Translation of Zirconium-89 Labeled Antibodies for Human Imaging

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Translation of Zirconium-89 labeled Antibodies for Human Imaging

by

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by

Catherine Fu
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I would like to thank Dr. Henry VanBrocklin and Joseph Blecha from the VanBrocklin group for all their guidance throughout the project. I thank Charles Truillet and Matthew Parker for their assistance in cell culturing. I also would like to thank Tony Huynh for his assistance in PET imaging.
The exquisite specificity and affinity for their target antigens makes radiolabeled antibodies attractive candidates for therapeutics and drug delivery agents. The aim of this study was to evaluate the potential utility of two clinically available antibodies, Certolizumab Pegol (Cimzia) and Pembrolizumab (Pembro) for immuno-PET imaging. **Methods:** Antibodies pembrolizumab and certolizuman pegol were labeled with $^{89}$Zr in high chemical and radiochemical purity. **Results:** $^{89}$Zr-DFO-Pembro and $^{89}$Zr-DFO-Cimzia have been prepared with high radiochemical purity (>95%) with good radiochemical yield (~70%). **Conclusion:** Results from this studies will provide requisite data for an IND submission to the FDA and ultimately clinical translation of $^{89}$Zr-DFO-Pembro and $^{89}$Zr-DFO-Cimzia.
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Introduction

Radiolabeled antibodies have been in research for diagnostic and therapeutic purposes for over 40 years. Antibodies, when labeled with radionuclides that emit positron rays, target disease associated antigens, permitting detection of the disease [11]. Their exquisite specificity and affinity for their target antigens makes radiolabeled antibodies attractive candidates for therapeutics and drug delivery agents.

Positron emission tomography (PET) with monoclonal antibodies (mAbs), also known as ‘immunoPET’, offers an appealing imaging method for non-invasive tumor detection by combining the high sensitivity of PET with the high antigen specificity of mAbs [1, 7]. At the intersection between PET imaging and controlled drug delivery, PET image-guided drug delivery can be used to quantitatively assess tumor-targeted therapeutic delivery, distribution, uptake and help predict patient subpopulations that are most likely to respond to a given treatment [10].

Immuno-PET requires an appropriate positron emitter with a half-life ($t_{1/2}$) that is compatible with the time needed to achieve optimal in vivo tumor-to-nontumor ratios. For intact full-length mAbs (e.g. 150-160kD mass for IgG) used for targeting solid tumors, this time is typically 2–4 d. Therefore, commonly used positron emitters such as $^{11}$C and $^{18}$F (half-lives of 20 and 110 min, respectively) are not suitable for labeling of intact full-length mAbs due to their half-lives being too short [4]. Longer-lived isotopes allow for imaging over a few days time to show the accumulation at the target and clearance from non-target tissues. Currently, some of the commonly used PET isotopes for antibody labeling include $^{124}$I ($t_{1/2} = 4.2$ d), $^{64}$Cu ($t_{1/2} = 12.7$ h), and $^{86}$Y ($t_{1/2} = 14.7$ h) [7]. However, each of these isotopes possesses shortcomings, ultimately
limiting their suitability for clinical imaging. For example, while $^{64}$Cu has been employed successfully as a radiolabel for antibodies in numerous pre-clinical studies in rodents, its 12.7 h half-life is often too short to prove effective under the slower pharmacokinetic conditions of imaging *in vivo*. Likewise, $^{86}$Y possess a half-life that is too short for human imaging and there are also added difficulties such as limited availability due to difficulty in production and purification of the isotope. $^{124}$I, in contrast, has a near ideal half-life for antibody-based imaging and successful radiolabeled antibodies have been produced, such as $^{124}$I labeled humanized A33 (huA33) for imaging colorectal cancer. However, the expense of the isotope, its relatively low resolution due to the high kinetic energy carried by positron emissions, and the significant dehalogenation of $^{124}$I-labeled antibodies *in vivo* combine to limit its clinical potential [9].

