Two patient studies of a companion diagnostic Immuno-Positron Emission Tomography (immunoPET) tracer for measuring human CA6 expression in cancer for antibody drug conjugate (ADC) therapy

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**Supplemental data**

**Materials and Methods**

**Cell Lines and Cell Culture**

The human cell line CA6 positive-WISH (Originally derived from human amnion tissue; ATCC®CCL-25™) was purchased from American Type Culture Collection (Manassas, VA), and the human ovarian carcinoma cell line A2780 (CA6 negative) was purchased from European Cell Culture Collection (Wiltshire, United Kingdom). WISH and A2780 cell were cultivated at 37°C in a humidified 5% CO₂ incubator using Eagle’s minimum essential medium or Roswell Park Memorial Institute 1640 medium, respectively, and supplemented with 10% heat-inactivated fetal bovine serum, 2 mM (2 mmol/L) L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen Life Technologies (Carlsbad, California), except for Eagle’s minimum essential medium, which was supplied by Lonza (Walkersville, MD).

**Flow Cytometry**

The binding affinities of both BFab and DOTA-BFab were analyzed using fluorescence-activated cell sorting assay (FACS: Aria III, BD Biosciences, San Jose, CA, USA) by collecting $10^4$ events of positively stained CA6 cells. Assays were performed by dissolving BFab or DOTA-BFab in a series of (1:3) dilutions from $3 \times 10^{-7}$ to $1 \times 10^{-10}$ M using 1% bovine serum albumin dissolved in phosphate-buffered saline (PBSA) and mixed with WISH or A2780 cells ($2 \times 10^6$) per well in a 96 well ELISA plate. All wells were filled to a final volume of 0.1 mL PBSA, then were gently mixed, and it were kept on ice for incubation. After 1 hour, cells were washed twice using PBSA and stained with mouse anti-human kappa mAb conjugated with Alexa Fluor 488 (1:50, Invitrogen Life Technologies) or anti-6X His tag antibody tagged with fluorescein isothiocyanate (1:100, Abcam, Cambridge, Mass) in 0.1 mL PBSA/well. These cells were kept on ice in the dark. After an hour-long incubation, stained cells were washed thrice using 200μL of PBSA for each time. After washing, cells were resuspended in 200μL of PBSA with 1% formaldehyde for
30 mins incubation, and then formaldehyde was washed off thrice using PBSA. Dead cells were excluded by staining with propidium iodide (10 µg/ml). Cell binding assays were performed at the Canary Center for Early Cancer Detection, a Stanford University School of Medicine Core Facility (Stanford, CA). FlowJo software version 10.1 (Tree Star, Ashland, OR) was used to measure the mean florescence intensity (MFI) associated with single cells. Background MFI was computed from cells stained with secondary antibody alone and subtracted from the test samples.

**Quality control tests methods listed in the table 1 column 2.**

**Test #1: Immunoconjugate purity.** [(DOTA)-BFab] purity was measured by SEC-HPLC (2000 column) connected with an inline UV280nm detector using 0.1M phosphate buffer (pH 7) as the mobile phase eluted at 1ml/min.

**Test #2: DOTA/BFab (c/a).** The number of DOTA chelators coupled per antibody (c/a) was estimated with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) by comparison with unmodified BFab and (DOTA)-BFab. BFab and DOTA-BFab samples were mixed with bovine serum albumin (BSA; 1 pM) as an internal standard. To provide the number of chelates per antibody the mass difference between the unmodified BFab and DOTA-BFab was divided by the mass of a single DOTA.

**Tests #3 and 4: Tracer appearance and pH.** The [64Cu]BFab (radiopharmaceutical) appearance was evaluated for the clarity and color of the solution by visual inspection and pH was measured using narrow-range pH paper (pH 6.5–7.5).

**Test #5: Radiopharmaceutical purity.** Purity was measured by SEC-HPLC (2000 column) connected with in line UV280nm/radioactive detector using 0.1M phosphate buffer (pH 7) as the mobile phase eluted at 1ml/min. The percent of radioactivity peak corresponding to (DOTA)-BFab was measured and reported as purity of the tracer.
**Test #6:** Radio chemical yield. (DOTA)-BFab labeling yield was determined by instant thin-layer paper chromatography (ITLC: Biodex Medical Systems, Inc., Shirley, NY) developed in saline solution (0.9% w/v NaCl in water).

**Test #7:** Specific activity. This was calculated from the ratio of the radioactive concentration ([⁶⁴Cu] bound to the DOTA-BFab) in the radiopharmaceutical solution over the concentration of the DOTA-BFab in the solution was chelated with the radioisotope i.e., Specific activity = Total radioactivity ([⁶⁴Cu] BFab) in solution (Ci) per mL/ concentration of DOTA-BFab (µmol) per mL.

