

General

The Anti-HER2, Imaging Agent Affibody molecule $Z_{\text{HER2:477}}$ and a bivalent Affibody protein Anti-HER2, unconjugated, $(Z_{\text{HER2:477}})_2$ were purchased from Affibody AB (Bromma, Sweden). Dimethyl sulfoxide (DMSO) and ethyl acetate were purchased from Fisher Scientific (Pittsburgh, PA). Dichloromethane, 2-(*tert*-butoxycarbonylaminoxy) acetic acid, triethylamine, N-(2-aminoethyl)maleimide trifluoroacetic acid (TFA) salt, N-hydroxybenzotriazole hydrate (HOBT), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethyl acetate, dithiothreitol (DTT), Tricine, 4-fluorobenzaldehyde (4-FBA), and all other standard synthesis reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All chemicals were used without further purification. Herceptin was obtained from Genentech Inc. (South San Francisco, CA). No-carrier-added ^{18}F -fluoride was produced by the $^{18}\text{O}(\text{p}, \text{n})^{18}\text{F}$ nuclear reaction on a GE PETtrace cyclotron at cyclotron facility, Molecular Imaging Program at Stanford (Stanford, CA).

A CRC-15R PET dose calibrator (Capintec Inc., Ramsey, NJ) was used for all radioactivity measurements. Reverse phase high performance liquid chromatography (RP-HPLC) was performed on a Dionex Summit HPLC system (Dionex Corporation, Sunnyvale, CA) equipped with a 170U 4-Channel UV-Vis absorbance detector and radioactivity detector (Carroll & Ramsey Associates, model 105S, Berkeley, CA). UV detection wavelengths were 218 nm, 254 nm and 280 nm for all the experiments. Analytical RP-HPLC columns (Vydac, Hesperia, CA. 218TP510-C18, 10 mm \times 250 mm) were used for analysis of labeled protein. The mobile phase was solvent A, 0.1% TFA/ H_2O , and solvent B, 0.1% TFA/acetonitrile. Matrix-assisted laser

desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS, model: Perseptive Voyager-DE RP Biospectrometer) (Framingham, MA) or an electrospray ionization time of flight mass spectrometer (ESI-TOF-MS, model: JMS-T100LC) (JEOL, Tokyo, Japan) was performed by the Mass Spectrometry Lab at GE Global Research (Niskayuna, NY). Nuclear Magnetic Resonance Spectroscopy (NMR, 400MHz, Bruker Avance AV400, Billerica, MA) was also used for characterization of the chemicals prepared. Alpha cyano-4-hydroxy-cinnamic acid (α -CHCA, prepared as 10 g/L in 33.3% CH₃CN : 33.3% EtOH : 33.3% H₂O : 0.1% TFA) were used as the solid matrix for MALDI. Human ovarian cancer SKOV3 cell line was obtained from the American Type Tissue Culture Collection (Manassas, VA). Female athymic nude mice (*nu/nu*) were purchased from Charles River Laboratories (Boston, MA).

Synthesis of Bifunctional Linker: Mal-AO

The bifunctional linker tert-butyl 2-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethylamino)-2-oxoethoxycarbamate (abbreviated as Mal-AO-Boc) was first prepared as follows: to a solution of 2-(*tert*-butoxycarbonylaminoxy)acetic acid (382 mg, 2 mmol) in anhydrous dichloromethane (20 mL) was added sequentially triethylamine (307 μ L, 2.2 mmol), N-(2-aminoethyl)maleimide-TFA salt (508 mg, 2 mmol), HOBt (306 mg, 2 mmol), and EDC (420 mg, 2.2 mmol). After being stirred for 24 hrs at room temperature, the reaction mixture was diluted with ethyl acetate (50 mL) and washed with saturated sodium bicarbonate solution (3 \times 30 mL), water (30 mL), and brine (30 mL). The organic layer was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated to a pale yellow solid, which was purified by column chromatography

(70% ethyl acetate in hexanes) to give the product as a white powder (500 mg, 80% of the theoretical yield). The Boc-protected bifunctional compound, 2-(aminooxy)-N-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)acetamide hydrochloride (abbreviated as Mal-AO) was subsequently prepared as follows: a solution of 9.3 mg of Mal-AO-Boc in 1 mL of 3 M HCl in MeOH was prepared and stirred at ambient temperature for 18 hours. The resulting solution was evaporated under vacuum to yield Mal-AO as a light yellow solid. The product was characterized using Liquid Chromatography-ESI-MS (LC-ESI-MS) and NMR. Compound Mal-AO $m/z = 214.11$ for $[M+H]^+$ ($C_8H_{11}N_3O_4$, Calculated MW = 213.07); 1H -NMR (400MHz, DMSO- d_6): δ 3.27 CH₂ (t, J= 4.0 Hz, 2), 3.49 CH₂ (t, J= 4.0 Hz, 2), 4.39 CH₂O (s, 2), 7.00 CH=CH (2, s). Yield: 80%.