<table>
<thead>
<tr>
<th>Nuclide</th>
<th>Emission</th>
<th>Half-life</th>
<th>Decay (% $\beta+$)</th>
<th>$\beta+$ Energy (keV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{64}$Cu</td>
<td>$\beta+$, $\beta$-</td>
<td>12.7 h</td>
<td>18</td>
<td>653</td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>$\beta+$</td>
<td>109 min</td>
<td>97</td>
<td>633</td>
</tr>
<tr>
<td>$^{89}$Zr</td>
<td>$\beta^+$, $\gamma$</td>
<td>78.4 h</td>
<td>23</td>
<td>897</td>
</tr>
<tr>
<td>$^{68}$Ga</td>
<td>$\beta+$</td>
<td>67.6 m</td>
<td>88</td>
<td>1899</td>
</tr>
<tr>
<td>$^{124}$I</td>
<td>$\beta+$</td>
<td>4.17 d</td>
<td>11</td>
<td>1534</td>
</tr>
</tbody>
</table>

Zirconium-89 ($^{89}$Zr) is a positron-emitting radionuclide with a desirable half-life of 78.4 h, which matches the pharmacokinetics of a majority of antibodies and has a low average positron energy ($\beta_{\text{max}} = 897$ keV), making it an ideal candidate for high resolution PET imaging of slow-accumulating biomolecules. In addition, $^{89}$Zr-based agents are safer to handle, inexpensive to produce and more stable *in vivo* making them better candidates for clinical applications [12]. However, a major disadvantage of $^{89}$Zr is the high-energy gamma emission at
909 keV, which may limit the radioactive dose that can be administered into patients [7]. As $^{89}\text{Zr}$ is a radiometal, direct labeling of the antibody is not possible. An appropriate chelator is needed to couple $^{89}\text{Zr}$ to the desired antibody. Several chelators have been used over the years but desferrioxamine B (DFO) is currently the most widely used chelator because of the stable bond formation of $^{89}\text{Zr}$ with the hydroxamate groups of this chelate [4]. Previous studies have demonstrated that this chelate exhibits rapid and efficient labeling, with good stability in regard to demetallation [1]. Generally, mAbs are conjugated with a bifunctional derivative of DFO via an amide linkage for subsequent labeling with $^{89}\text{Zr}$. The hydroxamate groups within DFO need to be temporarily blocked with Fe(III) before mAb conjugation. Subsequently, Fe(III) is removed by transchelation to ethylenediaminetetraacetic acid (EDTA) before the conjugate is exposed to $^{89}\text{Zr}$ [7]. Despite the success of this strategy, the multi-step procedure is both complicated and time-consuming, making it challenging to produce $^{89}\text{Zr}$-labeled mAbs in compliance with the current Good Manufacturing Practice (cGMP) for clinical investigations [7]. Thus a new developed chelate, $p$-isothiocyanatobenzyl-derivative of desferrioxamine D (Df-Bz-NCS), was introduced, and has enabled an efficient and rapid preparation of $^{89}\text{Zr}$-labeled mAbs (Fig. 1).

![Figure 1. Schematic representation of monoclonal antibody modification with Df-Bz-NCS and labeling with $^{89}\text{Zr}$.](image)

In order to translate a $^{89}\text{Zr}$-immuno-PET agent from preclinical development to clinical trials, the agent must pass several tests. Prerequisites include that the radioimmunoconjugate of
interest, or product, is stable and has similar binding and biodistribution characteristics to the unlabeled parental mAb. Imaging procedures should be standardized and validated in order to provide reliable quantification. Once all prerequisites are met, information on the product is manufacturing and characterization, quality control, and stability can be submitted in the Chemistry, Manufacturing, and Controls (CMC) portion of the Investigational New Drug (IND) application [Fig 2].