**Test #8:** Serum stability. The final dose of [⁶⁴Cu]BFab in PBS was performed by diluting nine-fold with normal human serum (Equitech-Bio, Kerrville, TX) and incubated at 37°C for 24 hours. This incubated mixture was analyzed by drawing 100 µL of the sample at 0 hour, 1 hour, and 8 hour after mixing, by analytical SEC-2000 HPLC (100% 0.1M phosphate buffer, 1 ml/min). Radioactivity signal corresponding to chromatogram such as [⁶⁴Cu]BFab, [⁶⁴Cu]-EDTA and any protein aggregation. Each peak signal was expressed as a percentage from total activity of all peak units.

**Test #9:** Bacterial endotoxin. Endotoxin tests were performed at room temperature with endotoxin free water, depyrogenated glassware, sterile tubes, syringes and pipette tips and sterile reagents. Radiopharmaceutical was diluted in series using 13 x 100 mm dilution tubes. The dilution range from the original concentration of product was within the Maximum Valid Dilution. It was computed based on the endo toxin sensitivity of the PTS cartridge. Endo toxin free water and radiopharmaceutical solution were mixed well before delivery into the endo safe cartridge. According to USP, the bacterial endotoxin’s limit for intravenous radiopharmaceuticals is determined by the following formula: 175.EU/V, where EU are Endotoxin units, and V is the volume of the dose.
**Test#10:** Radiopharmaceutical Sterility. The sterility of the final product (purified and passed through 0.22 µ filter) was determined by established procedure as recognized by the USP. Sterility of the radiopharmaceutical was tested in Laminar Flow Hood (LFH) using Fluid Thioglycollate Medium (FTM) and Tryptic Soybean Broth (TSB) tubes. Each of these tubes were marked with product name, lot number, and inoculation date. FTM media (1 mL) was mixed with 0.25 mL of radiopharmaceutical in FTM tubes using a sterile syringe. FTM tube marked as negative control did not receive radiopharmaceutical instead, 0.25 mL of sterile saline was added. The same procedure was repeated using TSB tubes and TSB media. FTM tubes were incubated at 30 - 35°C, and TSB tubes were incubated at 20 - 25°C. All these tubes were visually inspected every day for the appearance of any growth in the media tubes. The results were recorded in the sterility test score sheet for up to 14 days.

**Test#11:** Immunoreactivity. The cell binding immunoreactive fraction was calculated as previously described. WISH cells were used to determine the immunoreactivity. Briefly, WISH cells were grown in Eagle medium in Earle's BSS with non-essential amino acids, 1 mM sodium pyruvate, and 10% FBS. These cells were (> 90% viability as determined by hemocytometer) re-suspended in Hank's Balanced Salt Solution (HBSS; Gibco, Mountain View, CA #14025-092) of 5 x 10^7 cells/mL. These cells were aliquoted at various concentrations (10 concentrations from 6 x 10^4 to 6 x 10^6) in 200 µL of HBSS, in two sets of duplicate Eppendorf tubes (2 mL volume). In one set of tubes (non-blocking cells), cells were received just HBSS (50 µL) and in the other set of tubes (blocking of CA6 antigen), cells were mixed with unmodified BFab in HBSS (50 µL; 1mg/mL). Both sets of tubes were incubated on ice for 30 mins and pelleted. These pelleted cells were further washed twice and resuspended in 150 µL of HBSS, followed by addition of 50 µL of tracer ([^64Cu]BFab; 1 μCi) in HBSS and kept at room temperature for 90-120 minutes. After
incubation, and mild vortexing, 100 µL of aliquot was removed from each of the cell suspension into a Falcon® Round-Bottom Polystyrene Tubes (12 x 75 mm) for counting of total radioactivity. The remaining cells suspension (100 µL) in tubes were transferred into another Eppendorf tubes (Biopur, sterile, pyrogen-free) containing 200 µL of a 4:1 mixture of silicone oil (Catalogue #63148-52-7, Sigma Aldrich, St Louis, Missouri, MO) and mineral oil mixture (Catalogue #8042-47-5, Sigma Aldrich) and mixed well with addition of 200 µL of HBSS. These tubes were centrifuged at 16,000g for 5 minutes at room temperature. These tubes were frozen by submerging in liquid nitrogen and the bottom of these Eppendorf tubes were clipped (a guillotine-style dog nail clipper) into a Falcon® counting tube, which contained cell/oil mixtures that bound to the tracer. Both set of the tubes (total tracer activity and cell bound tracer activity) were counted in a gamma counter (Packard Cobra II Gamma counter, Ramsey, MN) using an energy window of 350-650 keV. From the radioactivity count, immunoreactivity (Kd) was computed by plotting total radioactivity/cell bound radioactivity ratio, against cell concentrations pelleted in each of the tubes. GraphPad software (Prism 6; version 6.02; San Diego, CA) was used to plot a non-linear curve fit with a “one site – total” binding model. Statistical significance was denoted as $P <0.05$ in any single-comparison statistical tests.
**Table 1S.** Two case studies: PET scanning and IHC tests performance sequence

