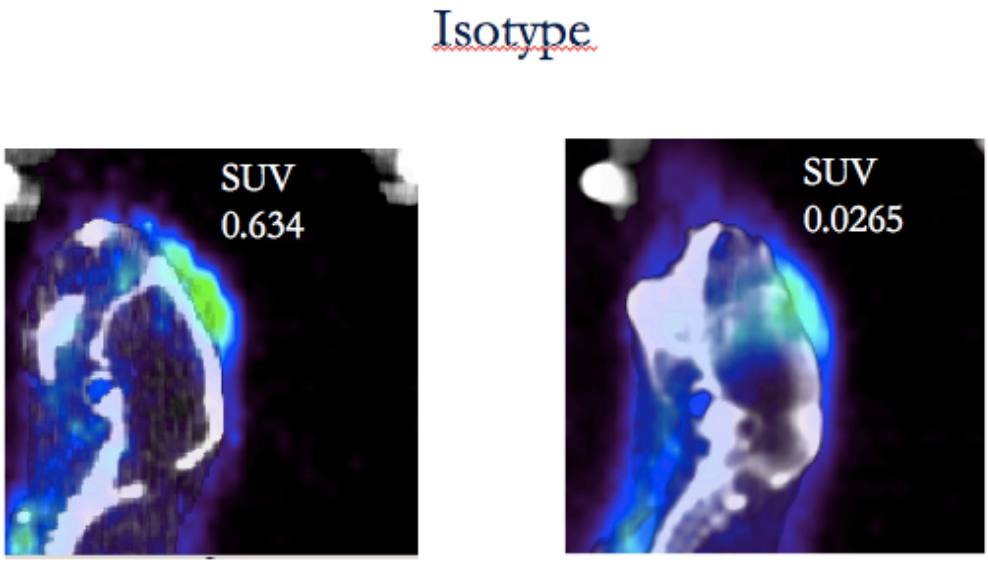


Figure 8: Mice injected with Isotype for Fresolimumab

Imaging the treatment response using fresolimumab as a potential therapeutic and imaging agent for BCBM can accelerate clinical translation of the antibody as a therapy. Imaging of the tumor model with the synthesized imaging probe provides evidence through PET imaging that the probe is being taken into the tumor tissue in the brain and surpassing the blood brain barrier. The tumor progression was characterized through serial diffusion weighted MRI imaging sessions, and the tumors maximum volume was seen two weeks after the mice were implanted with the 4T1-BR5 cells, while mice inoculated with PBS had normal brain after 2 weeks.

Conclusion

In the present study we evaluated the viability of a radiolabeled probe synthesized to target breast cancer brain metastasis. The radiolabeled probe was a ^{89}Zr-DFO-fresolimumab immuno-PET imaging agent that was synthesized with high radiochemical purity. We were able to identify that ^{89}Zr-DFO-fresolimumab was able to cross the compromised blood brain barrier of the tumor model and be localized with enough signal to show the brain tumor on a PET image. Together this study can be used to provide the baseline untreated model to be compared against mice with brain tumors after receiving treatment in future studies.

While this study provides strong evidence that the imaging probe could be used to localize BCBM, further work needs to be done to clarify the mechanism of action for the imaging probe. A study done with an isotype showed high variability in tracer uptake. To determine the specificity of the radiolabeled probe, a study can be done using a tumor with a TGF-β knockdown. By using a tumor with TGF-β knockdown, enhanced permeability and retention effect can be investigated. Therefore, this will indicate specificity of the probe determining the mechanism of action for tracer uptake. Although further studies need to be done regarding the mechanism of action, the results from this study are promising. They indicate that ^{89}Zr-DFO-fresolimumab can be localized in the brain, and can be used for therapy monitoring.

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