**Figure 2. Flow chart of prerequisites needed to submit in the Chemistry, Manufacturing, and Controls (CMC) portion of the Investigational New Drug (IND) application.**

The aim of this study is to evaluate the potential utility of two clinically available antibodies, CDP870 (certolizumab pegol) and Mk-3475 (pembrolizumab), for immuno-PET imaging. The mAb was radiolabeled with $^{89}$Zr and the *in vitro* binding characteristics of the radioligand were analyzed. Furthermore, storage stability was assessed and biodistribution and uptake were evaluated in rats. The collected data will be used in the drug product component of the CMC portion in the IND application for imaging agents.

Two biomolecules of interest are certolizumab pegol (Cimzia) and pembrolizumab (Pembro). Certolizumab pegol, marketed as Cimzia® by UCB in the US, is the only PEGylated anti-tumor necrosis factor alpha (TNFα) monoclonal antibody approved for the treatment of
rheumatoid arthritis (RA) and Crohn’s disease (CD), with a terminal half-life of 14 days [2]. The product is a humanized antigen-binding fragment (Fab’) that was subsequently PEGylated with a 40 kDa PEG moiety by reacting a C-terminal cysteine with a PEG–maleimide [3]. TNFα is a pro-inflammatory cytokine and a key mediator of the inflammation-induced joint damage that is associated with RA and the reduction in TNFα levels improves signs and symptoms of RA. Cimzia works by binding and neutralizing both soluble and transmembrane TNFα and inhibits signaling through the p55 and p75 TNFα receptors in vitro [2].

Pembrolizumab is a potent, highly selective, fully humanized immunoglobulin (Ig) G4-kappa monoclonal antibody against programmed death receptor 1 (PD-1). It is the first anti-PD-1 antibody to be approved by the US Food and Drug Administration for the treatment of patients with unresectable or metastatic melanoma [14] and has a terminal half-life of 27 days. PD-1 is an inhibitory receptor expressed on the surface of T-cells following their activation. When its main ligands (PD-L1 and PD-L2) bind to PD-1, it results in the inhibition of T-cell function. Thus, by binding to PD-1, pembrolizumab blocks its interaction with PD-L1 and PD-L2 thereby potentiating T-cell responses and reactivating anti-tumor immunity [13].

Methods and Materials

All chemicals, unless stated otherwise, were purchased from Sigma Aldrich (St. Louis, MO) and were used as received. OmniTrace® Ultra water from EMD (Wx0003-6) was used in all reactions. Df-Bz-NCS (DFO) was obtained from Macrocyclics (cat. no. B-705). Certolizumab pegol (Cimzia; 200 mg/mL) directed against tumor necrosis factor alpha (TNFα) was purchased from Merck (USA). Pembrolizumab directed against programmed cell death protein 1 (PD-1) was purchased from UCB (Georgia, USA). Radioactivity measurements were made using a
Capintec CRC-15PET Dose Calibrator (Capintec, Ramsey, NJ) with a calibration factor of 465 for $^{89}\text{Zr}$. $^{89}\text{Zr}$-radiolabeling reactions were monitored using silica gel impregnated glass-fiber instant thin-layer chromatography (ITLC-SG) paper (Gelman Sciences Inc., Ann Arbor, MI) and analyzed on a radio-TLC plate reader (Bioscan System AR-2000 Imaging Scanner) using Win-Scan Radio-TLC software version 2.2. $^{89}\text{Zr}$-oxalate was purchased from 3D Imaging (Little Rock, AR). Human embryonic kidney (HEK 293) cells were obtained from University of California-San Francisco Core Facilities (San Francisco, CA) and were grown by serial passage.

**Preparation of DFO-mAb**

Certolizumab pegol and pembrolizumab were premodified with Df-Bz-NCS (DFO) as previously described [15]. A three-fold molar excess of Df-Bz-NCS (in 20 μL DMSO) was added to mAb (1–6 mg) in 1 mL 0.1 M NaHCO$_3$ buffer, pH 9 and incubated for 30 min at 37° C. The ligand-antibody conjugate was purified by size exclusion chromatography using a PD-10 column (GE Healthcare Life Sciences) with 0.25 M sodium acetate/gentisic acid 5 mg/mL buffer (pH 5.5) as the mobile phase and the final bioconjugates were stored at -20°C.