| Tests                        | Imaging/Tests performance (Dates)                                                                 |
|------------------------------|--------------------------------------------------------------------------------------------------|
| Patient’s Cancer             | Ovarian                                                                                         | Breast                                                                 |
| IHC (Pre-imaging) for CA6+   | November 2016*                                                                                 | Not available                                                         |
|                              | [Eus-guided FNA of the peri-pancreatic LN tissue and positive for adenocarcinoma]                  |                                                                        |
| [¹⁸F]FDG                     | January 2017                                                                                   | September 2017                                                        |
|                              | [Possible paraortic LN reactive]                                                                 | [4 FDG-avid hepatic metastases; FDG-avid bilateral hilar metastases; an FDG-avid osseous metastasis at T5 vertebrae; and Possible FDG-avid lung metastases in lower left lobe ] |
| [⁶⁴Cu]BFab                   | January 2017                                                                                   | September 2017                                                        |
|                              | [Tracer was well tolerated, no adverse effects at administered dose, and no safety concerns]     | [Tracer was well tolerated, no AE’s at administered dose, and no safety concerns] |
| IHC for CA6+ score           | January 2017                                                                                   | September 2017                                                        |
|                              | [Biopsy proven disease in omentum, myometrium, serosa, fallopian tube, and ovaries] **Figure 2** | [Liver core biopsy positive for metastatic carcinoma, consistent with a breast primary origin] **Figure 2S.** |

*: IHC Test performed for CA6 on March 2017
Table 2S. Two case studies:
Two patients’ safety data before and after [⁶⁴Cu]BFab tracer injections

| Tests                        | Baseline | 1h    | 24h   | One week |
|------------------------------|----------|-------|-------|----------|
| **Patient 1 (Ovarian)**      |          |       |       |          |
| Blood pressure               | 121/79   | 120/74| 109/66| 93/49    |
| Electrocardiogram (EKG)      | Normal   | Normal| Normal| Normal   |
| Oxygen Levels (%)            | 99       | 100   | 100   | 100      |
| Heart rate (bpm)             | 84       | 68    | 70    | 72       |
| **Complete blood count**     |          |       |       |          |
| Hemoglobin (g/dL)            | 10.4g    | 9.9   | 8     |
| WBC (k/µL)                   | 6.2      | 5.4   | 6.6   |
| Platelets (k/µL)             | 209      | 191   | 146   |
| **Patient 2 (Breast cancer)**|          |       |       |          |
| Blood pressure               | 140/101  | 130/86| 130/90| 120/78   |
| Electrocardiogram (EKG)      | Normal   | Normal| Normal| Normal   |
| Oxygen Levels (%)            | 98       | 98    | 99    | 100      |
| Heart rate (bpm)             | 97       | 75    | 75    | 78       |
| **Complete blood count**     |          |       |       |          |
| Hemoglobin (g/dL)            | 11.5     | 11.8  | 12    |
| WBC (k/µL)                   | 3.6      | 3.7   | 2.9   |
| Platelets (k/µL)             | 168      | 177   | 172   |
Figure 1S. Tracer uptake (SUV) by key healthy tissues from both patients at 1 and 24 hours post tracer injections.

Post-treated breast cancer metastasis to liver
Figure 2S: **CA6-expression of breast cancer patient biopsies.** Immunohistochemistry was carried out to evaluate CA6 expression in the breast cancer metastasis to liver. Representative tissue specimens from the liver (post-treatment of chemotherapy, surgery, and adjuvant-chemotherapy). Tissues were stained for CA6 expression (top panels) using the validated anti-human CA6 antibody SAR566658 (Internal reagent from Sanofi) and hematoxylin eosin saffron (HES) staining (bottom panels) for the evaluation of cell morphology. IHC analysis for post-treatment liver tissue specimens indicated weak H-score (35) for CA6 expression. Original magnification was indicated on images as 10x, scale bar = 25 mm.

**References:**

1. Ilovich O, Natarajan A, Hori S, et al. Development and Validation of an Immuno-PET Tracer as a Companion Diagnostic Agent for Antibody-Drug Conjugate Therapy to Target the CA6 Epitope. *Radiology* 2015; 276: 191-198. DOI: 10.1148/radiol.15140058.

2. Natarajan A, Gowrishankar G, Nielsen CH, et al. Positron emission tomography of 64Cu-DOTA-Rituximab in a transgenic mouse model expressing human CD20 for clinical translation to image NHL. *Mol Imaging Biol* 2012; 14: 608-616. DOI: 10.1007/s11307-011-0537-8.