Synthesis of Aminooxy Functionalized Affibody [$Z_{HER2:477}$ -ONH₂ and ($Z_{HER2:477}$)₂-ONH₂]

The general procedure for preparing the aminooxy-Affibody is as follows: the Affibody molecule [$Z_{HER2:477}$ or ($Z_{HER2:477}$)₂] was dissolved with freshly degassed phosphate buffer (pH 7.4) at a concentration of approximately 1 mg/mL. The disulfide linkage in the Affibody was reduced by the addition of DTT to the Affibody solution to a 20 mM concentration. The reaction mixture was vortexed for 2 hours and eluted through a NAP-5 column (GE Healthcare, Piscataway, NJ) to remove excess of DTT reagent. The column was previously equilibrated with degassed phosphate buffer. Same buffer was used for elution of the Affibody sample (the isolation yield was over 79%). The reduced Affibody was collected and the Mal-AO in DMSO was added (10-20 equivalents per equivalent of the Affibody). After vortexing for 2 hours, the reaction mixture was

dialyzed overnight using Slide-A-Lyzer cassette of molecular weight cutoff (MWCO) 3.5 KDa (Pierce Biotechnology, Rockford, IL) and MilliQ water (Millipore, Billerica, MA) as exchange solvent. The dialyzed sample was concentrated using Microcon or Amicon Ultra centrifuge filters MWCO 5 KDa (Millipore, Billerica, MA). Characterization of the $Z_{\text{HER2:477}}\text{-ONH}_2$ and $(Z_{\text{HER2:477}})_2\text{-ONH}_2$ was confirmed using MALDI-TOF-MS or LC-ESI-MS. The purities of the products were confirmed by HPLC. The starting Affibody molecules were not observed for the reaction solution using MS analysis, demonstrating that the reaction of the Affibody conjugation was quantitative.

Synthesis of 4-FBA Conjugated Aminooxy-Affibody [FBO- $Z_{\text{HER2:477}}$ and FBO- $(Z_{\text{HER2:477}})_2$]

To 92 μL of an aqueous solution of ammonium acetate (50 mM, pH 4) was added a 7 μL aliquot of a 0.5 mM stock solution of $Z_{\text{HER2:477}}\text{-ONH}_2$ or $(Z_{\text{HER2:477}})_2\text{-ONH}_2$ in phosphate buffered saline (PBS, pH 7.4). A 1 μL aliquot of a stock solution of 3.4 molar 4-FBA in DMSO was then added. The resulting reaction mixture was thoroughly mixed and incubated for either 0.5 to 1 hour at 70 °C or overnight at room temperature. The reaction mixture was then immediately diluted to 4 mL using MilliQ water, and concentrated to a volume of less than 100 μL using an Amicon Centrifugal Ultrafiltration unit equipped with a Millipore MWCO 5 KDa filter. This water dilution-concentration procedure was repeated additional two additional times in order to completely remove excess 4-FBA. Characterization of the resulting purified FBO- $Z_{\text{HER2:477}}$ and FBO- $(Z_{\text{HER2:477}})_2$ was achieved using either MALDI-TOF-MS or LC-ESI-MS. The purities of the bioconjugates were confirmed by HPLC. Similarly, the absence of starting $Z_{\text{HER2:477}}\text{-ONH}_2$ and $(Z_{\text{HER2:477}})_2\text{-ONH}_2$ was confirmed by HPLC.

ONH₂ or (Z_{HER2:477})₂-ONH₂ for the reaction solution using MS analysis demonstrated that the conjugation reaction was quantitative.

***In Vitro* Biacore Analysis of Affibody Bioconjugates**

Binding interactions between the binders [Z_{HER2:477}-ONH₂, (Z_{HER2:477})₂-ONH₂, FBO-Z_{HER2:477}, and FBO-(Z_{HER2:477})₂] and the HER2/neu antigen were measured in vitro using surface plasmon resonance (SPR) detection on a Biacore™ 3000 instrument (GE Healthcare, Piscataway, NJ). The extracellular domain of the Her2/neu antigen was obtained as a conjugate with the Fc region of human IgG (Fc-Her2) from R&D Systems (Minneapolis, MN) and covalently attached to a CM-5 dextran-functionalized sensor chip (GE Healthcare, Piscataway, NJ) pre-equilibrated with HBS-EP buffer (0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, 0.005% v/v surfactant P20) at 10 µL/min and subsequently activated with EDC and NHS. The Fc-HER2 (5 µg/ml) in 10 mM sodium acetate (pH 5.5) was injected onto the activated sensor chip until the desired immobilization level (~3000 Resonance Units) was achieved (2 min). Residual activated groups on the sensor chip were blocked by injection of ethanolamine (1 M, pH 8.5). Any non-covalently bound conjugate was removed by repeated (5×) washing with 2.5 M NaCl, 50 mM NaOH. A second flow cell on the same sensor chip was treated identically, except with no Fc-HER2 immobilization, in order to serve as a control surface for refractive index changes and non-specific binding interactions with the sensor chip. Prior to the kinetic study, binding of the target analyte was tested on both surfaces and a surface stability experiment was performed to ensure adequate removal of the bound analyte and regeneration of the sensor chip following treatment with 2.5 M NaCl, 50 mM

NaOH. SPR sensorgrams were analyzed using the BIAevaluation software (GE Healthcare, Piscataway, NJ). The robustness of the kinetic model was determined by evaluation of the residuals and standard error for each of the calculated kinetic parameters, the “goodness of the fit” ($\chi^2 < 10$), and a direct comparison of the modeled sensorgrams to the experimental data. SPR measurements were collected at eight analyte concentrations (0-100 nM protein) and the resulting sensorgrams were fitted to a 1:1 Langmuir binding model.