**Preparation of $^{89}\text{Zr}$-labeled DFO-mAb**

Subsequently, DFO-mAb was labeled with $^{89}\text{Zr}$ at 37° C in a volume of 1 mL for 30 min; in a reaction vial 9 μL 2 M Na$_2$CO$_3$ were added to 18 μL $^{89}\text{Zr}$-oxalate (~2 mCi) solution, followed by 200 μL 0.5 M HEPES buffer (pH 7.0). Reaction vial solution (100 μL) was added to each DFO-mAb (1–3 mg). Finally, $^{89}\text{Zr}$-DFO-mAb was purified by size exclusion chromatography (PD10
column) using 0.25 M sodium acetate/gentisic acid 5 mg/mL buffer (pH 5.5) as the mobile phase [4].

**HPLC/ITLC Analysis**

The conjugates were analyzed by instant thin-layer chromatography (ITLC) for radiolabeling efficiency and radiochemical purity, and by high-performance liquid chromatography (HPLC) via UV/RAD detection.

ITLC analyses of $^{89}$Zr-labeled DFO-mAb was performed on silica gel impregnated glass fiber sheets with 20 mM citrate buffer (pH 5.0) as the mobile phase. Free $^{89}$Zr, taken up by the citrate buffer in the mobile phase, migrated along the ITLC strip, while $^{89}$Zr-mAb complexes remained at the origin.

HPLC monitoring of the final products was performed on a Jasco HPLC system using a Biosep SEC-s3000 size exclusion column (300 x 7.8 mm; Phenomenex) with 100 mM sodium phosphate buffer (pH 6.8) at a flow rate of 1 mL/min.

**Determination of Chelate-to-mAb Ratio**

The average number of chelates per mAb was determined using an isotopic dilution assay following a procedure developed at Memorial Sloan Kettering Cancer Center [1]. A 50 mL stock solution of 5mM ZrCl$_4$ (58.5 mg) was made up in 1M oxalic acid. $^{89}$Zr oxalate solution in 1M
oxalic acid (~300 μCi) was added to the stock solution to achieve a radioactive concentration of 8μCi/μL working solution. Solutions of DFO-mAb (n = 4) were prepared containing 153 μL of 1M ammonium acetate and 20 μL of the conjugated antibody solution (0.5 nmol of Cimzia and 1.5 nmol of Pembro). The 89Zr/Zr working solution (5 μL) was added to each sample and neutralized with 2.25 μL of 2M sodium carbonate. The solutions were incubated at room temperature for 2 hr. After incubation, a volume of 50 μL of 5 mM DTPA was added to each sample and left to incubate for 10 min. Samples were analyzed using ITLC and the average number of chelates per antibody was calculated from the ratio of bound vs unbound 89Zr multiplied by the ratio of the moles of Zr4+ to the moles of antibody.

**Evaluation of the Dose Stability**

To assess the storage stability of 89Zr-DFO-mAb, labeled mAbs were stored at -20°C in 0.25 M sodium acetate/gentisic acid 5 mg/mL buffer (pH 5.5). Final activity concentration was between 150 – 200 μCi. At various time points (0 hr, 120 hr, 168 hr), aliquots were taken and analyzed by ITLC. The stability of the complex was measured by the percentage of intact 89Zr that remained at the origin of the ITLC strip.

**Transfecting HEK 293 Cells**

In preparation for the immunoreactivity assay, human embryonic kidney (HEK) 293 cells were transfected with plasmid DNA of TNFα. For each 10 cm plate (n=3), 62 μg of DNA was diluted with 1.25 mL of Opti-MEM® I Reduced Serum Media. Also, 225 μL of Lipofectamine LTX®
Reagent was diluted in Opti-MEM® I Reduced Serum Media. Combine both solutions together and incubate for 5 min at room temperature. After 5 minute incubation, add 2.5 mL of the DNA-Lipofectamine LTX® Reagent complexes directly to each plate containing cells and mix gently. Incubate the cells at 37°C in a CO₂ (5%) incubator for 18-24 h post-transfection before assaying for transgene expression.

**Micro-PET Imaging**

Male and female Sprague Dawley rats were used for the imaging studies. The animals were cared for and used at the University of California (UCSF) facilities. All studies were carried out under a protocol reviewed and approved by the UCSF IACUC. All doses (200-300 μCi) were administrated as intravenous bolus injections into a catheterized tail vein, followed by a saline flush. Multiple time-point PET imaging data measuring the temporal distribution of the radiotracer $^{89}$Zr-DFO-Cimzia and $^{89}$Zr-DFO-Pembro were acquired at 30 min post-injection for 15 min, 4 hr post-injection for 15 min, 24 hr post-injection for 20 min and 96 hr post-injection for 60 min. PET were acquired in list-mode, histogramed, and reconstructed using 2D OS-EM algorithm that includes CT-based attenuation correction. Data collected from these time points will be used to measure the organ dosimetry for each antibody.

**Results and Discussion**

**Preparation of $^{89}$Zr-labeled DFO-mAb**
89Zr-Df-Bz-NCS-mAb was prepared as shown in [Fig 1]. First, DFO is coupled to the lysine groups of a mAb. This was achieved by the addition of a three-fold molar excess of DFO-NCS to the mAb solution, a reaction pH of 9.0, and incubation for 30 min at 37°C. Following purification, DFO-mAbs were labeled with 89Zr-oxalate in 0.5 M HEPES buffer (pH 7.0). The radio-ITLC of the crude radiolabeling mixture of 89Zr-DFO-Cimzia revealed some unbound 89Zr that eluted at the solvent front (Fig. 3B) while there was no such peak in 89Zr-DFO-Pembro (Fig. 3A). However, after purifying the 89Zr-DFO-Cimzia construct via size exclusion chromatography, the radiochemical purity of the 89Zr-DFO-Cimzia was >95% (Fig. 4B). The final radiochemical yield of the purified 89Zr-DFO-Cimzia was 69% and 71% for 89Zr-DFO-Pembro.

Figure 3. Representative radio-TLC chromatograms of the crude radiolabeling mixture of (A) 89Zr-DFO-Pembro and (B) 89Zr-DFO-Cimzia. Radio-TLCs were run on silica strips using an eluent of 20 mM citrate buffer, pH 5.0.
Figure 4. Representative radio-TLC chromatograms of the purified product of (A) $^{89}$Zr-DFO-Pembro and (B) $^{89}$Zr-DFO-Cimzia. Radio-TLCs were run on silica strips using an eluent of 20 mM citrate buffer, pH 5.0.

Determination of Chelate-to-mAb Ratio

Isotopic dilution assays revealed an average of 1.5±0.8 and 0.86±0.6 DFOs per mAb for Pembro and Cimzia, respectively (Equ. 1).

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

Equation 1. Determining Chelate-to-mAb Ratio.

HPLC Analysis

HPLC analysis was used to confirm radiochemical purity of the products. Commercial grade of each mAb were injected and analyzed via UV detection, followed by injection and analysis of the conjugated mixture of both mAbs. The peaks of the pure mAb and the DFO conjugated mAb
aligned, as seen in (Fig. 5), confirming that the DFO-mAbs are indeed the desired product. Also seen in (Fig. 5B, 5C) is a second UV peak that is present in both DFO-mAb mixtures. Later analysis reveals that the peak is 0.25 M sodium acetate/gentisic acid buffer (pH 5.5), in which the DFO-mAbs are stored (data no shown). The purified radiolabeled products of $^{89}$Zr-DFO-Cimzia and $^{89}$Zr-DFO-Pembro were also analyzed on HPLC (Fig. 6). The peak of $^{89}$Zr-DFO-Cimzia elutes earlier when compared to $^{89}$Zr-DFO-Pembro. This is due to the size difference between the two biomolecules, Pembro being a full size IgG while Cimzia is a Fab’ and one-third of the size of Pembro. The gentisic acid peak is not present because only radioactivity is being detected and shown.

Figure 5. Representative HPLC of (A) Cimiza, (B) DFO-Cimzia, (C) Pembro, and (D) DFO-Pemrbo. HPLC were run using 100 mM sodium phosphate buffer (pH 6.8) at a flow rate of 1 mL/min as the solvent.
Figure 6. Representative Radio-HPLC of (A) $^{89}\text{Zr}$-DFO-Pembro and (B) $^{89}\text{Zr}$-DFO-Cimzia. HPLC were run using 100 mM sodium phosphate buffer (pH 6.8) at a flow rate of 1 mL/min as the solvent.

Evaluation of the Dose Stability

$^{89}\text{Zr}$-labeled DFO-mAb were stored in 0.25 M sodium acetate/gentisic acid buffer (pH 5.5) at -20°C over several days to evaluate the storage stability (Fig 7). The zirconium dissociated at day 5 was 0.1% for $^{89}\text{Zr}$-DFO-Pembro and 1% for $^{89}\text{Zr}$-DFO-Cimzia, and at day 7 was 6% for $^{89}\text{Zr}$-DFO-Pembro and 11.6% for $^{89}\text{Zr}$-DFO-Cimzia, respectively.
Figure 7. Time curve of dose storage stability for $^{89}$Zr-DFO-Pembro and $^{89}$Zr-DFO-Cimzia.

**Micro-PET/CT Imaging**

Sagittal PET/CT images of $^{89}$Zr-DFO-Pembro (180 $\mu$Ci, 3 mg of mAb, in ~300 $\mu$Ci 0.25 M sodium acetate/gentisic acid buffer (pH 5.5)) and $^{89}$Zr-DFO-Cimzia (209 $\mu$Ci, 1.5 mg of mAb, in ~300 $\mu$Ci 0.25 M sodium acetate/gentisic acid buffer (pH 5.5)) recorded in Sprague Dawley rats at 24 h and 48 h are presented (Fig. 8). PET images were taken in order to determine normal distribution of the radiotracers. Activity can be seen in the heart, liver, lungs, and kidneys at 24 h for $^{89}$Zr-DFO-Cimzia. At 48 h, there is less activity in the heart and lungs with more accumulation in the liver and kidneys. In $^{89}$Zr-DFO-Pembro, there was very little activity throughout the rat, with some in heart, liver, lungs, and kidneys at 24 h. Activity decreased in all organs at 48 h in $^{89}$Zr-DFO-Pembro. There was no activity seen in the bones, revealing no free $^{89}$Zr in either image.
Conclusion

In the present study we evaluated the radiolabeling of two current antibody-based drugs with $^{89}$Zr for immunoPET imaging. $^{89}$Zr-DFO-Pembro and $^{89}$Zr-DFO-Cimzia have been prepared with high radiochemical purity (>95%) with good radiochemical yield (~70%). The number of DFOs per biomolecule was determined to be 0.85 and 1.5. Radiolabeled immunoconjugates remained stable for up to 7 days at -20°C. Future works to be done include evaluation of immunoreactivity and biodistribution experiments for dosimetry. Together all of these studies will provide requisite data for an IND submission to the FDA and ultimately clinical translation of $^{89}$Zr-DFO-Pembro and $^{89}$Zr-DFO-Cimzia.

Figure 8. PET/CT images of (A) $^{89}$Zr-DFO-Cimzia and (B) $^{89}$Zr-DFO-Pembro at 24 h and 48 h after injection.
References


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